

DESCRIPTION

Title of Invention: CAPPED RNA AND METHOD FOR PRODUCING SAME,
APPARATUS FOR PRODUCING PROTEIN, AND METHOD FOR PRODUCING PROTEIN

5 Technical Field

[0001]

The present invention relates to a capped RNA having a cap structure and a method for producing the same, an apparatus for producing a protein, and a method for producing a protein.

10

Background Art

[0002]

Eukaryotic mRNA and the like has been known to have a 5'-capped structure in which 7-methylguanosine forms 5'-5' bond with the 5' end of RNA via a triphosphate bond. The cap structure has been known to promote translation of mRNA, and a target protein can be efficiently synthesized by artificially introducing the cap structure into mRNA in a protein expression system or the like.

20 [0003]

The present inventors have so far developed a method for producing a capped RNA which is RNA having the 5' end modified with a cap, the method including reacting an activated capping compound with a monophosphate RNA having the 5' end monophosphorylated (see, for example, PTL 1). By this method, a cap structure can be introduced into the 5' end of the RNA by a chemical method. A general RNA having a cap structure at the 5' end of the main chain of the RNA is shown in Fig. 1(A).

[0004]

30 On the other hand, the present inventors have developed a circular RNA that encodes a protein, has a full-length base composed by number of 3 multiples, larger than 102 or more, has at least one start codon, does not have a terminator codon (stop codon) in the same reading frame as the start codon, and further
35 does not contain an IRES (see, for example, PTL 2 and NPL 1).

This circular RNA does not have a cap structure, but can perform rolling-circle-type protein translation with high efficiency.

[0005]

As described above, there are a linear RNA and a circular
5 RNA, and the linear RNA generally has one cap structure at the 5'
end of the main chain, whereas the circular RNA is not known to
have a cap structure. In general, it is known that a translation
reaction is initiated from a start codon immediately after the
introduction of a cap structure at the 5' end in the linear RNA.
10 On the other hand, in the circular mRNA having no 5' end, since a
cap structure cannot be introduced, it is common to introduce an
IRES sequence in order to initiate a translation reaction. When
two proteins are also encoded in a linear mRNA, in order to start
a translation reaction in the second coding region, a method of
15 introducing an IRES sequence is known (see, for example, NPL 2).
In this literature, IRES is incorporated into eToehold, and
eToehold is designed to be inactive until it is activated by a
sense-antisense interaction with specific tRNA.

20 Citation List

Patent Literature

[0006]

PTL 1: WO2021/172204A (Abstract, Claim 1, and the like)

PTL 2: WO2013/118878A (Abstract, Claim 1, paragraph 0004,
25 and the like)

Non-patent Literature

[0007]

NPL 1: "Successful Mass Synthesis of Protein in Human
Cultured Cells ~Realization of Endless Rotary Protein Synthesis
30 Reaction Using Circular mRNA~", Japan Science and Technology
Agency, Press Release, November 10, 2015, Japan Science and
Technology Agency (JST), Nagoya University, RIKEN (joint
announcement), <ULR:
<https://www.jst.go.jp/pr/announce/20151110/index.html#ZU1>>

35 NPL 2: "RNA-responsive elements for eukaryotic

translational control", Evan M. Zhao et al., nature
biotechnology, October 28, 2021,
<URL:<https://doi.org/10.1038/s41587-021-01068-2>>

5 Summary of Invention

Technical Problem

[0008]

In the linear RNA as in PTL 1, it is possible to chemically
introduce one cap structure at the 5' end, but further
10 improvement for improving translational activity is required.

[0009]

In the circular RNA having no cap structure as in PTL 2,
the rate at which translation initiation factors constituting a
ribosome binds to RNA is slow, and there is room for further
15 improvement in translational activity.

[0010]

An object of the present invention is to provide a capped
RNA having various types of structures not conventionally used
and having translational activity, and a method for producing the
20 same. Another object of the present invention is to provide a
method for producing a protein and an apparatus for producing a
protein capable of synthesizing a protein using such a capped
RNA.

Solution to Problem

25 [0011]

[1] A capped RNA including:

a translated region beginning with a start codon and
encoding a protein; and

an upstream region upstream of the start codon, a branch
30 part having a cap structure being provided in the upstream
region.

[0012]

[2] The capped RNA, wherein the branch part has at least one
structure of (a) to (c) below:

35 (a) a capped probe having a sequence complementary to a

sequence of a part or all of the upstream region and having the cap structure is hybridized to the upstream region;

(b) a branched chain branching from a main chain constituting the RNA is provided in a part of the upstream region
5 and the cap structure is provided in the branched chain; or

(c) the RNA is a linear RNA and has a branch structure in which the 5' end of a main chain of the upstream region is branched into two or more branches, the cap structure being provided in each branch.

10 [0013]

[3] The capped RNA according to [2], wherein

in the (a), the RNA is a circular RNA, and

a capped probe having a sequence complementary to a sequence of a part or all of the upstream region of the circular
15 RNA and having a cap structure is hybridized to the upstream region of the circular RNA.

[0014]

[4] The capped RNA according to [2], wherein

in the (a), the RNA is a linear RNA, and

20 a capped probe having a sequence complementary to a sequence of a part or all of the upstream region of the linear RNA and having a cap structure is hybridized to the upstream region of the linear RNA.

[0015]

25 [5] The capped RNA according to [2], wherein

in the (b), the RNA is a circular RNA, and

a branched chain branching from a main chain constituting the RNA is provided in a part of the upstream region of the circular RNA, and the cap structure is provided in the branched
30 chain.

[0016]

[6] The capped RNA according to [2], wherein

in the (b), the RNA is a linear RNA, and

a branched chain branching from a main chain constituting
35 the RNA is provided in a part of the upstream region of the

[7] The capped RNA according to [2], which has a structure represented by Formula (3) below in the (b):

$$\begin{array}{c}
 \text{m}^7\text{G}-\text{ppp}-\text{Nuc}-\text{O}-\text{P}(=\text{O})(\text{OH})-\text{O}-(\text{CH}_2)_m-\text{C}(=\text{O})-\text{NH} \\
 | \\
 (\text{CH}_2)_m \\
 | \\
 \text{O}-\text{P}(=\text{O})(\text{OH})-\text{RNA2} \\
 | \\
 (\text{CH}_2)_m \\
 | \\
 \text{RNA1}-\text{O}-(\text{CH}_2)_m-\text{C}(=\text{O})-\text{NH}
 \end{array} \quad (3)$$

where m^7G represents a cap, and p represents a phosphate group; Nuc represents a polynucleotide in which one or more natural or non-natural nucleotides are linked; m represents an integer of 1 to 5, and a plurality of m's may be the same as or different from each other; RNA1 and RNA2 represent RNAs constituting a main chain, and RNA1 and RNA2 are linked to a portion of a branched structure at the 3' end side and the 5' end side, respectively; and a RNA of Formula (3) may be a circular RNA in which the 5' end of RNA1 and the 3' end of RNA2 are linked to form a circular structure, or may be a linear RNA without the 5' end of RNA1 and the 3' end of RNA2 being linked.

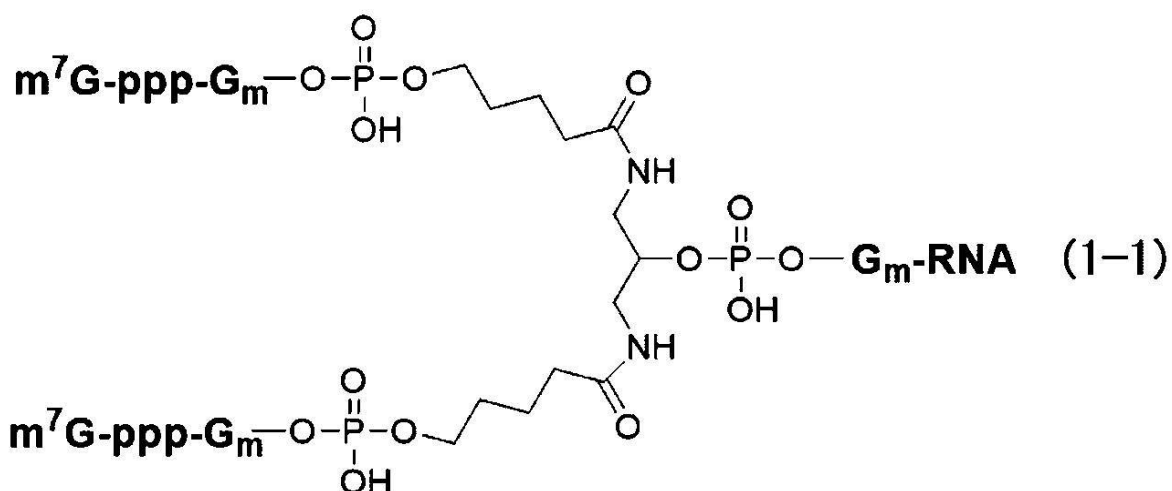
[8] The capped RNA according to [2], which has a structure represented by Formula (1) or Formula (2) below in the (c):

[Chemical Formula 2]

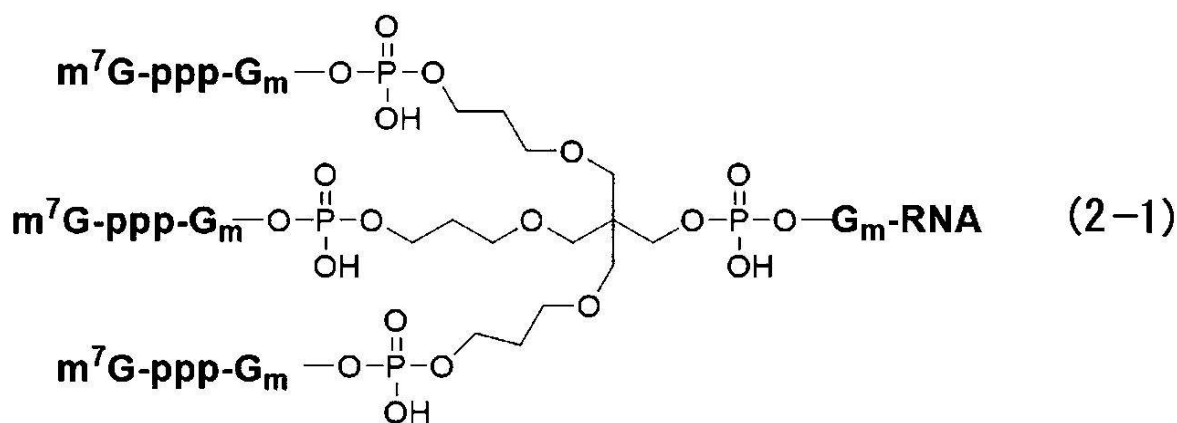
$$\begin{array}{c}
 m^7G\text{-}ppp\text{-}Gm\text{-}O\text{-}\overset{\overset{O}{\parallel}}{\underset{\underset{HO}{|}}{P}}\text{-}O\text{-}(CH_2)_n\text{-}O \\
 m^7G\text{-}ppp\text{-}Gm\text{-}O\text{-}\overset{\overset{O}{\parallel}}{\underset{\underset{HO}{|}}{P}}\text{-}O\text{-}(CH_2)_n\text{-}O \\
 m^7G\text{-}ppp\text{-}Gm\text{-}O\text{-}\overset{\overset{O}{\parallel}}{\underset{\underset{HO}{|}}{P}}\text{-}O\text{-}(CH_2)_n\text{-}O
 \end{array}
 \left[\begin{array}{c} \\ \\ \end{array} \right]
 (CH_2)_n\text{-}O\text{-}\overset{\overset{O}{\parallel}}{\underset{\underset{HO}{|}}{P}}\text{-}Gm\text{-}RNA \quad (2)$$

10 [0019]

[Chemical Formula 4]



[Chemical Formula 5]



[0020]

- 5 [10] The capped RNA according to [1], wherein the RNA has a plurality of translated regions.

[0021]

[11] A method for producing the capped RNA according to [1], the method including at least one of steps (a) to (c) below:

- 10 (a) hybridizing a capped probe having a sequence complementary to a sequence of a part or all of the upstream region and having a cap structure to the upstream region; or
- (b) synthesizing the RNA having a branched chain branching from a main chain constituting the RNA in a part of the upstream region, and then binding the cap structure to the branched chain;
- 15 or
- (c) with the RNA being a linear RNA, synthesizing RNA having a branch structure in which the 5' end of a main chain of

the upstream region is branched into two or more branches, and then binding the cap structure to each branch.

[0022]

[12] An apparatus for producing a protein, including:

5 the capped RNA according to [1]; and

 an expression system of a eukaryotic cell capable of expressing the protein encoded by the translated region using the capped RNA as a template.

[0023]

10 [13] A method for producing a protein, including adding the capped RNA according to [1] to an expression system of a eukaryotic cell capable of expressing the protein encoded by the translated region using the capped RNA as a template to express the protein.

15 [0024]

[14] A detection device of a target RNA having a cap structure at the 5' end of RNA, the detection device including:

 a detection probe containing a circular RNA having a translated region beginning with a start codon and encoding a
20 labeled protein and an upstream region upstream of the start codon, the upstream region having a sequence complementary to a sequence of a part or all of the target RNA to be detected;

 a means for hybridizing the target RNA to the upstream region of the circular RNA by adding the circular RNA to a sample
25 containing the target RNA to generate a capped RNA;

 an expression system of a eukaryotic cell capable of expressing the labeled protein by adding the capped RNA; and

 a means for detecting the labeled protein expressed in the expression system.

30 [0025]

[15] A detection method of a target RNA having a cap structure at the 5' end of RNA, the detection method including:

 a step of preparing a detection probe containing a circular RNA having a translated region beginning with a start codon and
35 encoding a labeled protein and an upstream region upstream of the

start codon, the upstream region having a sequence complementary to a sequence of a part or all of the target RNA to be detected;

a step of hybridizing the target RNA to the upstream region of the circular RNA by adding the circular RNA to a sample

5 containing the target RNA to generate a capped RNA;

a step of adding the capped RNA to an expression system of a eukaryotic cell capable of expressing the labeled protein to express the labeled protein; and

10 a step of detecting the labeled protein expressed in the expression system.

[0026]

[1 (Pre)] A capped RNA having a cap structure in an untranslated region of RNA having a translated region beginning with a start codon and encoding a protein and the untranslated region upstream
15 of the start codon, the capped RNA including at least one structure of (a) to (c) below:

(a) a capped probe having a sequence complementary to a sequence of a part or all of the untranslated region and having a cap structure is hybridized to the untranslated region;

20 (b) a branched chain branching from a main chain constituting the RNA is provided in a part of the untranslated region and the cap structure is provided in the branched chain; or

(c) the RNA has a branch structure in which the 5' end of a
25 main chain of the untranslated region is branched into two or more branches, the cap structure being provided in each branch.

[0027]

[9 (Pre)] A method for producing the capped RNA according to [1 (Pre)], the method including at least one of the following steps

30 (a) to (c):

(a) hybridizing a capped probe having a sequence complementary to a sequence of a part or all of the untranslated region and having a cap structure to the untranslated region; or

(b) synthesizing the RNA having a branched chain branching
35 from the main chain constituting the RNA in a part of the

untranslated region, and then binding the cap structure to the branched chain; or

(c) synthesizing RNA having a branch structure in which the 5' end of a main chain of the untranslated region is branched into two or more branches, and then binding the cap structure to each branch.

[0028]

[12 (Pre)] A detection device of a target RNA having a cap structure at the 5' end of RNA, the detection device including:

a probe containing a circular RNA having a translated region beginning with a start codon and encoding a labeled protein and an untranslated region upstream of the start codon, the untranslated region having a sequence complementary to a sequence of a part or all of the target RNA to be detected;

a means for hybridizing the target RNA to the untranslated region of the circular RNA by adding the circular RNA to a sample containing the target RNA to generate a capped RNA;

an expression system of a eukaryotic cell capable of expressing the labeled protein by adding the capped RNA; and

a means for detecting the labeled protein expressed in the expression system.

[0029]

[13 (Pre)] A detection method of a target RNA having a cap structure at the 5' end of RNA, the detection method including:

a step of preparing a probe containing a circular RNA having a translated region beginning with a start codon and encoding a labeled protein and an untranslated region upstream of the start codon, the untranslated region having a sequence complementary to a sequence of a part or all of the target RNA to be detected;

a step of hybridizing the target RNA to the untranslated region of the circular RNA by adding the circular RNA to a sample containing the target RNA to generate a capped RNA;

a step of adding the capped RNA to an expression system of a eukaryotic cell capable of expressing the labeled protein to

express the labeled protein; and

a step of detecting the labeled protein expressed in the expression system.

Advantageous Effects of Invention

5 [0030]

According to the present invention, it is possible to provide a capped RNA having various types of structures not conventionally used and having translational activity, and a method for producing the same. Another object of the present
10 invention can provide a method for producing a protein and an apparatus for producing a protein capable of synthesizing a protein using such a capped RNA.

Brief Description of Drawings

15 [0031]

Fig. 1 is a view showing an outline of a capped RNA (hybridization type) of the present invention.

Fig. 2 is a view showing an outline of a capped RNA (branched chain type) of the present invention.

20 Fig. 3 is a view showing base sequences and the like of a circular RNA and a capped probe used in this experiment in Examples.

Fig. 4 is a view showing the sequences of a template DNA and transcribed RNA in Examples.

25 Fig. 5 is a view showing the results of synthesis experiment of a capped probe in Examples.

Fig. 6 is a view showing the results of synthesis experiment of a linear RNA in Examples.

30 Fig. 7 is a view showing the results of circularization experiment of a linear RNA in Examples.

Fig. 8 is a view showing the results of relative evaluation of translational activity in Examples.

35 Fig. 9 is a view showing the evaluation results of the translational activity in the case of using various probes in Examples.

Fig. 10 is a view showing the evaluation results of the translational activity depending on the presence or absence of a polyA sequence in Examples.

Fig. 11 is a view showing the results of evaluating the
5 translational activity by changing the ratio of a probe RNA in Examples.

Fig. 12 is a schematic view showing the synthesis scheme of a capped branched-chain-containing RNA oligonucleotide in Examples.

10 Fig. 13 is a view showing a chemical capping reaction of a branched chain in Examples.

Fig. 14 is a view showing the synthesis reaction of a circular RNA in Examples.

Fig. 15 is a view showing evaluation of translational
15 activity of capped branched-chain-containing circular mRNA in human-derived cultured cells (HeLa) in Examples.

Fig. 16 is a view showing the structure of a 107-base-long RNA including a synthesized branch structure in Examples.

Fig. 17 is a view showing the evaluation results of the
20 translational activity in HeLa cells including a synthesized 107-base-long RNA with branch structure/a plurality of cap structures in Examples.

Fig. 18 is a view showing the results of HPLC analysis of synthesized RNA in Examples.

25 Fig. 19 is a view showing the results of absorbance measurement of deprotected RNA and the results of electrophoresis of a capped RNA in Examples.

Fig. 20 is a view showing the results of absorbance measurement of a capped RNA in Examples.

30 Fig. 21 is a view showing the sequence of RNA after annealing in Examples.

Fig. 22 is a view showing the results of evaluating the structure and translational activity of bicistronic mRNA in Examples.

35 Fig. 23 is a view showing the overall structure of multi-

uORF-mRNA and the sequence of probe RNA in Examples.

Fig. 24 is a view showing the overall structure and the sequence of multi-uORF-mRNA in Examples.

Fig. 25 is a view showing results of HPLC analysis of
5 multi-uORF-mRNA in Examples.

Fig. 26 is a view showing results of HPLC analysis of multi-uORF-mRNA in Examples.

Fig. 27 is a photograph of denaturing PAGE of multi-uORF-mRNA in Examples and is a view showing capping efficiency.

10 Fig. 28 is a view showing evaluation results of the translational activity of multi-uORF-mRNA in Examples.

Fig. 29 is a view showing the sequence of a plasmid fragment containing IRES in Examples.

Fig. 30 is a view showing the evaluation results of the
15 translational activity of capped circular RNA and IRES in Examples.

Fig. 31 is a view showing the experimental results showing that the translational activity of a circular mRNA in which a cap structure is introduced into a branched chain is greatly improved
20 as compared with that in which a cap structure is not introduced.

Fig. 32 is a view showing the experimental results showing the relationship between the branched chain length of a circular mRNA in which a cap structure is introduced into a branched chain and translational activity (that the branched chain length is
25 variable).

Fig. 33 is a view showing the experimental results showing that the translational activity of a circular mRNA having a cap structure in a branched chain is higher than that of mRNA or the like containing an EMCV-derived IRES sequence.

30 Fig. 34 is a view showing the results of synthesis of ATF4-Renilla mRNA by in vitro transcription.

Fig. 35 is a view showing the results of chemical synthesis of RNA.

Fig. 36 is a view showing the results of chemical capping
35 of RNA.

Fig. 37 is a view showing the results of synthesis of circular NanoLuc mRNA by in vitro transcription.

Fig. 38 is a view showing the results of circularization reaction of circular NanoLuc mRNA.

5 Fig. 39 is a view showing the results of csRNA with respect to circular Nluc mRNA.

Fig. 40 is a view showing the results of chemical synthesis of RNA.

10 Fig. 41 is a view showing the results of chemical capping of RNA.

Fig. 42 is a view showing the results of chemical capping of RNA.

Fig. 43 is a view showing the sequences and secondary structures of ATF4-Renilla mRNA and ATF4-csRNA.

15 Fig. 44 is a view showing a translation reaction in HeLa cells of ATF4-Renilla mRNA and ATF4-csRNA5 to csRNA8.

Fig. 45 is a view showing the experimental results of a translation reaction in a HeLa cell extract of csRNA into which chemical modification was introduced.

20 Fig. 46 is a view showing the results of a translation reaction in HeLa cells of csRNA and a circular mRNA into which LNA modification was introduced.

Description of Embodiments

25 [0032]

A capped RNA of the present invention has a translated region beginning with a start codon (for example, AUG) and encoding a protein and an upstream region upstream (on the 5' end side) of the start codon. This upstream region may be an
30 untranslated region (5'UTR) or may be within the reading frame of another open reading frame (ORF). This capped RNA has a branch part having a cap structure in the upstream region. Here, the "branch part having a cap structure" includes various forms in which the cap structure is branched from the RNA of the main
35 chain. This includes, for example, a molecule such as a nucleic

acid having a cap structure (DNA, RNA, or the like) hybridized to RNA of the main chain, a molecule in which nucleotides constituting the main chain are branched, a molecule covalently bound to nucleotides constituting the main chain, and the like.

5 Note that, in the case of the circular RNA, 3'UTR and polyA are also included in the upstream region, but the branch part is preferably located at 5'UTR or uORF close to the start codon.

[0033]

Specifically, the capped RNA has at least one of the
10 following structures (a) to (c):

(a) a capped probe having a sequence complementary to a sequence of a part or all of an upstream region and having a cap structure is hybridized to the upstream region (hereinafter, referred to as "hybridization type");

15 (b) a structure in which a branched chain branching from the middle of a main chain constituting the RNA is provided in a part of an upstream region and a cap structure is provided in the branched chain (hereinafter, referred to as "branched chain type"); and

20 (c) a structure which has a branch structure in which the 5' end of a main chain of an upstream region is branched into two or more branches, the cap structure being provided in each branch (hereinafter, referred to as "multi-capped type").

Note that, the capped RNA of the present invention includes
25 not only a natural RNA (that is, a polynucleotide to which nucleotides composed of ATP, GTP, CTP, and UTP are linked) but also a non-natural nucleic acid strand (modified nucleic acid strand) in which an mC4 linker, 2'OMe, LNA, or the like is introduced into a nucleotide of the RNA. Hereinafter, each type
30 of capped RNA will be described.

[0034]

1. Hybridization type

Hereinafter, the hybridization-type capped RNA will be described. (B) of Fig. 1 shows a hybridization-type capped RNA in
35 a linear RNA and (C) of Fig. 1 shows a hybridization-type capped

RNA in a circular RNA with the structures described in Examples, respectively. In both the linear RNA and the circular RNA, a capped probe, which is RNA having a cap structure, is hybridized with an upstream region (for example, an untranslated region
5 (5'UTR)) of RNA encoding a protein to be translated (hereinafter, referred to as "coding RNA").

[0035]

The coding RNA has a translated region ("Nano Luc" region in the drawing) beginning with a start codon and encoding a
10 protein and an upstream region (for example, an untranslated region ("5'UTR" region) upstream (on the 5' end side) of the start codon. In the present embodiment, there may be a stop codon at the 3' end of the translated region, and an untranslated region (3'UTR) downstream (on the 3' end side) of the stop codon.
15 The 3'UTR contains a polyA sequence. The coding RNA is linear in the linear RNA, and has a circular structure in which the 5' end and the 3' end of the linear RNA are linked in the circular RNA. Note that, in the present embodiment, an embodiment in which the coding RNA includes a stop codon is shown, but the present
20 invention is not limited thereto, and for example, as in PTL 2 and NPL 1, the coding RNA may not have a stop codon. In the case of not including a stop codon, as disclosed in these prior arts, when a circular coding RNA having the number of bases of the entire length as a multiple of 3, which is 102 or more, is used
25 as a circular RNA, a target protein can be generated with high translation efficiency by rotary protein translation.

[0036]

The coding RNA may have only one translated region or two or more translated regions. When there are two or more translated
30 regions, a capped probe having an upstream region on the upstream side of each translated region and hybridizing to any of the upstream regions can be used. In this case, the translational activity of the protein in the translated region starting from the start codon immediately below the upstream region is
35 improved. It may have a normal cap structure at the 5' end of the

main chain of the coding RNA. In the case of having a normal cap structure at the 5' end, the translation efficiency of the capped RNA is further improved by using the capped probe in combination with the cap structure.

5 [0037]

The capped probe is composed of RNA shorter than the coding RNA, and has a base sequence complementary to a sequence of a part or all of 5'UTR of the coding RNA. The position at which the capped probe hybridizes in 5'UTR of the coding RNA is not particularly limited, but in the case of having a Kozak sequence, the position is preferably on the upstream side thereof. The length of the capped probe is not particularly limited, but is preferably about 10 to 50 bases (nt). The capped probe may have a cap bound to the 5' end or a cap bound to the 3' end. The capped probe may have a polyA sequence on the 3' end side. The length (number of bases) of the polyA sequence is not particularly limited, and is preferably 10 to 50 bases. The capped probe may have a locked nucleic acid (LNA) in which 2' carbon and 4' carbon of ribose are crosslinked, a C4 linker, or the like.

20 [0038]

In the capped RNA, the capped probe is hybridized to 5'UTR of the coding RNA. Here, "hybridize" means to hybridize under stringent conditions. The "stringent conditions" refer to conditions under which a specific hybrid is formed and a non-specific hybrid is hardly formed. Examples of RNA that "hybridizes under stringent conditions" include RNA having a certain or more homology to a sequence complementary to a sequence in a predetermined range of 5'UTR of the coding RNA. The homology in this case is, for example, usually 60% or more, preferably 80% or more, more preferably 90% or more, particularly preferably 95% or more, and most preferably 100%, in the BLAST algorithm.

[0039]

Next, a method for producing a hybridization-type capped RNA will be described. A coding RNA constituting a capped RNA can

35

be produced by using DNA having a sequence complementary to RNA having a target sequence and transcribing the DNA with RNA polymerase. Alternatively, the coding RNA may be chemically synthesized using a DNA synthesizer or the like. A circular
5 coding RNA can be produced by producing a linear coding RNA and then circularizing the linear coding RNA by connecting ends using T4 RNA ligase 2 or the like.

[0040]

The probe RNA can be produced by the following method.

10 First, a non-capped RNA as a base is produced by an RNA solid phase synthesis method or the like using a phosphoramidite, a phosphorylation reagent, a 2'-OMe RNA amidite, an LNA amidite, a C4 linker amidite, a CPG column, or the like. Next, this RNA is reacted with m⁷GDP, 2-nitroimidazole, or the like to cap a
15 phosphorylated RNA. Details of this reaction can be referred to PTL 1.

[0041]

The capped RNA can be produced by annealing the coding RNA and the probe RNA. The ratio (molar ratio) between the coding RNA
20 and the probe RNA during annealing is preferably in a range of 1 : 1 to 1 : 20, and more preferably in a range of 1 : 5 to 1 : 15 from the viewpoint of translational activity. The annealing temperature can be 80°C or higher, preferably 90°C or higher, and the reaction time can be 1 to 30 minutes, preferably about 3 to
25 10 minutes.

[0042]

The hybridization-type capped RNA can add a cap structure at any position of 5'UTR regardless of whether the coding RNA is linear or circular. In RNA having a cap structure, the rate at
30 which the translation initiation factor constituting a ribosome binds to the coding RNA is improved as compared with RNA having no cap structure, and as a result, the translational activity is improved. Alternatively, in a hybridization-type capped RNA, it is possible to enhance translation or induce a translation
35 phenomenon from an arbitrary position by presenting a cap by

hybridization with a probe in a circular RNA or a linear RNA.

[0043]

In the linear RNA, by using a capped probe, a translation reaction can be induced from, for example, the second and
5 subsequent start codons that are not originally used as a reading frame of protein synthesis. Thereby, translation can be induced from any RNA position by the capped probe.

[0044]

A circular RNA having a cap structure has not been known so
10 far, but, in the hybridization-type capped RNA of the present invention, a cap structure can also be introduced into a circular RNA. This makes it possible to significantly improve the translational activity as compared with a circular RNA having no cap structure.

15 [0045]

2. Branched chain type

Hereinafter, the branched chain type capped RNA will be described. (a) of Fig. 2 shows a branched chain type capped RNA in a linear RNA and (b) of Fig. 2 shows a branched chain type
20 capped RNA in a circular RNA with the structures described in Examples, respectively. In both the linear RNA and the circular RNA, the upstream region of the coding RNA has a branched chain branching from the main chain constituting the coding RNA, and the cap structure is provided in this branched chain.

25 [0046]

The overall configuration of the coding RNA is as described above in "1. Hybridization type". In the branched chain type, a part of 5'UTR of the coding RNA has a branched chain branching from the main chain, and has a cap structure at the 5' end of
30 this branched chain. The 5' end of the main chain of the coding RNA is phosphorylated in a linear RNA. The coding RNA may have only one translated region or two or more translated regions. When the coding RNA is linear, the coding RNA may have a normal cap structure at the 5' end.

35 [0047]

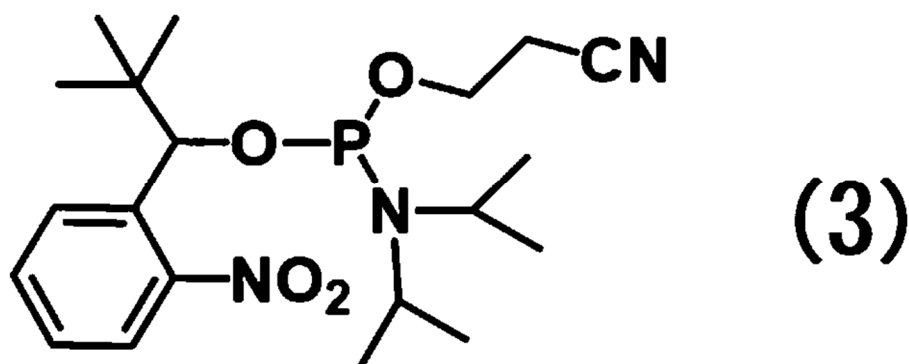
$$m^7G\text{-ppp-Nuc-O-P(=O)(OH)-O-(CH}_2)_m\text{-C(=O)-NH-} \begin{array}{c} \text{(CH}_2)_m \\ | \\ \text{---O-P(=O)(OH)-RNA2} \end{array} \quad (3)$$

5 where m^7G represents a cap, and p represents a phosphate group;
Nuc represents a polynucleotide in which one or more natural or
non-natural nucleotides are linked; m represents an integer of 1
to 5, and a plurality of m's may be the same as or different from
each other; RNA1 and RNA2 represent RNAs constituting a main
10 chain, and RNA1 and RNA2 are linked to a portion of a branched
structure at the 3' end side and the 5' end side, respectively;
and a RNA of Formula (3) may be a circular RNA in which the 5'
end of RNA1 and the 3' end of RNA2 are linked to form a circular
structure, or may be a linear RNA without the 5' end of RNA1 and
15 the 3' end of RNA2 being linked.

Next, a method for producing a branched chain type capped RNA will be described. A coding RNA having a branched chain in 5'UTR can be synthesized using a normal phosphoramidite reagent used for RNA synthesis, a phosphoramidite reagent having a branched chain, and a chemical phosphorylation reagent, and using an automated nucleic acid synthesizer. Examples of the phosphoramidite having a branched chain may include 1-[5-(4,4'-dimethoxytrityloxy)pentylamide]-3-[5-levulinyloxypentylamide]-propyl-2-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite used in Examples described below. Examples of the chemical phosphorylation reagent may include Compound 1 (Formula (3))

below) of Examples described below.

[Chemical Formula 7]



[0049]

5 The sequence of 5'UTR of the coding RNA is synthesized from the upstream side using a normal phosphoramidite reagent or device, and a branched chain can be introduced at a desired position by using a phosphoramidite reagent having a branched chain at a position where the branched chain is to be introduced.

10 Since a hydrophobic protective group is introduced into the 5' end of the branched chain of the coding RNA by a chemical phosphorylation reagent, this hydrophobic protective group is decomposed by photo-irradiation or the like to form a phosphate group at the 5' end of the branched chain. Then, the 5' end of

15 the branched chain is capped using a heteroaromatic compound such as 7-methylguanosine 5'-diphosphate imidazolide salt (Im-m⁷GDP) or 2-nitroimidazole. Finally, the 5' end of the main chain of the coding RNA is phosphorylated using T4 polynucleotide kinase or the like. Thereby, a linear capped RNA is obtained. A circular

20 coding RNA can be produced by producing a linear capped RNA and then cyclizing the linear capped RNA by connecting ends using T4 RNA ligase 2 or the like.

[0050]

25 The branched chain type capped RNA can add a cap structure at any position of 5'UTR regardless of whether the coding RNA is linear or circular, similarly to the hybridization-type capped RNA. When the coding RNA has two or more translated regions, the

translational activity of the translated region can be improved by introducing a branched chain cap into 5'UTR located upstream of a target translated region. Thereby, for example, a translation reaction can be induced from a start codon that is not used as a reading frame for protein synthesis. In the branched chain type capped RNA, a cap structure can be introduced even in a circular RNA that does not conventionally have a cap structure. This makes it possible to significantly improve the translational activity as compared with a circular RNA having no cap structure.

[0051]

As shown in Examples described below, the branched chain type capped RNA tends to have higher translational activity and lower immunogenicity in a circular RNA than in a linear RNA.

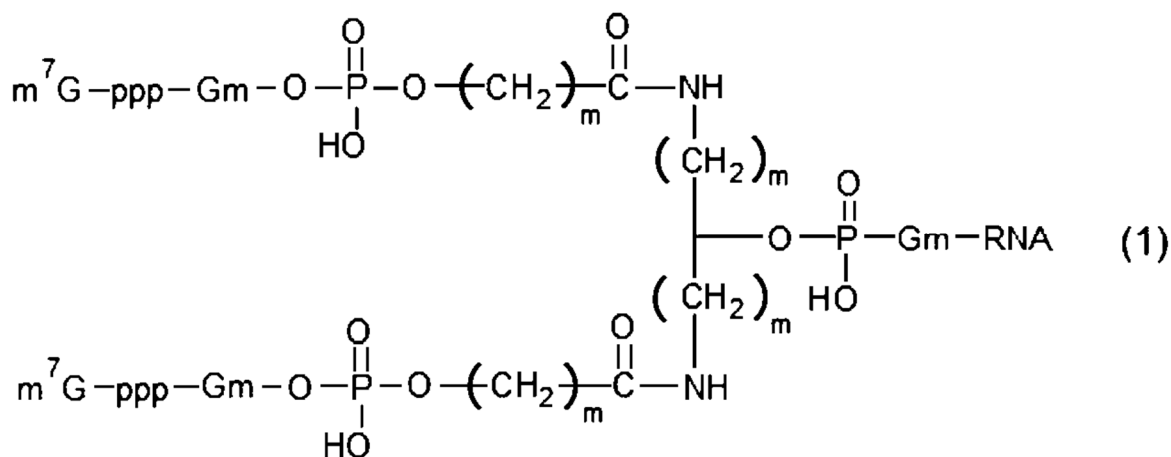
The closer the position of the cap in the upstream region is to the start codon, the higher the translational activity tends to be. The position of the branch in the upstream region (that is, the position of the structure between RNA1 and RNA2 in Formula (3)) is not particularly limited, but since a cap structure is provided at the end of the branch, the translational activity tends to increase as the position of the branch is closer to the start codon. The position of the branch is preferably within a range of 1 to 50 nt, more preferably within a range of 1 to 30 nt, and particularly preferably within a range of 1 to 15 nt, on the 5' side based on the start codon.

The chain length of the branched portion of the branched chain (that is, the length of the polynucleotide of Nuc in Formula (3)) is not particularly limited, but the translational activity tends to decrease when the chain length is too long or too short. Here, the chain length of the branched chain is defined as a distance between the branched point and the 5' cap. The chain length of the branched chain is preferably within a range of 1 to 20 nt, more preferably within a range of 3 to 10 nt, and particularly preferably within a range of 5 to 8 nt. When the chain length is too short, the distance between the position

3. Multi-capped type

[0053]

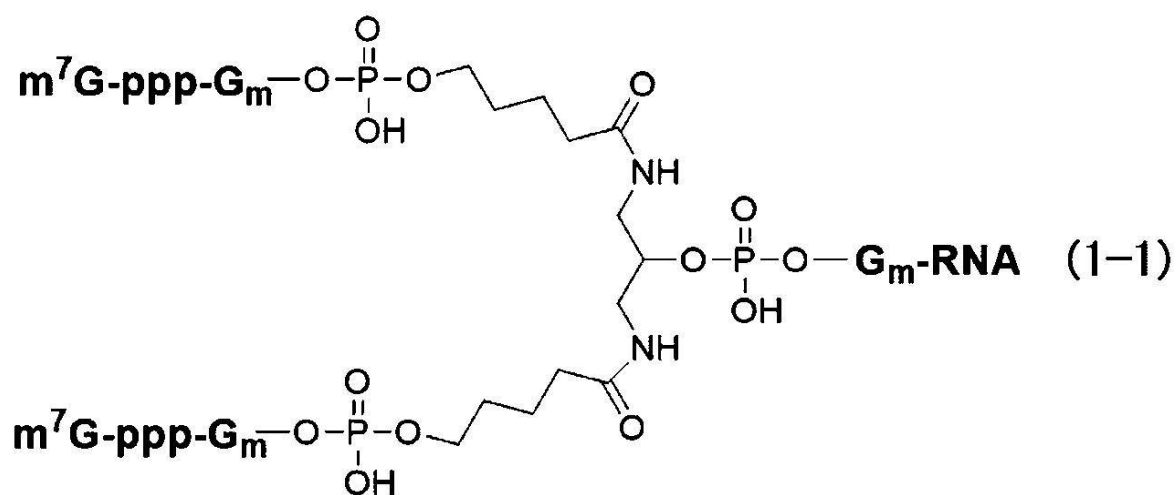
[Chemical Formula 8]



20 [Chemical Formula 9]

[0054]

[Chemical Formula 10]



[Chemical Formula 11]

Next, a method for producing a multi-capped type capped RNA will be described. RNA before being capped can be synthesized using a normal phosphoramidite reagent used for RNA synthesis, a phosphoramidite reagent having a branched chain, and a chemical phosphorylation reagent, and using an automated nucleic acid synthesizer. Examples of the phosphoramidite having a branched chain may include 1,3-bis-[5-(4,4'-dimethoxytrityloxy)pentylamide]propyl-2-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite (two-branch type), and tris-2,2,2-[3-(4,4'-dimethoxytrityloxy)propyloxymethyl]ethyl-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite (three-branch type) used in Examples described below. Examples of the chemical phosphorylation reagent may include Compound 1 (Formula (3)) of Examples described below.

A branch structure is formed at the 5' end using a phosphoramidite reagent having a branched chain, and the sequence of 5'UTR of the coding RNA is synthesized from the upstream side using a normal phosphoramidite reagent or device, thereby synthesizing a coding RNA having a plurality of branches at the 5' end. A hydrophobic protective group is introduced into the 5' end of each branch of the coding RNA by a chemical phosphorylation reagent, and this hydrophobic protective group is decomposed by photo-irradiation or the like to form a phosphate group at the 5' end of the branched chain. Then, the 5' end of

each branch of the branch structure is capped using a heteroaromatic compound such as 7-methylguanosine 5'-diphosphate imidazolide salt (Im-m⁷GDP) or 2-nitroimidazole. Thereby, a linear capped RNA in which a cap is bound to each branch is
5 obtained.

[0057]

Since the multi-capped type capped RNA has a plurality of caps at the 5' end of the main chain, the translation initiation factor constituting a ribosome is more likely to bind to the cap
10 as compared with a normal capped RNA in which the cap is one, and the translational activity can be greatly improved by the multi-ligand effect.

[0058]

4. Apparatus and method for producing protein

15 An apparatus for producing a protein of the present invention includes the above-described capped RNA, and an expression system of a eukaryotic cell capable of expressing the protein encoded by the translated region using the capped RNA as a template. In a method for producing a protein of the present
20 invention, a capped RNA is added to an expression system of a eukaryotic cell to express a protein. Examples of the eukaryotic cell may include fungi such as yeast and filamentous fungi, plant cells, insect cells, and animal cells of human and the like, and specific examples thereof may include mouse L-cells, Ehrlich
25 ascites cancer cells, HeLa cells, CHO cells, and *Saccharomyces cerevisiae*. The expression system of a eukaryotic cell may be either a cell expression system or a cell-free expression system. The introduction of the capped RNA into the cell expression
30 system can be performed by a method such as a calcium phosphate method, a DEAE dextran method, a lipofection method, or an electroporation method. In the cell expression system, after the capped RNA is introduced into the cell, the cell is cultured under conditions such as an appropriate temperature, the cell is lysed after the culture, and then a target protein is separated
35 and purified. In the cell-free expression system, lysates of

eukaryotic cells can be used.

[0059]

5. Detection device and detection method of target RNA

As described above, since the circular RNA does not have a
5 cap structure, the circular RNA is not translated by itself, but
a protein encoded by the circular RNA can be translated by
hybridizing RNA having a cap structure. By utilizing this
property, a target RNA can be detected using the circular RNA as
a detection probe. Examples of the target RNA may include RNA
10 derived from eukaryotes, viruses, and the like. Hereinafter, a
detection device and a detection method of a target RNA of the
present invention will be described.

[0060]

The detection device of a target RNA of the present
15 invention is a device that detects a target RNA having a cap
structure at the 5' end of RNA. This detection device includes a
detection probe containing a circular RNA, a means for generating
a capped RNA, an expression system of a eukaryotic cell, and a
means for detecting a labeled protein.

20 [0061]

The detection probe has a translated region beginning with
a start codon and encoding a labeled protein and an upstream
region upstream of the start codon. This upstream region has a
sequence complementary to a sequence of a part or all of a target
25 RNA to be detected. Examples of the labeled protein may include
fluorescently labelled proteins such as luciferase and green
fluorescent protein (GFP), and enzyme-linked proteins such as
horseradish peroxidase (HRP) and alkaline phosphatase (AP) used
in Examples described below.

30 [0062]

The means for generating a capped RNA is a means for
hybridizing the above-described detection probe (circular RNA)
and a target RNA. For this means, a normal reagent or device for
annealing RNA can be used. Examples of such a reagent may include
35 buffers such as Tri-HCl. Examples of the device may include a

device such as a heat block or a thermal cycler that heats the reagent to 80°C or higher and then cools the reagent to room temperature.

The expression system of a eukaryotic cell is a system
5 capable of expressing the labeled protein encoded by the translated region using the capped RNA generated above as a template, and examples thereof may include the HeLa cells described above.

[0063]

10 As the means for detecting the labeled protein, various devices can be used depending on the type of the labeled protein. Examples of such a means may include a fluorescence measuring device when the labeled protein is a fluorescently labeled protein, and a luminescent substrate and a fluorescence measuring
15 device when the labeled protein is an enzyme-linked protein.

[0064]

The detection method of a target RNA of the present invention can be performed using the above-described detection device. First, a detection probe containing the circular RNA is
20 prepared, and added to a sample containing a target RNA to generate a capped RNA. Next, the capped RNA is added to an expression system of a eukaryotic cell capable of expressing a labeled protein to express a labeled protein, and then the expressed labeled protein is detected. By such a method, the
25 target RNA can be efficiently detected.

Examples

[0065]

Hereinafter, the present invention will be specifically
30 described by way of Examples; however, the object of the present invention is not limited to these Examples. In the following Examples, the expression "%" is on a mass basis (mass percent) unless otherwise particularly specified.

[0066]

35 1. Capped RNA (hybridization type)

In the following, experiments on a capped RNA of a hybridization type are shown. Fig. 3 is a view showing base sequences and the like of a circular RNA and a capped probe used in this experiment. The upper left view in the drawing shows the structure of the capped RNA. As the circular RNA (coding RNA), the RNA has the 5'-side upstream region (untranslated region (denoted as "5'UTR") in the drawing) and a Kozak sequence (denoted as "Kozak") on the downstream side, and has a region encoding Nano Luc luciferase ("Nano Luc"), a stop codon ("stop"), the 3'-side untranslated region ("3'UTR"), and a polyA sequence ("polyA₃₀"). The capped probe in the drawing has a cap structure (m⁷G) at the 5' end, has a sequence complementary to a partial region of 5'UTR of the circular RNA, and has polyA₃₀ at the 3' end. In the capped RNA, the capped probe is hybridized to 5'UTR of the circular RNA.

[0067]

The upper right side in the drawing shows the arrangement of the capped probe used in this example. As the capped probe, three types of "5' cap probe" having a cap structure at the 5' end, "3' cap probe" having a cap structure at the 3' end, and "5' cap probe with polyA" having a cap structure at the 5' end and having polyA at the 3' end were used. The lower part in the drawing shows the sequence of the circular RNA. Bolded regions represented by "ggcgc... cgc" are the portions where the capped probe hybridizes.

[0068]

(1) Probe RNA synthesis

RNA synthesis was performed on a 0.2 μmol scale, and an NTS M-2-MX DNA/RNA synthesizer or an NRs-4A10R7 DNA/RNA synthesizer manufactured by NIHON TECHNO SERVICE CO., LTD. was used. As the phosphoramidite used for RNA solid phase synthesis, rA(5'-DMT-2'-TOM-ribo-Adenosine (n-acetyl) OP), rC(5'-DMT-2'-TOM-ribo-Cytidine (n-acetyl) OP), rG(5'-DMT-2'-TOM-ribo-Guanosine (n-acetyl) OP), and U(5'-DMT-2'-TOM-ribo-Uridine OP) (ChemGenes) were used. As the phosphorylation reagent, 5' phosphate-ON Reagent (ChemGenes)

was used. As the 2'-OMe RNA amidite, 2'-OMe-C-CE-phosphoramidite and 2'-OMe-G(iBu)-CE-phosphoramidite (ChemGenes) were used. As the LNA amidite, MBN-Ganosine(N,N-DMF)-phosphoramidite (ChemGenes) and MBN-5'-Methyl-Cytidine(N-bz)-phosphoramidite (ChemGenes) were used. As the C4 linker amidite, one synthesized according to the previous report (Angew. Chem. Int. Ed. 2011, 50, 8922-8926) was used. As the CPG, 3'-TOM-ribo Cytidine (n-acetyl) 2'-lcaa CPG 1000Å, 3'-TOM-ribo Adenosine (n-acetyl) 2'-lcaa CPG 1000Å (ChemGenes), and 3'-phosphate ON lcaa CPG 1000A were used.

[0069]

After the synthesis of RNA, the CPG was transferred to a screw tube with 1.5 mL loop (SARSTEDT AG & Co. KG), 1.0 mL of 40% aq. methylamine/28% aq. ammonia (1 : 1) was added, and the mixture was incubated at 65°C for 20 minutes. The mixture was filtered using a Millex LH 0.45 µm filter, washed with sterile water (500 µL), and dried and solidified on a centrifugal evaporator. 1.0 mL of 1 M TBAF in THF was added and dissolved, and the mixture was allowed to stand still at room temperature overnight. 1.0 mL of a 1 M Tris-HCl buffer (pH 7.5) was added, and the organic solvent was distilled off with a centrifugal evaporator. Desalination was performed using a NAP-25 column (GE Healthcare). RNA was purified by reverse phase HPLC.

[0070]

HPLC (HITACHI) conditions; Column: hydrosphere C18 column (4.6 x 250 mm; YMC), Mobile phase: A) 50 mM TEAA buffer (pH 7.0), 5% acetonitrile, B) acetonitrile, Gradient: 0 to 20 min B conc. 0% to 20%, Flow rate: 1.0 mL/min

[0071]

RNA synthesized by 15% denatured polyacrylamide gel electrophoresis (PAGE) (acrylamide : bisacrylamide = 19 : 1 (wt/wt), 7.5 M urea) was analyzed. For PAGE, a TBE buffer (89 mM Tris-borate, 2 mM EDTA, pH 8.3) was used. Analysis by LC/MS was performed. LC/MS (agilent) conditions; ACQUITY UPLC BEH C18 Column, 0.3 mL/min, A; 8.6 mM TEA/100 mM HFIP solution, B; MeOH,

0 to 30%/20 min

[0072]

Information on the sequence, molecular weight, and the like of the synthesized RNA is shown in the following table (RNA1 to RNA12 correspond to SEQ ID NOS: 1 to 12).

[Table 1]

Sequence name	Sequence	Molecular weight	Measured value
RNA1	pGCGUCACCUAAUAUGCGCC	6382.8	6383.7
RNA2	pG-C4-GCGUCACCUAAUAUGCGCC	6880.1	6881.0
RNA3	pC4-GCGUCACCUAAUAUGCGCC	6534.9	6535.8
RNA4	GCGUCACCUAAUAUGCGCC-C4-p	6534.9	6535.2
RNA5	pCGCCGUGAACUAUCUCCUA	6382.8	6383.7
RNA6	pGCGUCACCUAAUAUGCGCCAAAAAAAAAAAAAAAAAAAAAAAAAAAA	16259.1	16261.4
RNA7	pG-C4-GCGUCACCUAAUAUGCGCCAAAAAAAAAAAAAAAAAAAAAAAAAAAA	16756.4	16759.7
RNA8	pC4-GCGUCACCUAAUAUGCGCCAAAAAAAAAAAAAAAAAAAAAAAAAAAA	16411.2	16414.3
RNA9	p-m _n G _n CGUCACCUAAUAUGCGCCAAAAAAAAAAAAAAAAAAAAAAAAAAAA	16287.1	16289.5
RNA10	pC4-mGmCGUCACCUAAUAUGCGCCAAAAAAAAAAAAAAAAAAAAAAAAAAAA	16439.2	16441.5
RNA11	pGCGUCACCUAAUAUGCGCCAAAAAAAAAAAAAAAAAAAAAAAAAAAA	16373.2	16375.2
RNA12	pC4-GCGUCACCUAAUAUGCGCCAAAAAAAAAAAAAAAAAAAAAAAAAAAA	16525.3	16528.3

C4; C4 linker; m_n; 2'OMe, Underlined; LNA

[0073]

(2) Capping reaction of phosphorylated RNA

The mixture was mixed in a 1.5 mL tube so as to have a final concentration of 20 μ M RNA and 10 mM CaCl₂, respectively, and the mixture was freeze-dried and solidified and then dissolved in DMSO. Im (m⁷GDP)·Na (Hongene Biotech) dissolved in DMSO was added thereto so as to have a final concentration of 10 mM and 2-nitroimidazole was added thereto so as to have a final concentration of 10 mM. This reaction solution was incubated at 55°C for 3 hours. The reaction solution was diluted two-fold with sterile water, and then isopropanol precipitation was performed. HPLC purification and analysis by LC/MS were performed.

[0074]

HPLC conditions; Column: hydrosphere C18 column (4.6 x 250 mm; YMC), Mobile phase: A) 50 mM TEAA buffer (pH 7.0), 5% acetonitrile, B) acetonitrile, Gradient: 0 to 20 min B conc. 0% to 20%, Flow rate: 1.0 mL/min

LC/MS (agilent) conditions; ACQUITY UPLC BEH C18 Column,

0.3 mL/min, A; 8.6 mM TEA/100 mM HFIP solution, B; MeOH, 0 to 30%/20 min

The HPLC-purified capped RNA was precipitated using isopropanol and sodium acetate (pH 5.2).

5 [0075]

Information on the sequence, molecular weight, and the like of the synthesized capped RNA is shown in the following table (capped RNA1 to RNA12 correspond to SEQ ID NOS: 13 to 24).

[Table 2]

Sequence name	Sequence	Molecular weight	Measured value
Capped RNA1	m7GpppGCGUCACCUUAAUAUGCGCC	6823.0	6822.9
Capped RNA2	m7GpppG-C4-GCGUCACCUUAAUAUGCGCC	7320.3	7320.2
Capped RNA3	m7Gppp-C4-GCGUCACCUUAAUAUGCGCC	6975.1	6974.9
Capped RNA4	GCGUCACCUUAAUAUGCGCC-C4-pppm7G	6975.1	6975.0
Capped RNA5	m7GpppCGCCGUGGAACUAUCUCCUA	6823.0	6822.9
Capped RNA6	m7GpppGCGUCACCUUAAUAUGCGCCAAAAAAAAAAAAAAAAAAAAAAAAAAAA	16699.3	16701.0
Capped RNA7	m7GpppG-C4-GCGUCACCUUAAUAUGCGCCAAAAAAAAAAAAAAAAAAAAAAAAAAAA	17196.6	17198.7
Capped RNA8	m7Gppp-C4-GCGUCACCUUAAUAUGCGCCAAAAAAAAAAAAAAAAAAAAAAAAAAAA	16851.4	16853.1
Capped RNA9	m7Gppp-mCGUCACCUUAAUAUGCGCCAAAAAAAAAAAAAAAAAAAAAAAAAAAA	16727.4	16728.7
Capped RNA10	m7Gppp-C4-mGmCGUCACCUUAAUAUGCGCCAAAAAAAAAAAAAAAAAAAAAAAAAAAA	16879.4	16881.3
Capped RNA11	m7GpppGCGUCACCUUAAUAUGCGCCAAAAAAAAAAAAAAAAAAAAAAAAAAAA	16813.5	16814.9
Capped RNA12	m7Gppp-C4-GCGUCACCUUAAUAUGCGCCAAAAAAAAAAAAAAAAAAAAAAAAAAAA	16965.5	16966.8

10 C4; C4 linker, m; 2'OMe, Underlined; LNA

[0076]

Fig. 5 is a view showing the results of synthesis experiment of a capped probe. (A) of Fig. 5 shows the synthesis scheme of the capped probe. The chemically synthesized monophosphorylated RNA was freeze-dried with calcium chloride. Im (m⁷GDP) ·Na (chemical capping reagent) and 2-nitroimidazole were added and reacted in a DMSO solution. (B) of Fig. 5 shows the results of the capped probe, and the RNA subjected to capping reaction was analyzed by PAGE and stained by SYBR Green II. As the capping reaction proceeded, the single base band shifted up due to the addition of methylguanine. As a result of PAGE analysis, it is found that the capping reaction proceeded with an efficiency of about 80 to 90%.

[0077]

25 (3) Synthesis of linear RNA by transcription reaction

The linear RNA was transcriptionally synthesized by T7 RNA polymerase using double-stranded DNA as a template. The upper part of Fig. 4 shows the sequence of the template DNA for the transcription synthesis of RNA (SEQ ID NO: 32) and the lower part shows the sequence of the transcribed RNA (SEQ ID NO: 31). The template double-stranded DNA was obtained by amplifying pNL-TK1.1 (Promega) by PCR reaction using PrimeSTAR HS DNA Polymerase (Takara Bio) (2.5 ng/ μ L DNA vector, 1 $\times$ PrimeSTAR buffer, 0.2 mM dNTPs, 0.5 μ M primers, 0.025 units/ μ L polymerase).

[0078]

A transcription reaction solution (template DNA 2.5 ng/ μ L, 1 $\times$ T7 RNA Polymerase buffer (Takara) (40 mM Tris-HCl (pH 8.0), 8 mM MgCl₂, 2 mM spermidine), 2.5 U/ μ L T7 RNA polymerase (Takara), 5 mM DTT, 2 mM ATP, 2 mM UTP, 2 mM CTP, 0.4 mM GTP, 2 mM GMP) was prepared and incubated at 37°C for 2 hours. Recombinant DNase (Takara) was added to the reaction solution at 0.1 U/ μ L, and the mixture was incubated at 37°C for 30 minutes to decompose the template DNA.

After the reaction, phenol/chloroform extraction was performed. Subsequently, ultrafiltration was performed using Amicon Ultra 10K (Millipore) to perform alcohol precipitation.

[0079]

Fig. 6 is a view showing the results of synthesis experiment of a linear RNA. A) of Fig. 6 shows the results of synthesis of double-stranded DNA as a template for a linear RNA by PCR reaction, and is a photograph of a gel analyzed on an agarose gel after the PCR reaction and stained by Gel Red. B) of Fig. 6 shows the results of transcriptional synthesis of the linear RNA and is a photograph of a gel analyzed by PAGE after the transcription reaction and stained by SYBR Green.

[0080]

(4) Cyclization reaction using T4 RNA ligase 2

The transcriptionally synthesized linear RNA was cyclized using T4 RNA ligase 2 (New England Biolabs) and 20-nt DNA (guide DNA). 0.5 μ M linear RNA and 1.0 μ M guide DNA were annealed in T4

RNA ligase 2 buffer (50 mM Tris-HCl (pH 7.5), 2 mM MgCl₂, 1 mM DTT and 0.4 mM ATP), then 0.1 U/μL T4 RNA ligase 2 and 10% PEG8000 were added, and the mixture was incubated at 37°C for 1 hour. The circular RNA of the ligated product and the linear RNA of the raw material were isolated by 5% denaturing PAGE.

[0081]

(5) Confirmation of cyclization using RNase R

RNA (0.18 μg/μL) was incubated in RNase R (1 unit/μL; Epicenter), 20 mM Tris-HCl (pH 8.0), 0.1 M KCl, and 0.1 mM MgCl₂ at 37°C for 10 minutes. The reaction solution was analyzed by 5% denaturing PAGE.

[0082]

Fig. 7 is a view showing the results of cyclization experiment of a linear RNA. A) of Fig. 7 shows the synthesis scheme of the circular RNA. The transcriptionally synthesized linear RNA was annealed to the guide DNA, and a circular RNA was synthesized using T4 RNA ligase 2. B) of Fig. 7 shows the confirmation result of the circularized form, and RNase R was added to the RNA subjected to the circularization reaction and was incubated. Analysis by PAGE and staining by SYBR Green II were performed. The transcriptionally synthesized linear RNA was cyclized using T4 RNA ligase 2. The identification of circularized forms was performed by PAGE analysis with the action of an enzyme (RNase R) that selectively degrades the linear RNA. The efficiency of circularization was about 60%. The linear RNAs of the cyclized form and the raw material were cut out from the gel, and isolated and purified.

[0083]

(6) Annealing

0.2 μM capped probe and 0.1 μM linear or 0.1 μM circular RNA were annealed in a buffer (10 mM Tris-HCl (pH 7.5), 50 mM NaCl) (90°C, 3 min).

[0084]

(7) Evaluation of intracellular translational activity

HeLa cells (RIKEN Cell Bank) were cultured in Dulbecco's

modified Eagle's medium (DMEM; WAKO) added with 10% fetal bovine serum (FBS; Invitrogen) (37°C, 5% CO₂). HeLa cells were seeded in a 96-well plate the day before transfection (1.0×10⁴ cell/well). The next day, the medium was removed and replaced with 100
5 μL/well Opti-MEM (registered trademark) (Thermo Fisher SCIENTIFIC). 0.15 μL Lipofectamine (registered trademark) MessengerMAX (registered trademark) and 20 ng mRNA were diluted in 10 μL Opti-MEM (registered trademark) and introduced into cells. After incubation at 37°C for 5 hours, 20 μL/well 1×Cell
10 Lysis Buffer (Promega) was added to lyse the cells. Luminescence measurement was performed using Nano-Glo (registered trademark) luciferase assay (registered trademark) (Promega).

[0085]

Fig. 8 is a view showing the results of relative evaluation
15 of translational activity. A) of Fig. 8 shows the result of the translational activity of the linear RNA, and B) of Fig. 8 shows the result of the translational activity of the circular RNA. As the capped RNA probe, "capped RNA8" was used, and as the non-capped RNA probe, "RNA8" was used. The translational activity of
20 a sample in which the RNA probe was not hybridized is relatively evaluated as "1". For any RNAs, the capped RNA was annealed and transfected into HeLa cells. After 5 hours, the cells were lysed and luminescence of NanoLuc luciferase was measured. From these results, when both the linear RNA and the circular RNA were
25 hybridized with the capped RNA, about 40 times the translational activity promoting effect was observed as compared with the case of the RNA alone. In the mRNA hybridized with phosphorylated RNA having no cap structure, the translational activity promoting effect was not observed. This showed that the translation
30 initiation stage was promoted in a cap-dependent manner.

[0086]

Fig. 9 is a view showing the evaluation results of the translational activity in the case of using various probes. A) of
Fig. 9 shows the result of the translational activity of the
35 linear RNA, and B) of Fig. 9 shows the result of the

translational activity of the circular RNA. For any RNAs, the capped RNA was annealed and transfected into HeLa cells. After 5 hours, the cells were lysed and luminescence of NanoLuc luciferase was measured. From these results, when both the linear
5 RNA and the circular RNA were hybridized with the capped RNA, the translational activity was promoted (capped RNA1 to RNA4, RNA6 to RNA12). In the capped RNA having a sequence not complementary to mRNA, since the translation promoting effect was not observed (capped RNA5), it was shown that the capped RNA was hybridized
10 with mRNA to promote translation. When a C4 linker and a polyA sequence were introduced into the cap structure, the promoting effect was increased (capped RNA8 to RNA10).
[0087]

Fig. 10 is a view showing the evaluation results of the
15 translational activity depending on the presence or absence of a polyA sequence. In the drawing, the upper part (Linear) shows the results using a linear RNA, and the lower part (Circular) shows the results using a circular RNA. "Cap probe" indicates the result of annealing the capped probe, and "p probe" indicates the
20 result of annealing the non-capped phosphate-terminated probe. From these graphs, it is found that the translational activity is improved by annealing the capped probe in both cases of a linear RNA and a circular RNA as compared with a case where the probe is not capped. It is also found that the translational activity is
25 slightly improved in the case of adding the polyA as compared with the case of not adding the polyA.
[0088]

Evaluation of intracellular translational activity was performed in the same manner as in the above "(6) Evaluation of
30 intracellular translational activity" except that the concentration of the capped probe was changed to two kinds (0.2 μ M, 1 μ M). The results are shown in Fig. 11. Fig. 11 is a view showing the results of evaluating the translational activity by changing the ratio of a probe RNA. From this drawing, it is found
35 that the translational activity is higher at mRNA : probe = 1 :

10 than at mRNA : probe = 1 : 2.

[0089]

2. Cap-containing circular RNA (branched chain type)

(1) Chemical synthesis, purification, and chemical capping of RNA

5 Fig. 12 is a schematic view showing the synthesis scheme of a capped branched-chain-containing RNA oligonucleotide.

Hereinafter, a capped branched-chain-containing RNA oligonucleotide was synthesized based on this synthesis scheme.

[0090]

10 (a) RNA chemical synthesis

An oligoribonucleotide having a 54-base-long main chain and containing a branched chain phosphorylated at the 5' end was synthesized according to a conventional method with an automated nucleic acid synthesizer (NR-2A 7MX, NIHON TECHNO SERVICE CO., LTD.) using Compound 1 (Formula (3) above: see Japanese Patent Application No. 2021-111420), a commercially available phosphoramidite reagent [amidite for RNA synthesis is a product of ChemGenes (ANP-3201, ANP-3202, ANP-3203, ANP-3205), Asymmetric Doubler (Lev) Phosphoramidite (10-1981) purchased from Glen Research for branched chain introduction], and a solid phase carrier (3'-TOM-ribo Uridine CPG 1000A, ChemGenes). In order to synthesize a circular RNA not containing a branched chain, a 54-base-long RNA sequence composed only of a main chain was synthesized, and the 5' end thereof was phosphorylated by
25 Compound 1.

[0091]

After completion of the synthesis of RNA, 1 mL of a mixed solution of equal amounts of a 40% methylamine aqueous solution and concentrated ammonia water was added to the solid phase
30 carrier, and heated at 65°C for 20 minutes. The supernatant was filtered through a Millex LH filter (0.45 µm, Merck), and then dried under reduced pressure using a centrifugal evaporator. A 1 M tetrabutylammonium fluoride/THF solution was further added to the residue, and the mixture was incubated overnight at room
35 temperature. After desalination using a NAP-25 gel filtration

column (Merck), RNA contained in the eluate was recovered by an alcohol precipitation method. This was redissolved in ultrapure water, and the target product was isolated and purified by reverse phase HPLC [system, Hitachi Chromaster (Pump 5110 and
5 others, Hitachi High-Tech Science Corporation); column, YMC Triart Bio C4 (250 mm × 10 mm I.D., YMC CO., LTD.); Solvent A, 50 mM triethylammonium acetate (pH 7.0), 5% acetonitrile; Solvent B, acetonitrile; linear gradient 0 to 20% Solvent B (0 to 20 min); flow rate: 3 mL/min; detection wavelength, 260 nm; column
10 temperature, 50°C].

[0092]

The protective group on the phosphate group of the obtained target product was removed by irradiation with 365-nm light. MAX-305 light source device (Asahi Spectra Co., Ltd.) was used for
15 photo-irradiation. An RNA solution (74 μM RNA, 1 mM Tris-HCl (pH 7.5), 0.1 mM EDTA) was added to each well of a transparent 96-well multi-well plate in an amount of 50 μL per well, and the plate was irradiated with light of 365 nm at a light quantity of 4 mW/cm² for 10 minutes. After irradiation, the RNA was
20 recovered, and isolated and purified by alcohol precipitation. All the RNAs before and after deprotection of the phosphate group were analyzed by an LC/MS system (Agilent) to confirm that the target product was obtained [molecular weight theoretical value of protected phosphate group: 20,110.4, observed value: 20,114.1
25 (+3.7): molecular weight theoretical value of deprotected phosphate group: 19,919.2, observed value: 19,921.7 (+2.5)].

[0093]

(b) Capping reaction to 5' end of branched chain

7-Methylguanosine 5'-diphosphate imidazolide sodium salt
30 (Im-m⁷GDP) was reacted with the phosphate group at the 5' end of the branched chain to obtain a capped product (see Japanese Patent Application No. 2021-111420). To 2 nmol (26 μL) of a raw material RNA containing a phosphate group at the 5' end of the branched chain, 5 μL of a 200 mM calcium chloride aqueous
35 solution was added, and the mixture was frozen with liquid

nitrogen and then freeze-dried. After 3 hours, 45 μ L of dimethyl sulfoxide (DMSO) was added to and dissolved in a mixture of dried RNA and calcium chloride, 50 μ L of an Im-m⁷GDP/DMSO solution and 5 μ L of a 200 mM 2-nitroimidazole/DMSO solution were added thereto, and the mixture was warmed at 55°C for 3 hours. 122 μ L of a 0.541 M sodium acetate (pH 5.2) aqueous solution was added, a part thereof was fractionated, and the efficiency of the capping reaction was evaluated. Each 5 pmol of the raw material and the RNA after the reaction was analyzed by 10% denaturing PAGE containing 7.5 M urea as a denaturant, and the band shift of the capped product was confirmed.

[0094]

Fig. 13 is a view showing a chemical capping reaction of a branched chain. (a) of Fig. 13 shows a schematic view of the reaction (SEQ ID NO: 33). (b) of Fig. 13 shows the analysis results in 10% denaturing PAGE of the RNA before and after the reaction (containing 7.5 M urea as a denaturant). The same molar amount of RNA (5 pmol) was analyzed from the raw material RNA before the capping reaction and the reaction solution. The gel after electrophoresis was stained by a nucleic acid staining reagent SYBR Green II, and the bands were visualized. The product of the capping reaction was observed at a higher electrophoresis position than the raw material, and the reaction yield calculated from the band intensity ratio was 78%.

[0095]

220 μ L of 2-propanol was added to the solution after adding the aqueous sodium acetate solution, and the mixture was cooled overnight at -30°C and centrifuged (20,000 g, 25 min) to obtain a pellet. This was redissolved in ultrapure water, and the target product was isolated and purified by reverse phase HPLC [system, Hitachi LaChrom Elite (Pump L-2130 and others, Hitachi High-Tech Science Corporation); column, YMC Hydrosphere C18 (250 mm $\times$ 4.6 mm I.D., YMC CO., LTD.); Solvent A, 50 mM triethylammonium acetate (pH 7.0), 5% acetonitrile; Solvent B, acetonitrile; linear gradient 0 to 20% Solvent B (0 to 20 min); flow rate: 1

mL/min; detection wavelength, 260 nm; column temperature, 50°C].
[0096]

The target product was recovered from the eluate by alcohol precipitation to obtain 1.185 nmol of a capped RNA (isolation
5 yield: 59%). The capped RNA was analyzed by an LC/MS system (Agilent), and it was confirmed that the target product was obtained [molecular weight theoretical value: 20,360.4, observed value: 20,361.2 (+0.8)].

[0097]

10 (c) Phosphorylation of 5' end of main chain using T4 polynucleotide kinase

For two types of RNA, one in which a cap structure was added to a phosphate group of a branched chain and one in which a cap structure was not added, the 5' end of the main chain was
15 enzymatically phosphorylated under the same conditions. The composition of the phosphorylation reaction is shown below. 8 µM RNA, 1 mM ATP, 50 mM Tris-HCl (pH 8.0), 10 mM magnesium chloride, 5 mM dithiothreitol, 0.2 U/µL T4 polynucleotide kinase (Takara Bio).

20 [0098]

After 50 µL of this reaction solution was incubated at 37°C for 1 hour, deproteinization was performed using a mixed solution of equal amounts of TE saturated phenol : chloroform, and RNA was recovered by alcohol precipitation. The RNA after phosphorylation
25 was analyzed by an LC/MS system (Agilent) to confirm that the target product was obtained [molecular weight theoretical value of non-capped RNA: 19,993.3, observed value: 20,002.3 (+9.0): molecular weight theoretical value of capped RNA: 20,434.4, observed value: 20,441.4 (+7.0)].

30 [0099]

(2) Synthesis of transcribed RNA

A PCR reaction product obtained using a commercially available plasmid DNA containing the coding sequence of NanoLuc luciferase and pNL1.1.TK (Promega) as a template and two
35 synthetic oligo-DNAs (5'-

CCCGGATCCTAATACGACTCACTATAGGTCTTCACACTCGAAGATTTTC-3' (SEQ ID NO:
25)/5'-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCTAGAATTACGCCAGAATGCG-3'
(SEQ ID NO: 26)) as a primer was prepared as a transcription
template. The composition of the PCR solution was as follows. 1
5 ng/ μ L pNL1.1.TK, 0.3 μ M primers, 0.2 mM dNTPs, 1.5 mM $MgSO_4$, 1 $\times$ PCR
Buffer for KOD -Plus- Neo, 0.02 U/ μ L KOD-Plus-Neo (Toyobo Co.,
Ltd.). The reaction solution was subjected to the following
thermal cycling conditions by using a T100 thermal cycler (Bio-
Rad). 94°C, 2 minutes $\rightarrow$ [98°C, 10 seconds $\rightarrow$ 55°C, 30 seconds $\rightarrow$
10 72°C, 70 seconds] $\times$ 25 cycles.
[0100]

The reaction solution (50 μ L $\times$ 8 pieces) was collected, 0.4
mL of a mixed solution of equal amounts of TE saturated phenol :
chloroform was added thereto, vigorously mixed, and centrifuged
15 (20,000 $\times$ g, 1 min) to separate an aqueous layer. 0.4 mL of
chloroform was further added, and the mixture was vigorously
mixed and centrifuged (20,000 $\times$ g, 1 min) to separate an aqueous
layer. 40 μ L of a 3 M aqueous sodium acetate solution (pH 5.2)
and 440 μ L of 2-propanol were added thereto, and the mixture was
20 cooled overnight at -30°C and then centrifuged (20,000 $\times$ g, 20 min)
to obtain DNA of a target product as a pellet. The pellet was
redissolved in 200 μ L of ultrapure water, the absorbance (A_{260}) of
the solution at 260 nm was measured using NanoDrop2000
spectrometer, and the DNA concentration of the solution was
25 calculated (A_{260} 5.632; DNA concentration: 281.6 ng/ μ L).
[0101]

A 550-base-long RNA transcript whose 5' end was
phosphorylated was prepared using the DNA obtained above as a
template and T7 RNA polymerase. The composition of the reaction
30 solution was as follows. 15 ng/ μ L template DNA, 2 mM ATP, 2 mM
UTP, 2 mM CTP, 0.5 mM GTP, 2 mM GMP, 40 mM Tris-HCl (pH 8.0), 8
mM $MgCl_2$, 2 mM spermidine, 5 mM DTT, 0.001 U/ μ L Pyrophosphatase
(New England Biolabs, M2403), 2.34 ng/ μ L T7 RNA polymerase. 1 mL
of the reaction solution was incubated at 37°C for 2 hours, 10 μ L
35 of DNase I (5 U/ μ L, Takara Bio) was then added, and the mixture

was further incubated at 37°C for 20 minutes.

[0102]

After the reaction solution was extracted with an equal amount of a mixed solution of equal amounts of saturated phenol :
5 chloroform, and deproteinization was performed by chloroform, 100 μ L of a 3 M aqueous sodium acetate solution (pH 5.2) and 1.1 mL of 2-propanol were added to the aqueous layer, and the mixture was centrifuged to obtain a crude RNA purified product as a precipitate. In order to remove nucleotides and the like
10 contained in the obtained crude RNA purified product, the RNA was further isolated and purified by reverse phase HPLC [Hitachi LaChrom Elite (Pump L-2130 and others, Hitachi High-Tech Science Corporation); column, YMC Hydrosphere C18 (250 mm $\times$ 4.6 mm I.D., YMC CO., LTD.); Solvent A, 50 mM triethylammonium acetate (pH
15 7.0), 5% acetonitrile; Solvent B, acetonitrile; linear gradient 5 to 15% Solvent B (0 to 20 min); flow rate: 1 mL/min; detection wavelength, 260 nm; column temperature, 50°C], and finally 420 μ g of the RNA transcript was obtained.

[0103]

20 (3) Circular RNA synthesis (ligase ligation, isolation)

A chemically synthesized RNA fragment whose main chain portion was composed of 54 bases and a transcriptionally synthesized RNA having a length of 550 bases were annealed in the presence of two types of template DNAs, and ligated and cyclized
25 using an enzyme T4 RNA ligase 2. The sequences of the two types of template DNAs each composed of 30 bases were as follows.

Splint_1, 5'-ACCTTAATATGCGCCTTTTTTTTTTTTTTTT-3' (SEQ ID NO: 27); Splint_2, 5'-TCGAGTGTGAAGACCATGGTGGCTTTACCC-3' (SEQ ID NO: 28).

30 [0104]

The composition of the enzyme reaction solution was as follows.

1 μ M chemically synthesized RNA, 0.5 μ M transcriptionally synthesized RNA, 2 μ M Splint DNA_1, 2 μ M Splint DNA_2, 20 (w/v)%
35 PEG6000, 0.05 μ g/ μ L T4 RNA ligase 2.

[0105]

The mixture was heated at 90°C for 3 minutes and slowly cooled to room temperature over about 20 minutes before adding PEG6000 with a stock solution dose of 60 (w/v)% each and 1 µg/µL of T4 RNA ligase 2. Thereafter, PEG6000 and ligase were added so as to have the final concentration shown above, and the mixture was incubated at 37°C for 1 hour. 0.4 mL of a mixed solution of equal amounts of TE saturated phenol : chloroform was added to 320 µL of the reaction solution, vigorously mixed, and centrifuged (20,000×g, 1 min) to separate an aqueous layer. 0.4 mL of chloroform was further added, and the mixture was vigorously mixed and centrifuged (20,000×g, 1 min) to separate an aqueous layer. 40 µL of a 3 M aqueous sodium acetate solution (pH 5.2) and 440 µL of 2-propanol were added thereto, and the mixture was cooled overnight at -30°C and then centrifuged (20,000×g, 20 min).

[0106]

The obtained pellet was dissolved in 10 µL of ultrapure water and separated by denaturing polyacrylamide gel electrophoresis (PAGE) [6% polyacrylamide (acrylamide/N,N'-methylenebisacrylamide, 19/1) containing 7.5 M urea as a denaturant and 25 (v/v)%formamide was prepared using a TBE buffer (89 mM Tris-boric acid, 2 mM EDTA)], a gel part containing a target cyclized RNA was cut out and crushed, and then ultrapure water was added thereto for extraction. RNA contained in the gel extract was recovered by an alcohol precipitation method and redissolved in ultrapure water. The RNA concentration was calculated based on the absorbance at 260 nm. The isolation yield of the circular RNA calculated based on the amount of the transcribed RNA used was 5 to 6%. The purity of the circular RNA after purification was confirmed by denaturing PAGE analysis.

[0107]

Fig. 14 is a view showing the synthesis reaction of a circular RNA. A) of Fig. 14 shows a schematic view of the reaction. Circled P represents a phosphate group at the 5' end of

the RNA strand. b) of Fig. 14 shows the analysis results of a ligase ligation reaction product in 6% denaturing PAGE (containing 7.5 M urea as a denaturant, 25 (v/v)% formamide). The gel after electrophoresis was stained by a nucleic acid staining reagent SYBR Green II, and the bands were visualized.

[0108]

(4) Translation reaction in HeLa cells

HeLa cells (RIKEN Cell Bank) were seeded at 1×10^4 cells per well of a 96-well multi-well plate the day before, and 10 ng of RNA per well was introduced into HeLa cells based on the recommended conditions of a transfection reagent (Lipofectamine MessengerMAX, Thermo). After 3 hours from transfection, the supernatant was replaced with Dulbecco's Modified Eagle Medium (Wako) medium containing 10 (w/w)% fetal bovine serum (Hyclone), and the cells were further cultured under the conditions of 37°C and 5% CO₂ for 24 hours, and then lysed. The amount of NanoLuc luciferase contained in the lysate was measured using Nano-Glo (registered trademark) Luciferase Assay System (Promega) and Mithras LB 940 plate reader (Berthold).

[0109]

Fig. 15 is a view showing evaluation of translational activity of capped branched-chain-containing circular mRNA in human-derived cultured cells (HeLa). Fig. 15 is a view showing the results of a translation reaction using a circular RNA. (a) of Fig. 15 is a schematic view showing the structures of three types of synthesized circular mRNAs (604 bases in length).

(b) of Fig. 15 is a graph showing the results of comparing the translational activity in HeLa cells of three kinds of RNAs (unbranched, branched without a cap structure, branched with a cap structure) encoding NanoLuc luciferase. HeLa cells were seeded at 1×10^4 cells per well of a 96-well multi-well plate the day before, and 10 ng of RNA per well was introduced into cells based using a transfection reagent (Lipofectamine MessengerMAX, 0.15 μ L per well). The cells were cultured for 24 hours, then lysed, and the amount of NanoLuc luciferase contained in the

lysate was evaluated using Nano-Glo (registered trademark) Luciferase Assay System (Promega).

(c) of Fig. 15 is a photograph showing the results of modified polyacrylamide electrophoresis analysis of the circular RNA used in translational activity measurement. 50 ng of RNA was heated and denatured in a loading solution containing formamide, and then electrophoresed using a 5% polyacrylamide gel containing 7.5 M urea as a denaturant. The gel after electrophoresis was stained by a nucleic acid staining reagent SYBR Green II, and the bands of the RNA were visualized.

[0110]

From these drawings, it is found that the translational activity of the cap-containing circular RNA (Branch(+) Cap(+)) is 2000 times or more higher than that of the circular RNA (Branch(-)) having neither a branched chain nor a cap.

[0111]

3. Multi-capped RNA

(1) Synthesis of multi-capped RNA

An RNA strand and a non-nucleotide chain introduction site were synthesized using a commercially available RNA amidite (TOM-protected) reagent and Trebler Phosphoramidite, Symmetric Doubler Phosphoramidite, and Spacer Phosphoroamidite 9 (all manufactured by Glen Research) with an automated nucleic acid synthesizer. At this time, the 5' end was phosphorylated on a synthesizer using a chemical phosphorylation reagent Nb-CPR (Compound 1: Formula (3)). The deprotected and purified RNA was reacted with a chemical capping reagent (Im-m⁷GDP) to introduce a cap structure of mRNA.

[0112]

Fig. 16 is a view showing the structure of a 107-base-long RNA including a synthesized branch structure. The sequence is as follows. "p" at the 5' end indicates that it is phosphorylated, and "Gm" indicates that the 2'-hydroxyl group of the G nucleotide is methylated. "X" indicated three non-nucleotide linker structures. The structure of each different 5' end vicinity site

is shown in the drawing.

5' _p _G_m _X _G_m _AGCCACCAUGGACUACAAGGACGACGACGAUAAGAUAUCGACUAUA
AAGACGACGACGAUAAACACCACCACCACCACCACUGAAAAAAAAAAAAAAAAAAAAAA_3'
[0113]

5 (2) Evaluation of translational activity

Fig. 17 is a view showing the evaluation results of the translational activity in 107-base-long HeLa cells including a synthesized branch structure/a plurality of cap structures. RNA was introduced into HeLa cells seeded on a 12-well plate at 1×10^5 cells per well the day before by a lipofection method (Lipofectamine MessengerMAX reagent), the cells were cultured for 6 hours and then lysed, and the amount of the expressed peptide contained in the lysate was quantified by ELISA (immobilized anti-FLAG antibody HRP complex using an anti-His tag antibody as an immobilized antibody and an anti-FLAG antibody HRP complex as a detection antibody). As a result, in RNA including three cap structures, about 1.4 fold improvement in translational activity was confirmed as compared with the unmodified control sequence.
[0114]

20 4. Bicistronic mRNA (linear type: Fluc-Nluc-based)

(1) Preparation of Fluc-Nluc plasmid DNA by HiFi DNA assembly

PCR was performed using the following plasmid DNA and primer, and then the template plasmid was digested with DpnI, thereby synthesizing Fluc (Firefly luciferase) DNA, Nanoluc (Nano luciferase: Nluc) DNA, and vector DNA having a homologous sequence of 20 bases at the end. 20 μ L of a reaction solution (100 ng/ μ L Fluc DNA, 100 ng/ μ L Nluc DNA, 100 ng/ μ L vector DNA, 1 $\times$ NE Builder (registered trademark) HiFi DNA Assembly Master Mix (NEW ENGLAND BioLabs), scaled-up with MQ water) was prepared, incubated at 50°C for 1 hour, and then cooled on ice. 2 μ L of the reaction solution was transformed into E. coli competent cells (ECOS (registered trademark) Competent E. coli JM109 (NIPPON GENE CO., LTD.)) and applied to an LB agar medium (containing 100 μ g/mL ampicillin sodium). After overnight culture at 37°C, colonies were picked and added to 5 mL of an LB liquid medium

(containing 50 µg/mL of ampicillin sodium). The mixture was cultured overnight at 37°C and 160 rpm, and the next day, the plasmid was extracted with Wizard (registered trademark) Plus SV Minipreps DNA Purification Systems (Promega) to obtain a target
5 plasmid DNA.

[0115]

(2) Preparation of 3×FLAG-Fluc-Nluc plasmid DNA by HiFi DNA assembly

PCR was performed using the following plasmid DNA and
10 primer to synthesize 3×FLAG DNA and pGEM-Fluc-Nluc vector having a homologous sequence of about 20 bases at the end. The PCR product was analyzed by 0.8% agarose gel electrophoresis. A target band was cut out and purified by gel extraction using Wizard (registered trademark) SV Gel and PCR Clean-Up System
15 (Promega). 10 µL of a reaction solution (20 nM 3×FLAG DNA, 4 nM pGEM-Fluc-Nluc vector, 1×NE Builder (registered trademark) HiFi DNA Assembly Master Mix, scaled-up with MQ water) was prepared, incubated at 50°C for 1 hour, and then cooled on ice. 5 µL of the reaction solution was transformed into E. coli competent cells
20 (ECOS (registered trademark) Competent E. coli JM109 (NIPPON GENE CO., LTD.)) and applied to an LB agar medium (containing 100 µg/mL ampicillin sodium). After overnight culture at 37°C, colonies were picked and added to 5 mL of an LB liquid medium (containing 50 µg/mL of ampicillin sodium). The mixture was
25 cultured overnight at 37°C and 180 rpm, and the next day, the plasmid was extracted with Wizard (registered trademark) Plus SV Minipreps DNA Purification Systems (Promega) to obtain a target plasmid DNA.

[0116]

30 (3) Synthesis of transcription template DNA of 3×FLAG-Fluc-Nluc mRNA by PCR

50 µL of a PCR reaction solution (0.3 µM Forward primer, 0.3 µM Reverse primer, 1 ng/µL 3×FLAG-Fluc-Nluc plasmid DNA, 0.2 mM dNTPs, 1.5 mM MgSO₄, 1×PCR Buffer, 0.02 U/µL KOD-plus-Neo
35 (TOYOBO)) was prepared, and a template DNA was synthesized by

PCR. The PCR product was analyzed by 0.8% agarose gel electrophoresis. A target band was cut out and purified by gel extraction using Wizard (registered trademark) SV Gel and PCR Clean-Up System (Promega).

5 [0117]

(4) Synthesis of 3×FLAG-Fluc-Nluc mRNA by in vitro transcription

A transcription reaction solution (10 ng/μL template DNA, 1×T7 RNA Polymerase buffer (Takara) (40 mM Tris-HCl (pH 8.0), 8 mM MgCl₂, 2 mM spermidine), T7 RNA polymerase (Takara) 2.5 U/μL, 5
10 mM DTT, 2 mM ATP, 2 mM UTP, 2 mM CTP, 2 mM GTP or 0.4 mM GTP and 1.6 mM ARCA) was prepared and incubated at 37°C for 1 hour. As the non-capped mRNA, 2 mM GTP was used, and as the capped mRNA, 0.4 mM GTP and 1.6 mM ARCA were added to the reaction solution and synthesized. Recombinant DNase I (TaKaRa) was added to the
15 reaction solution at 0.3 U/μL, and the mixture was incubated at 37°C for 15 minutes to decompose the template DNA. After the reaction, purification was performed by phenol/chloroform extraction and alcohol precipitation.

[0118]

20 (5) Chemical synthesis of RNA

5'-GACAGUCUGCUCGAAGCGGCCGUCUAGAAGCGTCACCTTAATATGCGCC-3'

(SEQ ID NO: 30)

RNA was synthesized with an automated nucleic acid synthesizer using Universal UnyLinker Support 2000Å CPG
25 (ChemGenes). Monophosphorylation of the 5' end was performed by synthetic amidite having a photoprotective group at the 5' end (Compound 1 described above). The synthesis of Compound 1 was performed based on the contents of "Onda, K. Master Thesis. Master thesis (Nagoya university) 2021". After the synthesis of
30 Compound 1, the CPG was transferred to a screw tube with 1.5 mL loop (SARSTEDT AG & Co. KG), 1.0 mL of 40% aq. methylamine/28% aq. ammonia (1 : 1) was added, and the mixture was incubated at 65°C for 1 hour. The mixture was allowed to cool to room temperature, then filtered using a Millex LH 0.45 μm filter
35 (Merck, Millipore), washed with sterile water (500 μL), and dried

and solidified on a centrifugal evaporator. 1.0 mL of 1 M TBAF in THF was added and dissolved, and the mixture was allowed to stand still at room temperature overnight. 1.0 mL of a 1 M Tris-HCl buffer (pH 7.5) was added, and the organic solvent was distilled
5 off with a centrifugal evaporator. Desalination was performed using a NAP-25 column (GE Healthcare). Purification of phosphorylated synthetic amidite-added RNA was performed by reverse phase HPLC. After acetonitrile and the TEAA buffer were removed with a centrifugal evaporator, the absorbance of the
10 nucleic acid aqueous solution at 260 nm was measured to determine the concentration. Conditions of HPLC are shown below. The results of HPLC are shown in Fig. 18.

Column: Triart C4 (YMC) 10×250 mm, Mobile phase: A) 50 mM TEAA buffer (pH 7.0), 5% acetonitrile, B) acetonitrile, Gradient:
15 0 to 20 min B conc. 0% to 40%, 20 to 30 min B conc. 80%, Flow rate: 3.0 mL/min

[0119]

(6) Deprotection of RNA by photo-irradiation

Approximately 100 μ M of RNA in a 96-wel plate was
20 irradiated with light at a wavelength of 365 nm at an intensity of 4000 μ W/cm² for 10 minutes using MAX-305 (Asahi Spectra Co., Ltd.). The RNA solution after photo-irradiation was subjected to purification by confirming removal of a photoprotective group by reverse phase HPLC under the same conditions as those for
25 purification of chemically synthesized RNA. After acetonitrile and the TEAA buffer were removed with a centrifugal evaporator, the absorbance of the nucleic acid aqueous solution at 260 nm was measured to determine the concentration. The results of the absorbance measurement are shown in Fig. 19(a).

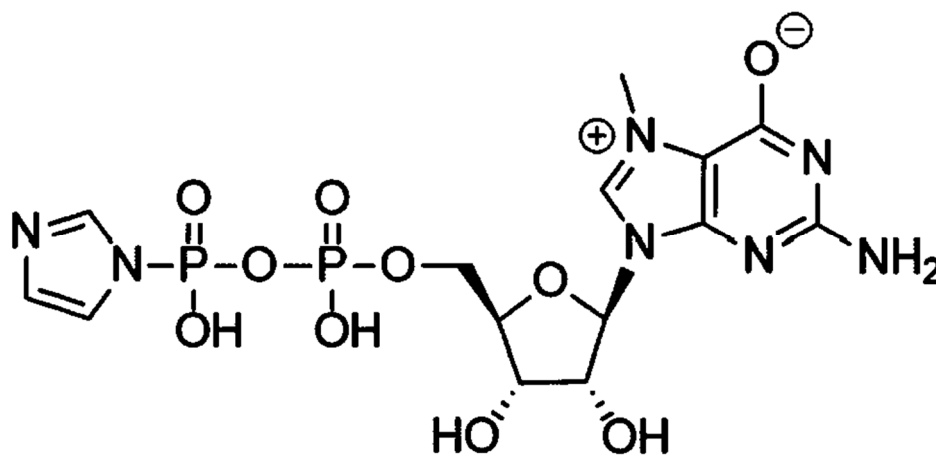
30 [0120]

(7) Chemical capping of RNA

The mixture was mixed in a 1.5 mL tube so as to have a final concentration of 10 μ M RNA and 10 mM CaCl₂, respectively, and the mixture was freeze-dried and solidified. A capping
35 reagent (structure shown in the following formula) dissolved in

DMSO was added thereto so as to have a final concentration of 10 mM and 2-nitroimidazole was added thereto so as to have a final concentration of 10 mM, and the mixture was scaled-up to 20 μ L with DMSO. This reaction solution was incubated at 55°C for 3
5 hours. The reaction solution was diluted 10 times with sterile water, then 5 pmol of RNA was mixed with 2 $\times$ formamide loading solution, and analysis was performed by 8% denaturing PAGE. The mixture was electrophoresed at a constant current of 20 W for 1.5 hours, stained by SYBR Green II, and the capping efficiency was
10 calculated from the band intensity. The reaction solution diluted with sterile water was ultrafiltered with Amicon 3K (Merck, Millipore). The absorbance of the obtained RNA solution at 260 nm was measured to determine the concentration. A photograph of a gel obtained by electrophoresis of the capped RNA is shown in
15 Fig. 19(b). The results of absorbance measurement of the capped RNA are shown in Fig. 20. As a result, the capping efficiency was 78%.

[Chemical Formula 12]



20 [0121]

(8) Annealing of mRNA and csRNA or sRNA

10 μ L of an annealing solution (0.1 μ M mRNA, 0.2 μ M csRNA or sRNA 2, 50 mM NaCl) was prepared using mRNA (3 $\times$ FLAG-Fluc-Nluc mRNA), csRNA (capped short RNA: capped RNA), and sRNA (short RNA:
25 non-capped RNA) obtained above, and heated at 80°C for 5 minutes. Thereafter, the mixture was gradually cooled until the temperature reached room temperature. The sequence of the RNA

after annealing (SEQ ID NO: 34) is shown in Fig. 21.

[0122]

(9) Transfection into HeLa cells and translation reaction

HeLa cells suspended in a 100 μ L D-MEM medium (FUJIFILM,
5 containing 10% FBS) at a concentration of 1×10^4 cells/well were
seeded in a 96-well plate the day before transfection. The next
day, the medium was replaced with a 90 μ L Opti-MEM (registered
trademark) (Thermo Fisher SCIENTIFIC), and transfected with
annealed mRNA-csRNA or mRNA-sRNA by using Lipofectamine
10 (registered trademark) MessengerMAX (registered trademark)
(Thermo Fisher SCIENTIFIC) (Lipofectamine (registered trademark)
MessengerMAX (registered trademark) 0.3 μ L, annealing solution 1
 μ L, scaled-up to 10 μ L with Opti-MEM (registered trademark)).
After incubation in an environment of 37°C and 5% CO₂ for 3.5
15 hours, the medium was replaced with 50 μ L of a 10% FBS-containing
D-MEM medium and incubated at 37°C for 3 hours. Luminescence
measurement was performed using Nano-Glo (registered trademark)
Dual-Luciferase (registered trademark) Reporter Assay System
(Promega), and translation efficiency was determined by
20 normalizing the luminescence amount of Nano luciferase with the
luminescence amount of Firefly luciferase. The results are shown
in Fig. 22. (a) of Fig. 22 shows the configuration of RNA, and
(b) of Fig. 22 shows the results of translation efficiency
corresponding thereto.

25 [0123]

In bicistronic mRNA, hybridization of the Cap-probe before
the start codon of the later coding region can induce translation
of the coding region downstream thereof. This indicates that
translation can be specifically induced in the coding region of
30 the latter half with or without a normal cap at the 5' end.

[0124]

5. Multi-uORF-mRNA (linear type: Rluc-based)

By binding a capped probe (RNA5 to RNA8 in Fig. 23(b)) to
mRNA having a plurality of coding regions (Figs. 23(a) and 24:
35 SEQ ID NO: 39), it was examined whether the translational

activity of the downstream coding region to which the probe was bound was improved. A sequence of luciferase is inserted downstream of ORF3 of mRNA having a total length of 1338 nt. A complementary probe (RNA1 to RNA8) was designed and synthesized upstream of ORF3. The translational activity was evaluated by the expression level of luciferase. Details of the experiment will be described below.

[0125]

(1) Synthesis of transcription template DNA of Rluc mRNA by PCR

10 50 μ L of a PCR reaction solution (0.3 μ M Forward primer, 0.3 μ M Reverse primer, 1 ng/ μ L Rluc plasmid DNA, 0.2 mM dNTPs, 1.5 mM $MgSO_4$, 1 $\times$ PCR Buffer, 0.02 U/ μ L KOD-plus-Neo (TOYOBO)) was prepared, and a template DNA was synthesized by PCR. The PCR product was analyzed by 1% agarose gel electrophoresis. A target band was cut out and purified by gel extraction using Wizard (registered trademark) SV Gel and PCR Clean-Up System (Promega).

[0126]

(2) Synthesis of Rluc mRNA by in vitro transcription

20 A transcription reaction solution (10 ng/ μ L template DNA, 1 $\times$ T7 RNA Polymerase buffer (Takara) (40 mM Tris-HCl (pH 8.0), 8 mM $MgCl_2$, 2 mM spermidine), 2.5 U/ μ L T7 RNA polymerase (Takara), 5 mM DTT, 2 mM ATP, 2 mM UTP, 2 mM CTP, 2 mM GTP or 0.4 mM GTP and 1.6 mM ARCA) was prepared and incubated at 37°C for 1 hour.

25 Recombinant DNase I (TaKaRa) was added to the reaction solution at 0.3 U/ μ L, and the mixture was incubated at 37°C for 15 minutes to decompose the template DNA. After the reaction, purification was performed by phenol/chloroform extraction, alcohol precipitation, and reverse phase HPLC. Conditions of HPLC are as follows. The results of HPLC preparative post-analysis are shown in Fig. 25.

30 Column: Hydrosphere C18 (HS12S05-2546WT, 250 $\times$ 4.6 mm I.D. S-5 μ m, 12 nm), Mobile phase: A) 50 mM TEAA buffer (pH 7.0), 5% acetonitrile, B) acetonitrile, Gradient: 0 to 20 min B conc. 0% to 20%, Flow rate: 1.0 mL/min, Column temperature: 50°C

35 [0127]

(3) Chemical synthesis of RNA

RNA was synthesized with an automated nucleic acid synthesizer using CPG and amidite shown below. After the synthesis, the CPG was transferred to a screw tube with 1.5 mL loop (SARSTEDT AG & Co. KG), 1.0 mL of 40% methylamine aq./28% ammonia aq. (1 : 1) was added, and the mixture was incubated at 65°C for 30 minutes. The mixture was allowed to cool to room temperature, then filtered using a Millex LH 0.45 µm filter (Merck, Millipore), washed with sterile water (500 µL), and dried and solidified on a centrifugal evaporator. 1.0 mL of 1 M TBAF in THF was added and dissolved, and the mixture was allowed to stand still at room temperature overnight. 1.0 mL of a 1 M Tris-HCl buffer (pH 7.5) was added, and the organic solvent was distilled off with a centrifugal evaporator. Desalination was performed using a NAP-25 column (GE Healthcare), and then purification was performed by reverse phase HPLC. The results of HPLC preparative post-analysis are shown in Fig. 26, and the results of LC-MS are shown in the following table.

<CPG>

_____ 3'-TOM ribo Adenosine (n-acetyl) 2'-lcaa CPG 1000Å (N-3201-10, ChemGenes)/2'-TOM ribo Cytidine (n-acetyl) 3'-lcaa CPG 1000Å (N-3202-10, ChemGenes)/2'-TOM ribo Guanosine (n-acetyl) CPG 1000Å (N-3203-10, ChemGenes)/2'-TOM ribo Uridine 2'-lcaa CPG 1000Å (N-3205-10, ChemGenes)

25 <Amidite>

_____ 5'-DMT- 2'-TOM ribo Adenosine (ANP-3201, ChemGenes)/2'-TOM ribo Cytidine (ANP-3202)/2'-TOM ribo Guanosine (ANP-3203, ChemGenes)/2'-TOM ribo Uridine (ANP-3205, ChemGenes)/5'-Phosphate-ON Reagent (CLP-1544, ChemGenes)

30 <Conditions of HPLC>

_____ Column: Hydrosphere C18 (HS12S05-2546WT, 250 × 4.6 mm I.D. S-5 µm, 12 nm), Mobile phase: A) 50 mM TEAA buffer (pH 7.0), 5% acetonitrile, B) acetonitrile, Gradient: 0 to 20 min B conc. 0% to 30%, Flow rate: 1.0 mL/min, Column temperature: room

35 temperature

[Table 3]

RNA5 (M.W.)	RNA6 (M.W.)	RNA7 (M.W.)	RNA8 (M.W.)
Calcd. 7465.4855	Calcd. 7466.4695	Calcd. 7422.3535	Calcd. 7438.3525
Obs. 7466.49	Obs. 7467.48	Obs. 7421.77	Obs. 7437.76

[0128]

(4) Chemical capping of RNA

5 The mixture was mixed in a 1.5 mL tube so as to have a
final concentration of 10 μ M RNA and 10 mM CaCl_2 , respectively,
and the mixture was freeze-dried and solidified. A capping
reagent (Chemical Formula 10 above) dissolved in DMSO was added
thereto so as to have a final concentration of 10 mM and 2-
10 nitroimidazole was added thereto so as to have a final
concentration of 10 mM, and the mixture was scaled-up to 50 μ L
with DMSO. This reaction solution was incubated at 55°C for 3
hours. After the reaction, purification was performed by Amicon
Ultra (0.5 mL, 3K, Merck Millipore) and alcohol precipitation,
15 analysis was performed by 20% denaturing PAGE, and the capping
efficiency was calculated from the band intensity. The results
are shown in Fig. 26.

[0129]

(5) Annealing of mRNA and csRNA or sRNA

20 10 μ L of an annealing solution (0.1 μ M mRNA, 1.0 μ M csRNA
or sRNA, 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 10 mM EDTA (pH
8.0)) was prepared using mRNA (Rluc mRNA), csRNA (capped short
RNA: capped RNA), and sRNA (short RNA: non-capped RNA) obtained
above, and heated at 80°C for 5 minutes. Thereafter, the mixture
25 was gradually cooled until the temperature reached room
temperature.

[0130]

(6) Transfection into HeLa cells and translation reaction

30 HeLa cells suspended in a 100 μ L D-MEM medium (FUJIFILM,
containing 10% FBS) at a concentration of 1×10^4 cells/well were
seeded in a 96-well plate the day before transfection. The next
day, the medium was replaced with a 90 μ L Opti-MEM (registered
trademark) (Thermo Fisher SCIENTIFIC), and transfected with

annealed mRNA-csRNA or mRNA-sRNA by using Lipofectamine
(registered trademark) MessengerMAX (registered trademark)
(Thermo Fisher SCIENTIFIC) (Lipofectamine (registered trademark)
MessengerMAX (registered trademark) 0.3 μ L, annealing solution 1
5 μ L, scaled-up to 10 μ L with Opti-MEM (registered trademark)).
After incubation in an environment of 37°C and 5% CO₂ for 3 hours,
the medium was replaced with 50 μ L of a 10% FBS-containing D-MEM
medium and incubated at 37°C for 3 hours. Luminescence
measurement was performed using Renilla Luciferase Assay System
10 (Promega). The results are shown in Fig. 28. From mRNA having no
cap structure ((a) of Fig. 28), it is found that all of RNA5 to
RNA8 have higher luminescence intensity, that is, higher
translational activity in the capped RNA (csRNA) than in the non-
capped RNA (sRNA). In both of the mRNA having no cap structure
15 ((a) of Fig. 28) and the mRNA having a cap structure ((b) of Fig.
28), the translational activity was improved particularly when
the cap probe 7 or 8 was bound.

[0131]

6. Comparison between capped circular RNA and
20 encephalomyocarditis virus (EMCV) IRES
(1) Preparation of plasmid containing IRES

First, 1 μ L of plasmid DNA containing 10 ng/ μ L of IRES was
prepared and placed in a 1.5 mL tube. 10 μ L of ECOS JM109
competent cells were placed thereto, and transformation was
25 performed according to the ECOS 6-minute protocol. Under an
alcohol lamp, the transformed sample was applied to an LB/agar
medium (containing 100 μ L/mL of ampicillin) and incubated in an
incubator set at 37°C overnight. After 15 hours, it was confirmed
that colonies were formed, and a single colony was added to 10 mL
30 of an LB/ampicillin liquid medium. Then, the mixture was shaken
overnight at 37°C. Thereafter, plasmid extraction was performed
using Wizard Plus SV Minipreps DNA Purification system. The
concentration of the plasmid was measured at an absorbance at 260
nm.

35 [0132]

(2) IRES fragment purification of purified plasmid with restriction enzyme

To 500 ng of the purified plasmid DNA containing IRES, 1 μ L of 10 $\times$ K buffer, 0.5 μ L of EcoRI (Takara), and 0.5 μ L of BamHI (Takara) were added, and MQ water was added so that the total amount was 10 μ L. The sample was incubated at 37°C for 1 hour, and then it was confirmed that restriction enzyme treatment could be performed by 1% agarose electrophoresis (100 V, 30 min). Since the 645 bp band is a sequence fragment containing IRES, the band was isolated and purified by Wizard Plus SV Minipreps DNA Purification system.

[0133]

(3) Preparation of fragment containing NanoLuc luciferase

To 10 ng of plasmid DNA in which 5'UTR of pNL1.1TK was shortened by 50 nt, 10 μ M to 25 pmol of each of a forward primer and a reverse primer was added. This sample was added to a PCR reaction solution (10 $\times$ buffer 5 μ L, 25 mM MgSO₄ 3 μ L, 2 mM dNTPs 5 μ L, KOD plus neo 1 μ L) to prepare a sample having a total amount of 50 μ L. Then, the adjusted sample was subjected to PCR under the conditions of 94°C for 2 minutes $\rightarrow$ (98°C for 10 seconds, 55°C for 30 seconds, 68°C for 3 minutes) $\times$ 25 $\rightarrow$ 68°C for 3 minutes $\rightarrow$ 4°C. The results were confirmed by 1% agarose electrophoresis (100 V, 30 min). To 48 μ L of the PCR reaction solution, 0.5 μ L of DpnI as a restriction enzyme was added in order to digest the plasmid before the reaction, and the mixture was incubated at 37°C for 1 hour. The PCR reaction solution was purified by a Wizard Plus SV Minipreps DNA Purification system.

[0134]

(4) In-fusion reaction

The IRES fragment and NanoLuc luciferase fragment obtained above were each measured at an absorbance at 260 nm. Then, 2.5 μ L of IRES fragment (13.5 ng/ μ L), 1.5 μ L of a linear vector containing NanoLuc luciferase (31.1 ng/ μ L), and 1 μ L of 5 $\times$ In-fusion HD Enzyme Premix (Takara) were mixed to have a total amount of 5 μ L. As a negative control sample, a sample was also

prepared in which 1.5 μ L of a linear vector containing NanoLuc luciferase (31.1 ng/ μ L) and 1 μ L of 5×In-fusion HD Enzyme Premix (Takara) were mixed, and the total amount was 5 μ L with MQ water. Then, the adjusted sample was incubated at 50°C for 15 minutes.

5 The incubated sample was subjected to the ECOS 6-minute protocol again, and then 50 μ L of ECOS JM109 competent cells were added thereto. This sample was applied to an LB/agar medium (containing 100 μ L/mL of ampicillin) under an alcohol lamp and incubated in an incubator set at 37°C overnight. After 15 hours, it was
10 confirmed that colonies were formed, and a single colony was added to 10 mL of an LB/ampicillin liquid medium. Then, the mixture was shaken overnight at 37°C. Thereafter, plasmid extraction was performed using Wizard Plus SV Minipreps DNA Purification system. The concentration of the plasmid was
15 measured at an absorbance at 260 nm.

[0135]

(5) Amplification of NanoLuc luciferase region from plasmid DNA

A PCR reaction solution (10×buffer 5 μ L, 25 mM MgSO₄ 3 μ L, 2 mM dNTPs 5 μ L, forward primer 10 μ M 1.5 μ L, reverse primer 10 μ M
20 1.5 μ L, plasmid DNA 0.37 μ L, KOD plus neo 1 μ L) was prepared so as to be 50 μ L. Then, the adjusted sample was subjected to PCR under the conditions of 94°C for 2 minutes → (98°C for 10 seconds, 55°C for 30 seconds, 72°C for 90 seconds) × 25 → 72°C for 3 minutes → 4°C. From the plasmid extracted by the above
25 operation, the required NanoLuc luciferase region and IRES sequence were cut out by PCR. The sequence of the excised DNA fragment (SEQ ID NO: 40) is shown in Fig. 29. The product amplified by PCR was electrophoresed on an agarose gel (1.0%, 1×TAE, 100 V, 30 min). The PCR product was purified by a Wizard
30 SV gel and PCR Clean-up System after PCR.

[0136]

(6) IVT (in vitro transcription) of purified DNA

The DNA (3'fragment, full length) purified above was subjected to IVT reaction. 600 μ L of a transcription reaction
35 solution (4000 ng of each purified DNA, 10×buffer 60 μ L, 50 mM

DTT 60 μ L, 20 mM ATP, CTP, UTP, GMP 60 μ L, 20 mM GTP 15 μ L, T7 RNA polymerase (Takara) 30 μ L, scaled-up with MQ water) was prepared and incubated at 37°C for 2 hours. Thereafter, this sample was electrophoresed by 5% denaturing PAGE (7.5 M urea, 1×TBE buffer, 26 mA, 60 min). Thereafter, the sample after electrophoresis was stained for 20 minutes using a stain prepared with 3 μ L of SYBR Green II and 30 mL of MQ water. Denaturing PAGE was photographed using ChemiDoc to confirm an IVT product.

[0137]

10 (7) Purification of IVT product

To 600 μ L of a 3'fragment and full-length IVT product, 20 μ L of DNase I (Takara) was added, and the mixture was incubated at 37°C for 15 minutes. Thereby, DNA remaining in the IVT reaction was removed. Next, 600 μ L of neutral phenol and 600 μ L of chloroform were added, and the mixture was centrifuged with a centrifuge at 14500 rpm for 15 seconds. Thereafter, 600 μ L of chloroform was added thereto, and centrifugation was performed in the same manner with a centrifuge. Thereby, proteins including an enzyme were removed. Next, centrifugation was performed at room temperature for 5 minutes using a centrifugal evaporator. Thereby, chloroform not suitable for Amicon was removed. Next, Amicon 10K was used. Thereby, low-molecular-weight one was removed. Finally, 100 μ L of isopropanol and 20 μ L of a 3 M aqueous sodium acetate solution (pH 5.2) were added to 100 μ L of the obtained mixture using Amicon, and the mixture was allowed to stand still at -30°C for 1 hour. Thereafter, centrifugation was performed with a centrifuge under the conditions of 14500 rpm, room temperature, and 30 minutes. The supernatant was removed so that the pellet obtained by centrifugation was not sucked, and 1 mL of 80% ethanol was then added and washed. After centrifugation for 2 minutes under the same conditions, the supernatant was removed and allowed to stand still for 5 minutes. Finally, it was dissolved by centrifugation with 200 μ L of MQ water three times to purify 3'fragment and full-length RNA.

35 [0138]

(8) HPLC purification of IVT product

As described above, the purification operation of the sample was performed after the IVT reaction, but double-stranded RNA, triphosphoric acid, and the like were not removed in this operation. These components produce effects such as a decrease in yield and translational activity when a ligation reaction or a translation reaction is verified in cells, and therefore, they were removed by HPLC. HITACHI HPLC system was used. The conditions were as follows: Hydrosphere C18: Solvent A, 50 mM TEAA (pH 7.0), 5% ACN; Solvent B, ACN; Gradient: 0 to 20% B/0 to 20 min; Flow rate, 1 mL/min; Column temperature, 50°C; Column size: 250 × 4.6 mm I.D, S-5 µL, 12 nm; detection wavelength, 260 nm. After fractionation by HPLC, acetonitrile was removed by concentration under reduced pressure. Then, after alcohol precipitation, the absorbance at 260 nm was measured and used for future experiments.

[0139]

(9) Isolation and purification of circular mRNA containing IRES

The target RNA was subjected to cyclization reaction under optimized conditions. The cyclization reaction was performed using 1 µM of a guide strand, 0.4 µM of 3'fragment, 0.4 µM of RNA, 10×T4 RNA ligase 2 buffer, 60% PEG, and 0.025 µg/µL of T4 RNA ligase 2. After electrophoresis with 6% dPAGE (7.5 µM urea, 1×TBE buffer, 30 mA, 180 min), bands thought to be a circular mRNA were cut with a cutter and placed in a 1.5 mL tube under ultraviolet radiation on a TLC plate. Thereafter, a total of 500 µL of MQ water was added to the tube and shaken with a shaker overnight. Then, after centrifugation at 14500 rpm for 1 minute at room temperature, the supernatant was recovered, 400 µL of MQ water was added to the remaining gel, and the same centrifugation was performed to recover the supernatant. Subsequently, the supernatant was filtered in two portions using Millex LCR 13 mm. Then, the sample after filtration was subjected to purification operation of circular mRNA by isopropanol precipitation. The concentration of the purified circular mRNA was measured at an

absorbance of 260 nm to prepare a sample for cell experiment.

[0140]

(10) Confirmation of circular mRNA by ribonuclease

To 10 ng of the circular mRNA purified by the above
5 isolation and purification operation, RNase R (Epicenter, 20
U/ μ L) was added so as to be 1 U/ μ L, and the mixture was scaled-up
to 5 μ L with MQ water and then incubated at 37°C for 15 minutes.
Then, the circular mRNA was purified by phenol/chloroform
extraction and isopropanol precipitation. Thereafter, the
10 resultant product was electrophoresed by 5% dPAGE (7.5 μ M urea,
1×TBE buffer, 30 mA, 240 min). The gel after electrophoresis was
stained by SYBR Green II for 30 minutes, and the electrophoresis
result was confirmed with ChemiDoc.

[0141]

15 (11) Seeding of human cultured cells

Human cultured cells (HeLa) cultured in the dish were
washed twice with 3 mL of PBS (Phosphate buffered saline, nacalai
tesque), and 1 mL of a 0.05% trypsin solution was added to
degrade the cells, then the cells were incubated for 1 minute.
20 Thereafter, 3 mL of the medium was added to stop tryptic
degradation. The sample was centrifuged (Multipurpose
refrigerated centrifuge EX-126, 200G, 3 min), the supernatant was
removed, then the sample was resuspended in a medium, and cell
counting was performed using 20 μ L of the supernatant.
25 Thereafter, the cells were adjusted to 10×10^4 cells with a medium,
and then 100 μ L each was added thereto to make 10×10^3 cells per
well, and then incubated for 24 hours under the conditions of
37°C and 5% CO₂.

[0142]

30 (12) Transfection of circular mRNA into human cultured cells

First, an experiment was performed in which a sample whose
translation amount was measured in human cultured cells was 5 ng
per well and 3 wells per sample. After removing the medium from
the incubated sample, the sample was washed once with 150 μ L of
35 PBS (Phosphate buffered saline, nacalai tesque). Cells were added

to a multi-well plate for cell culture (96 well). Then, human cultured cells were cultured in a multi-well plate for cell culture (D-MEM, high glucose, containing phenol red, 37°C, 5% CO₂). Lipofectamine Messenger MAX transfection reagent

5 (Invitrogen, 0.3 µL/well) and Opti-MEM (10 µL/well) were mixed with the cultured sample. 33 µL each of the mixed solution was added to the sample RNA. 89 µL each of Opti-MEM was added to each well, and then 11.3 µL each of the prepared RNA/Lipo reagent was added. After incubation under the conditions of 37°C and 5% CO₂
10 for 3 hours, the supernatant was removed, and the medium was replaced with D-MEM.

[0143]

(13) Luminescence measurement of circular mRNA in human cultured cells

15 Luminescence measurement of human cultured cells was performed 24 hours after the transfection. The medium was sucked from a multi-well plate for cell culture (96 well), and then washed once with 150 µL of PBS. Luminescence measurement of the sample after washing was performed using Nano Glo luciferase
20 Assay System (Promega). First, 20 µL of 1×Glo Lysis Buffer per well was added. This was incubated at room temperature for 5 minutes, and the lysate of each cell was added to a 96-well white plate in an amount of 2 µL per well. To each well, 0.1 µL of Nano Glo luciferase assay substrate and 10 µL of Nano-Glo luciferase
25 assay buffer were added. After the reagent was added, the measurement was performed with a plate reader (Comparative Example). The results are shown in Fig. 30.

An evaluation experiment using the above human cultured cells was also performed using the cap-containing circular RNA
30 (Branch(+) Cap(+)) in the above "2. Cap-containing circular RNA (branched chain type)". The results are also shown in the drawing. From this drawing, it was found that the expression level of luciferase in the cap-containing circular RNA was 10 times that in the method using the existing IRES.

35 [0144]

6. Synthesis of circular mRNA composed of two fragments of chemically synthesized RNA fragment (54 bases in length) and transcriptionally synthesized RNA fragment (550 bases in length) (Figs. 31 to 33)

5 An oligoribonucleotide having a 54-base-long main chain and containing a branched chain phosphorylated at the 5' end was synthesized according to a conventional method with an automated nucleic acid synthesizer (NR-2A 7MX or NRs-4A10R7NP, NIHON TECHNO SERVICE CO., LTD.) using a photodegradable protective group-
10 containing chemical phosphorylation reagent (Japanese Patent Application No. 2021-111420), a commercially available phosphoramidite reagent [product from ChemGenes (ANP-3201, ANP-3202, ANP-3203, ANP-3205) for RNA strand synthesis, Asymmetric Doubler (Lev) Phosphoramidite (10-1981) purchased from Glen
15 Research for branched chain introduction], and a solid phase carrier (3'-TOM-ribo Uridine CPG 1000A, ChemGenes). In order to synthesize a circular RNA not containing a branched chain, a 54-base-long RNA sequence (SEQ ID NO: 43) composed only of a main chain was synthesized. After completion of the synthesis, 1 mL of
20 a mixed solution of equal amounts of a 40% methylamine aqueous solution and concentrated ammonia water was added to the solid phase carrier, and heated at 65°C for 20 minutes to performed cutting-out from the solid phase single substance and deprotection of the nucleobase moiety. The supernatant was
25 filtered through a Millex LH filter (0.45 µm, Merck), and then dried under reduced pressure using a centrifugal evaporator. A 1 M tetrabutylammonium fluoride/THF solution was further added to the residue, and the mixture was incubated overnight at room temperature. After desalination using a NAP-25 gel filtration
30 column (Merck), RNA contained in the eluate was recovered by an alcohol precipitation method. This was redissolved in ultrapure water, and the target product was isolated and purified by reverse phase HPLC.

 [Hitachi LaChrom Elite or Chromaster system (Hitachi High-Tech
35 Science Corporation); column, YMC Triart Bio C4 (250 mm × 10 mm

I.D., YMC CO., LTD.); Solvent A, 50 mM triethylammonium acetate (pH 7.0), 5% acetonitrile; Solvent B, acetonitrile; linear gradient 0 to 20% Solvent B (0 to 20 min); flow rate: 3 mL/min; detection wavelength, 260 nm; column temperature, 50°C].

5 [0145]

The protective group on the phosphate group of the obtained target product was removed by irradiation with 365-nm light. MAX-305 light source device (Asahi Spectra Co., Ltd.) was used for photo-irradiation. An RNA solution was added to each well of a
10 transparent 96-well multi-well plate in an amount of 50 μ L per well, and the plate was irradiated with light of 365 nm at a light quantity of 4 mW/cm² for 10 minutes. After irradiation, the RNA was recovered, and isolated and purified by alcohol precipitation. All the RNAs before and after deprotection of the
15 phosphate group were analyzed by an LC/MS system (Agilent), and it was confirmed that the target product was obtained. Then, 7-methylguanosine 5'-diphosphate imidazolidine sodium salt (Im-m⁷GDP) was reacted with the phosphate group at the 5' end of the branched chain to obtain a capped RNA strand (WO2021172204A1). To
20 2 nmol (26 μ L) of a raw material RNA containing a phosphate group at the 5' end of the branched chain, 5 μ L of a 200 mM calcium chloride aqueous solution was added, and the mixture was frozen with liquid nitrogen and then freeze-dried. After 3 hours, 45 μ L of dimethyl sulfoxide (DMSO) was added to and dissolved in a
25 mixture of dried RNA and calcium chloride, 50 μ L of an Im-m⁷GDP/DMSO solution and 5 μ L of a 200 mM 2-nitroimidazole/DMSO solution were added thereto, and the mixture was warmed at 55°C for 3 hours. 122 μ L of a 0.541 M sodium acetate (pH 5.2) aqueous solution was added, a part thereof was fractionated, and the
30 efficiency of the capping reaction was evaluated. Each 5 pmol of the raw material and the RNA after the reaction was analyzed by 10% denaturing PAGE containing 7.5 M urea as a denaturant, and the band shift of the capped product was confirmed. 220 μ L of 2-propanol was added to the solution after adding the aqueous
35 sodium acetate solution, and the mixture was cooled overnight at

-30°C and centrifuged (20,000 g, 25 min) to obtain a pellet. This was redissolved in ultrapure water, and the target product was isolated and purified by reverse phase HPLC.

[Hitachi LaChrom Elite or Chromaster system (Hitachi High-Tech Science Corporation); column, YMC Hydrosphere C18 (250 mm × 4.6 mm I.D., YMC CO., LTD.); Solvent A, 50 mM triethylammonium acetate (pH 7.0), 5% acetonitrile; Solvent B, acetonitrile; linear gradient 0 to 20% Solvent B (0 to 20 min); flow rate: 1 mL/min; detection wavelength, 260 nm; column temperature, 50°C].

10 [0146]

The target product was recovered from the eluate by alcohol precipitation to obtain a capped RNA. As described above, the cap structure was introduced into the branched chain, and then the 5' end of the main chain RNA was phosphorylated using an enzyme T4 polynucleotide kinase. The composition of the phosphorylation reaction is shown below. 8 µM RNA, 1 mM ATP, 50 mM Tris-HCl (pH 8.0), 10 mM magnesium chloride, 5 mM dithiothreitol, 0.2 U/µL T4 polynucleotide kinase (Takara Bio). After this reaction was incubated at 37°C for 1 hour, deproteinization was performed using a mixed solution of equal amounts of TE saturated phenol : chloroform, and RNA was recovered by alcohol precipitation.

[0147]

The PCR reaction was performed by using a commercially available plasmid DNA containing the coding sequence of NanoLuc luciferase (Nluc) and pNL1.1.TK (Promega) as a template and two synthetic oligo-DNAs (5'-
25 CCCGGATCCTAATACGACTCACTATAGGTCTTCACACTCGAAGATTTTC-3' (SEQ ID NO: 25)/5'-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCTAGAATTACGCCAGAATGCG-3' (SEQ ID NO: 26)) as a primer. The composition of the reaction solution was as follows. 1 ng/µL pNL1.1. TK, 0.3 µM oligo DNA, 0.2 mM dNTPs, 1.5 mM MgSO₄, 1×PCR Buffer for KOD-Plus-Neo, 0.02 U/µL KOD -Plus- Neo (Toyobo Co., Ltd.). The reaction solution was subjected to the following thermal cycling conditions by using a T100 thermal cycler (Bio-Rad). 94°C, 2 minutes → [98°C, 10
30 seconds → 55°C, 30 seconds → 72°C, 70 seconds] × 25 cycles. The
35

reaction solution (50 μ L $\times$ 8 pieces) was collected, 0.4 mL of a mixed solution of equal amounts of TE saturated phenol : chloroform was added thereto, vigorously mixed, and centrifuged (20,000 $\times$ g, 1 min) to separate an aqueous layer. 0.4 mL of
5 chloroform was further added, and the mixture was vigorously mixed and centrifuged (20,000 $\times$ g, 1 min) to separate an aqueous layer. 40 μ L of a 3 M aqueous sodium acetate solution (pH 5.2) and 440 μ L of 2-propanol were added thereto, and the mixture was cooled overnight at -30°C and then centrifuged (20,000 $\times$ g, 20 min)
10 to obtain DNA of a target product as a pellet. A 550-base-long RNA transcript whose 5' end was phosphorylated was prepared using the DNA as a template and T7 RNA polymerase. The composition of the reaction solution was as follows.

15 15 ng/ μ L template DNA, 2 mM ATP, 2 mM UTP, 2 mM CTP, 0.5 mM GTP, 2 mM GMP, 40 mM Tris-HCl (pH 8.0), 8 mM MgCl₂, 2 mM spermidine, 5 mM DTT, 0.001 U/ μ L Pyrophosphatase (New England Biolabs, M2403), 2.34 ng/ μ L T7 RNA polymerase.

1 mL of the reaction solution was incubated at 37°C for 2 hours, 10 μ L of DNase I (5 U/ μ L, Takara Bio) was then added, and
20 the mixture was further incubated at 37°C for 20 minutes. After the reaction solution was extracted with an equal amount of a mixed solution of equal amounts of saturated phenol : chloroform, and deproteinization was performed by chloroform, a 1/10 amount of a 3 M aqueous sodium acetate solution (pH 5.2) and an equal
25 amount of 2-propanol were added to the aqueous layer, and the mixture was centrifuged to obtain a crude RNA purified product as a precipitate. The obtained RNA transcript was isolated and purified by reverse phase HPLC [Hitachi LaChrom Elite or Chromaster system (Hitachi High-Tech Science Corporation);
30 column, YMC Hydrosphere C18 (250 mm $\times$ 4.6 mm I.D., YMC CO., LTD.); Solvent A, 50 mM triethylammonium acetate (pH 7.0), 5% acetonitrile; Solvent B, acetonitrile; linear gradient 5 to 15% Solvent B (0 to 20 min); flow rate: 1 mL/min; detection wavelength, 260 nm; column temperature, 50°C], and finally 420 μ g
35 of the RNA transcript was obtained.

[0148]

A chemically synthesized RNA fragment whose main chain portion was composed of 54 bases and a transcriptionally synthesized RNA having a length of 550 bases were annealed in the presence of two types of template DNAs, and ligated and cyclized using an enzyme T4 RNA ligase 2. The sequences of the two types of template DNAs each composed of 30 bases were as follows.

Splint_1, 5'-ACCTTAATATGCGCCTTTTTTTTTTTTTTTT-3' (SEQ ID NO: 27);

Splint_2, 5'-TCGAGTGTGAAGACCATGGTGGCTTTACCC-3' (SEQ ID NO: 28).

The composition of the enzyme reaction solution was as follows. 1 μ M chemically synthesized RNA, 0.5 μ M transcriptionally synthesized RNA, 2 μ M Splint DNA_1, 2 μ M Splint DNA_2, 50 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 1 mM DTT, 400 μ M ATP, 10 (w/v)% PEG6000, 0.05 μ g/ μ L T4 RNA ligase 2. The mixture was heated at 90°C for 3 minutes and slowly cooled to room temperature over about 20 minutes before adding PEG6000 with a stock solution dose of 60 (w/v)% each and 1 μ g/ μ L of T4 RNA ligase 2. Thereafter, PEG6000 and ligase were added so as to have the final concentration shown above, and the mixture was incubated at 37°C for 1 hour. An equal amount of a mixed solution of equal amounts of TE saturated phenol : chloroform was added to the reaction solution, vigorously mixed, and centrifuged (20,000 $\times$ g, 1 min) to separate an aqueous layer. An equal amount of chloroform was further added, and the mixture was vigorously mixed and centrifuged (20,000 $\times$ g, 1 min) to separate an aqueous layer. A 1/10 amount of a 3 M aqueous sodium acetate solution (pH 5.2) in the aqueous layer and an equal amount of 2-propanol were added thereto, and the mixture was cooled at -30°C for 1 hour or more and then centrifuged (20,000 $\times$ g, 20 mi) to recover RNA. The obtained pellet was dissolved in a small amount of ultrapure water and separated by denaturing polyacrylamide gel electrophoresis (PAGE) [6% polyacrylamide (acrylamide/N,N'-methylenebisacrylamide, 19/1) containing 7.5 M urea as a denaturant and 25 (v/v)%formamide was prepared using a TBE buffer (89 mM Tris-boric acid, 2 mM EDTA)]. A gel part containing a

target circular mRNA was detected by a UV shadowing method and cut out, and a gel piece was crushed, and then a 50 mM aqueous sodium acetate solution (pH 5.2) was added to extract RNA from the gel. RNA contained in the extract was recovered by an alcohol precipitation method and redissolved in ultrapure water. The RNA concentration was calculated based on the absorbance at 260 nm. The purity of the circular RNA after isolation and purification was confirmed by denaturing PAGE analysis (> 90%).

[0149]

10 7. Synthesis of 5'-end capped linear mRNA composed of two fragments of chemically synthesized RNA fragment (54 bases in length) and transcriptionally synthesized RNA fragment (550 bases in length) (Fig. 33)

A 5' end phosphorylated chemically synthesized RNA oligomer
15 having a 54-base-long main chain and not containing a branched chain was synthesized using an automated nucleic acid synthesizer according to a conventional method, similarly to the chemical synthesis containing a branched chain. A chemical capping reagent 7-methylguanosine 5'-diphosphate imidazolide sodium salt (Im-
20 m⁷GDP) was reacted with the phosphate group at the 5' end thereof to obtain a capped RNA strand (WO2021172204A1). To 2 nmol (26 µL) of the 5' end phosphorylated RNA, 5 µL of a 200 mM calcium chloride aqueous solution was added, and the mixture was frozen with liquid nitrogen and then freeze-dried. After 3 hours, 45 µL
25 of dimethyl sulfoxide (DMSO) was added to and dissolved in a mixture of dried RNA and calcium chloride, 50 µL of an Im-m⁷GDP/DMSO solution and 5 µL of a 200 mM 2-nitroimidazole/DMSO solution were added thereto, and the mixture was warmed at 55°C for 3 hours. 122 µL of a 0.541 M sodium acetate (pH 5.2) aqueous
30 solution was added, a part thereof was fractionated, and the efficiency of the capping reaction was evaluated. Each 5 pmol of the raw material and the RNA after the reaction was analyzed by 10% denaturing PAGE containing 7.5 M urea as a denaturant, and the band shift of the capped product was confirmed. 220 µL of 2-
35 propanol was added to the solution after adding the aqueous

sodium acetate solution, and the mixture was cooled overnight at -30°C and centrifuged (20,000 g, 25 min) to obtain a pellet. This was redissolved in ultrapure water, and the target product was isolated and purified by reverse phase HPLC. [Hitachi LaChrom
5 Elite or Chromaster system (Hitachi High-Tech Science Corporation); column, YMC Hydrosphere C18 (250 mm × 4.6 mm I.D., YMC CO., LTD.); Solvent A, 50 mM triethylammonium acetate (pH 7.0), 5% acetonitrile; Solvent B, acetonitrile; linear gradient 0 to 20% Solvent B (0 to 20 min); flow rate: 1 mL/min; detection
10 wavelength, 260 nm; column temperature, 50°C].

The target product was recovered from the eluate by alcohol precipitation to obtain a capped RNA. The obtained capped RNA was ligated with T4 RNA ligase 2 in the presence of only Splint DNA_2 and the same transcribed RNA as that used in the synthesis of the
15 above-described branched chain-containing circular mRNA. The composition of the enzyme reaction solution was as follows. 1 µM chemically synthesized 5'-capped RNA, 0.5 µM transcriptionally synthesized RNA, 2 µM Splint DNA_2, 50 mM Tris-HCl (pH 7.5), 2 mM MgCl₂, 1 mM DTT, 400 µM ATP, 10 (w/v)% PEG6000, 0.05 µg/µL T4 RNA
20 ligase 2. The progress of the linking reaction was confirmed by denaturing polyacrylamide gel electrophoresis (PAGE) [6% polyacrylamide (acrylamide/N,N'-methylenebisacrylamide, 19/1) containing 7.5 M urea as a denaturant and 25 (v/v)%formamide was prepared using a TBE buffer (89 mM Tris-boric acid, 2 mM EDTA)],
25 and separation and purification were performed. A gel part containing a linear mRNA as a target conjugate was detected by a UV shadowing method and cut out, and a gel piece was crushed, and then a 50 mM aqueous sodium acetate solution (pH 5.2) was added to extract RNA from the gel. RNA contained in the extract was
30 recovered by an alcohol precipitation method and redissolved in ultrapure water. The RNA concentration was calculated based on the absorbance at 260 nm. The purity of RNA after isolation and purification was confirmed by denaturing PAGE analysis.

[0150]

35 8. Synthesis of linear and circular mRNAs containing internal

ribosome entry site (IRES) sequences (Fig. 33)

From a commercially available plasmid DNA pIRES Vector (Takara Bio 631605), a sequence including the IRES sequence was cut out at two restriction enzyme sites (EcoR I/BamH I), and this
5 was inserted into pNL1.1 TK Vector (Promega N1501) to prepare a plasmid DNA. The sequence of the present plasmid DNA (4396-bp, pIRES_NL) used thereafter as a template for the PCR reaction is as shown in SEQ ID NO: 41.

[0151]

10 The PCR reaction was performed by using the above-described pIRES_NL as a template and two synthetic oligo-DNAs (5'-CCCGGATCCTAATACGACTCACTATAGGCGCATATTAAGGTGACGCGT-3' (SEQ ID NO: 42)/5'-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCTAGAATTACGCCAGAATGCG-3' (SEQ ID NO: 43)) as a primer. The composition of the reaction
15 solution was as follows. 1 ng/μL pIRES_NL, 0.3 μM oligo DNA, 0.2 mM dNTPs, 1.5 mM MgSO₄, 1×PCR Buffer for KOD-Plus-Neo, 0.02 U/μL KOD-Plus-Neo (Toyobo Co., Ltd.). The reaction solution was subjected to the following thermal cycling conditions by using a T100 thermal cycler (Bio-Rad). 94°C, 2 minutes → 98°C, 10 seconds
20 → 55°C, 30 seconds → 72°C, 90 seconds] × 25 cycles. The reaction solution was deproteinized, and then DNA was recovered by alcohol precipitation. Using the obtained DNA as a template, a transcription reaction using T7 RNA polymerase was performed in the same manner as in the case of the IRES sequence-free sequence
25 to prepare a linear mRNA. The obtained linear mRNA was similarly purified using reverse phase HPLC. The circular mRNA containing the IRES sequence was subjected to a circularization reaction in the presence of only one splint DNA Splint DNA_1 in the same manner as the circular mRNA not containing IRES [0.5 μM
30 transcriptionally synthesized IRES-containing RNA, 2 μM Splint DNA_1, 50 mM Tris-HCl (pH 7.5), 2 mM MgCl₂, 1 mM DTT, 400 μM ATP, 10 (w/v)% PEG6000, 0.05 μg/μL T4 RNA ligase 2], and isolated and purified by denaturing PAGE in the same manner. The purity of the circular RNA after isolation and purification was confirmed by
35 denaturing PAGE analysis (> 90%).

[0152]

9. Experiment for evaluating translational activity of mRNA using cultured HeLa cells (Figs. 31 to 33)

Cultured HeLa cells (RIKEN Cell Bank) were seeded at 1×10^4 cells per well of a 96-well cell culture plate the day before. Here, 10 ng of RNA per well was introduced into HeLa cells based on the recommended conditions of the transfection reagent (Lipofectamine MessengerMAX, Thermo Fisher, 0.15 μ L/well). After 3 hours from transfection, the supernatant was replaced with Dulbecco's Modified Eagle Medium (DMEM, Wako Pure Chemical Industries, Ltd.) medium containing 10 (w/w)% fetal bovine serum (FBS, Hyclone), and the cells were further cultured under the conditions of 37°C and 5% CO₂ for 24 hours, and then lysed. The amount of NanoLuc luciferase protein contained in the lysate was measured using Nano-Glo (registered trademark) Luciferase Assay System (Promega) and Mithras LB 940 plate reader (Berthold).

[0153]

10. Experiment for evaluating translational activity of mRNA using experimental animal (mouse) (Fig. 33)

pH-responsive lipid (ALC-0315, MedChemExpress, Monmouth Junction, NJ, USA), phospholipid (1,2-distearoyl-sn-glycero-3-phosphocholine, DSPC, Fujifilm Wako, Osaka, Japan), cholesterol (Sigma Aldrich), and ALC-0159 (MedChemExpress) were prepared in an ethanol solvent at a molar ratio of 46.3 : 9.4 : 42.7 : 1.6. mRNA was dissolved in 50 mM of citrate buffer (pH 3). mRNA and lipid were mixed at a volume ratio of 2 : 1 in a microchannel (NanoAssembler (registered trademark) Spark, Precision Nanosystems, Vancouver, Canada). Thereafter, the mixture was diluted 40 times with PBS, and ultrafiltered with Amicon Ultra-15-30K centrifugal units (Merck Millipore). LNP was administered from the tail vein so that mRNA was 0.33 μ g/mouse. After 4 hours, each organ was collected. After homogenization in a passive lysis buffer (Promega), the amount of luciferase was quantified using Nano-Glo (registered trademark) Luciferase Assay System (Promega) and Lumat3 LB9508 luminometer (Berthold technologies, Bad

Wildbad, Germany).

[0154]

11. Experiment for evaluating immunogenicity of mRNA using cultured HEK293-NF kappa B reporter cells (Fig. 33)

5 NF- κ B reporter (Luc)-HEK293 cells were purchased from BPS Biosciences. On the day before transfection, trypsinized cells were seeded at 3×10^5 cells per well of a 48-well cell culture plate. Immediately before transfection, a growth medium (DMEM medium containing 10% FBS and 100 μ g/mL hygromycin B) was removed
10 and replaced with 240 μ L Opti-MEM (registered trademark) I Reduced Serum Medium (Thermo Fisher) per well. 20 ng of mRNA per well was mixed with 0.15 μ L Lipofectamine MessengerMAX (Thermo Fisher) and 10 μ L Opti-MEM I medium and added to the wells and cells were cultured for 3 hours. The supernatant was replaced
15 with a growth medium (250 μ L), and the cells were cultured for 24 hours in total. Cells were lysed using 50 μ L of Glo Lysis Buffer (Promega) per well. The total protein concentration in the cell lysate was measured using Pierce BCA Protein Assay Kit (Thermo Fisher), and the expression level of luciferase protein was
20 measured using One-Glo Luciferase Assay System (Promega). The expression level of luciferase was standardized by the total protein concentration, and the relative value of the activation amount of the NF- κ B information transduction pathway by each mRNA introduction was calculated.

25 [0155]

Fig. 31 shows the experimental results showing that the translational activity of a circular mRNA in which a cap structure is introduced into a branched chain is greatly improved as compared with that in which a cap structure is not introduced.
30 (a) shows a conceptual diagram showing a method for synthesizing a circular mRNA containing a 5'-capped structure in the branched chain. A chemically synthesized RNA fragment (SEQ ID NOS: 44 to 48) having a length of 54 bases and a transcriptionally synthesized RNA (SEQ ID NO: 49) were linked by enzyme ligase to
35 prepare a circular mRNA. The sequences and structures of

chemically synthesized RNA fragments and enzyme-synthesized RNA fragments are shown in the drawing. (b) shows the evaluation results of the translational activity of a circular mRNA synthesized by the method shown in (a) in cultured HeLa cells.

5 The circular mRNA was introduced into cultured HeLa cells by a lipofection method and cultured for 24 hours, then the cells were lysed, and the NanoLuc luciferase activity of the lysate was measured. The activities of circular mRNAs having branched chains of three different chain lengths were compared. It is found that
10 the translational activity of the circular mRNA having a cap structure in the branched chain was greatly improved as compared with that having no cap structure.

[0156]

Fig. 32 shows the experimental results showing the
15 relationship between the branched chain length of a circular mRNA in which a cap structure is introduced into a branched chain and translational activity (that the branched chain length is variable). (a) is a view showing the structure of chemically synthesized RNA fragments (SEQ ID NOS: 48, 50, and 51) having a
20 5'-capped structure in the branched chain. Three types of RNA strands having different branched chain lengths and sequences were prepared. (b) shows the evaluation results of the translational activity of a circular mRNA synthesized using the fragment shown in (a) as a substrate in cultured HeLa cells. This
25 is the result of comparing a circular mRNA prepared using an RNA strand before introducing a cap structure at the 5' end of the branched chain and the activity thereof, and showing the importance of introducing a cap structure at the end of the branched chain. The circular mRNA was introduced into cultured
30 HeLa cells by a lipofection method and cultured for 24 hours, then the cells were lysed, and the NanoLuc luciferase activity of the lysate was measured. When the activities of circular mRNAs having branched chains of three different chain lengths were compared, the circular mRNA having a 6-base-long branched chain
35 showed the highest translational activity although the activities

were not significantly different.

[0157]

Fig. 33 shows the experimental results showing that the translational activity of a circular mRNA having a cap structure in a branched chain is higher than that of mRNA or the like containing an EMCV-derived IRES sequence. All RNAs contain a common Nano luciferase coding sequence, 3'UTR, and poly A30 tail. (a) is a schematic view showing the sequence and structure of the used mRNA. (b, c) show the evaluation results of the translational activity in cultured HeLa cells. The mRNA was introduced into cultured HeLa cells by a lipofection method and cultured for 24 hours (b) or 18, 24, 48, and 72 hours (c), then the cells were lysed, and the NanoLuc luciferase activity of the lysate was measured. (d) shows the results of measuring the expression level in the mouse spleen. (e) shows the experimental results of evaluating immunogenicity of mRNA in a HEK293-NF kappa B-luciferase reporter cell line. mRNA was introduced into cells by a lipofection method.

[0158]

From (b-e), the translational activity of the circular mRNA (E) having a cap structure in the branched chain was higher than that of linear mRNA (A) and circular mRNA (B) containing EMCV-IRES, linear mRNA (C) having a cap structure, and circular mRNA (D) having no cap structure in both the human-derived cultured cell line (b) and the mouse animal experiment (d) (b). In the cultured cell line, the circular RNA (E) having a cap structure showed continuous translational activity for a longer time as compared with the linear mRNA (C) having a cap structure. This is an experimental result showing that the immunogenicity of the circular mRNA (E) having a cap structure in the branched chain is lower than that of the linear mRNA (C) having a cap structure.

[0159]

12. Translation reaction in HeLa cells of ATF4-Renilla mRNA and ATF4-csRNA5 to csRNA8 (Figs. 43 and 44)

(1) Synthesis of transcription template DNA of ATF4-Renilla mRNA

by PCR

A PCR reaction solution (0.3 μ M Forward primer, 0.3 μ M Reverse primer, 1 ng/ μ L plasmid DNA (psiCHECK2_ATF4-Renilla), 0.2 mM dNTPs, 1.5 mM MgSO₄, 1 $\times$ PCR Buffer for KOD-Plus-Neo (TOYOBO), 5 0.02 U/ μ L KOD-Plus-Neo (TOYOBO), scaled-up with MQ water) was prepared, and a template DNA was synthesized by a PCR method using a thermal cycler. As PCR conditions, the initial denaturation cycle of 94°C for 2 minutes, 98°C for 10 seconds, 56°C for 30 seconds, and 72°C for 60 seconds was set to 30 10 cycles. A 1% agarose gel (Agarose S (NIPPON GENE CO., LTD.), 1 $\times$ TAE buffer) was prepared, 6 $\times$ Loading Buffer (takara) was added to the PCR reaction solution and applied, and electrophoresis was performed at 100 V for 30 minutes. After staining by EtBr, a target band was cut out, and transcription-type DNA was extracted 15 and purified using Wizard (registered trademark) SV Gel and PCR Clean-Up System (Promega). The absorbance of the obtained DNA solution at 260 nm was measured to determine the concentration. [0160]

(2) Synthesis of ATF4-Renilla mRNA by in vitro transcription

20 In order to introduce a cap structure at the 5' end, an in vitro transcription reaction was performed with the following composition in which the ratio of ARCA and GTP was set to 4 : 1. A transcription reaction solution (10 ng/ μ L template DNA, 1 $\times$ T7 polymerase buffer (takara) (40 mM Tris-HCl (pH 8.0), 8 mM MgCl₂, 2 25 mM spermidine), 5 mM DTT, 2 mM ATP, 2 mM CTP, 2 mM UTP, 0.4 mM GTP, 1.6 mM ARCA, 2.5 U/ μ L T7 RNA polymerase, scaled-up with MQ water) was prepared. In the case of introducing no cap structure, a transcription reaction solution was prepared with the following composition (10 ng/ μ L template DNA, 1 $\times$ T7 polymerase buffer 30 (takara) (40 mM Tris-HCl (pH 8.0), 8 mM MgCl₂, 2 mM spermidine), 5 mM DTT, 2 mM ATP, 2 mM CTP, 2 mM UTP, 2 mM GTP, 2.5 U/ μ L T7 RNA polymerase, scaled-up with MQ water). After incubation at 37°C for 1 hour, Recombinant DNase I (takara) was added to the reaction solution at 0.1 U/ μ L, and the mixture was incubated at 35 37°C for 15 minutes to decompose the template DNA. After the

reaction, the protein was removed by phenol/chloroform extraction, and the mixture was concentrated using Amicon Ultra (0.5 mL, 10K) (Merck Millipore). Thereafter, purification was performed by alcohol precipitation and reverse phase HPLC.

5 Acetonitrile was removed with a centrifugal evaporator, and then alcohol precipitation was performed. The nucleic acid aqueous solution was subjected to absorbance measurement at 260 nm to determine the concentration. The results are shown in Fig. 34. [0161]

10 (3) Chemical synthesis of RNA

Synthesis was performed with an automated nucleic acid synthesizer using any one of Universal UnyLinker Support 2000Å CPG (ChemGenes), 3'-TOM ribo Adenosine (n-acetyl) 2'-lcaa CPG 1000Å (ChemGenes), 2'-TOM ribo Cytidine (n-acetyl) 3'-lcaa CPG 1000Å (ChemGenes), 2'-TOM ribo Guanosine (n-acetyl) CPG 1000Å (ChemGenes), and 2'-TOM ribo Uridine 2'-lcaa CPG 1000Å (ChemGenes). The 5' end was phosphorylated using 5'-Phosphate-ON Reagent (ChemGenes). After the synthesis, the CPG was transferred to a screw tube with 1.5 mL loop (SARSTEDT AG & Co. KG), 1 mL of 20 40% aq. methylamine/28% aq. ammonia (1 : 1) was added, and the mixture was incubated at 65°C for 30 minutes. After cooling on ice, the mixture was filtered using a Millex LH 0.45 µm filter, washed with 0.75 mL of MQ water, and dried and solidified on a centrifugal evaporator. 1 mL of 1 M TBAF in THF was added, and 25 the mixture was allowed to stand still at room temperature overnight. 1 mL of a 1 M Tris-HCl buffer (pH 7.5) was added, and the organic solvent was distilled off with a centrifugal evaporator. Desalination was performed using a NAP-25 column (GE Healthcare), and then purification was performed by alcohol 30 precipitation and reverse phase HPLC. After acetonitrile was removed with a centrifugal evaporator, alcohol precipitation was performed, and the concentration of the nucleic acid aqueous solution was determined from absorbance measurement at 260 nm. The results are shown in Fig. 35.

35 [Table 4]

LC-MS

<u>ATF4-sRNA1</u>	<u>ATF4-sRNA2</u>	<u>ATF4-sRNA3</u>	<u>ATF4-sRNA4</u>
Calcd. 7465.4855	Calcd. 7498.4675	Calcd. 7448.5025	Calcd. 7489.5115
Obs. 7466.46	Obs. 7499.49	Obs. 7449.53	Obs. 7490.41
<u>ATF4-sRNA5</u>	<u>ATF4-sRNA6</u>	<u>ATF4-sRNA7</u>	<u>ATF4-sRNA8</u>
Calcd. 7465.4855	Calcd. 7466.4695	Calcd. 7422.3535	Calcd. 7438.3525
Obs. 7466.49	Obs. 7467.48	Obs. 7421.77	Obs. 7437.76
<u>ATF4-sRNA8-polyA</u>	<u>ATF4-sRNA8-polyA_OMe</u>	<u>ATF4-sRNA8-polyA-LNA</u>	
Calcd. 18012.6081	Calcd. 18157.3559	Calcd. 18207.3309	
Obs. 18016.39	Obs. 18156.25	Obs. 18206.12	

[0162]

(4) Chemical capping of RNA

An aqueous RNA solution and an aqueous CaCl_2 solution were placed in a 1.5 mL tube, and then freeze-dried and solidified. A capping reagent dissolved in DMSO and 2-nitroimidazole were added thereto, and the mixture was scaled-up with DMSO. The composition of the final capping reaction solution is as follows (20 μM RNA, 10 mM CaCl_2 , 10 mM capping reagent, 10 mM 2-nitroimidazole, scaled-up with DMSO). The reaction solution was incubated at 55°C for 3 hours, and then purified by Amicon Ultra (Merck Millipore, 0.5 mL, 3K) and alcohol precipitation, and the concentration of the nucleic acid aqueous solution was determined from absorbance measurement at 260 nm. Thereafter, analysis by denaturing PAGE was performed. 3 pmol of RNA was mixed with 2×formamide loading solution and loaded onto the gel after heating at 80°C for 5 minutes. The gel was electrophoresed at 25 mA for 2 hours or 20 mA for 2.5 hours, stained by SYBR Green II, and then photographed by ChemiDoc. The capping efficiency was calculated from the band intensity. The results are shown in Fig. 36.

[Table 5]

LC-MS

<u>ATF4-csRNA1</u>	<u>ATF4-csRNA2</u>	<u>ATF4-csRNA3</u>	<u>ATF4-csRNA4</u>
Calcd. 7904.6991	Calcd. 7937.6811	Calcd. 7887.7161	Calcd. 7928.7251
Obs. 7905.57	Obs. 7938.40	Obs. 7888.59	Obs. 7929.46
<u>ATF4-csRNA5</u>	<u>ATF4-csRNA6</u>	<u>ATF4-csRNA7</u>	<u>ATF4-csRNA8</u>
Calcd. 7904.6991	Calcd. 7905.6831	Calcd. 7861.5671	Calcd. 7877.5661
Obs. 7905.50	Obs. 7906.58	Obs. 7861.10	Obs. 7876.90
<u>ATF4-csRNA8-polyA</u>	<u>ATF4-csRNA8-polyA_OMe</u>	<u>ATF4-csRNA8-polyA-LNA</u>	
Calcd. 18456.2994	Calcd. 18596.5694	Calcd. 18646.5444	
Obs. 18456.01	Obs. 18596.14	Obs. 18645.35	

[0163]

(2) Annealing and luminescence measurement

An annealing solution (0.1 μ M ATF4_Renilla mRNA, 1 μ M csRNA
 5 (SEQ ID NOS: 52 to 55), 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM
 EDTA (pH 8.0)) was prepared and heated at 80°C for 5 minutes.
 Thereafter, annealing of mRNA and csRNA was performed by
 gradually cooling until the temperature reached room temperature.
 HeLa cells suspended in 100 μ L of 10% FBS-containing D-MEM (Wako)
 10 at 1.5×10^4 cells/well were seeded in a 96-well plate the day
 before transfection. The next day, the medium was removed and
 transfected with annealed mRNA by using Lipofectamine (registered
 trademark) MessengerMAX (registered trademark) (Thermo Fisher
 SCIENTIFIC) (Lipofectamine (registered trademark) MessengerMAX
 15 (registered trademark) 0.3 μ L, annealing solution 1 μ L, scaled-up
 to 100 μ L with Opti-MEM (registered trademark) (Thermo Fisher
 SCIENTIFIC)). After incubation in an environment of 37°C and 5%
 CO₂ for 3 hours, the medium was removed and 100 μ L of 10% FBS-
 containing D-MEM (Wako) was added. After incubation again in an
 20 environment of 37°C and 5% CO₂ for 3.5 hours, the medium was
 removed, and 20 μ L of 1 $\times$ Renilla Luciferase Assay Lysis Buffer
 (Promega) was added thereto for lysis. 10 μ L of lysate was
 mixed with 30 μ L of Renilla Luciferase Assay Reagent (Promega)
 and luminescence measurement was performed.

25 [0164]

(a) of Fig. 43 shows the sequence of newly designed csRNA. The complementary strand region with mRNA is indicated by underline. The sequences were all described in the 5' → 3' direction. (b) shows the secondary structure of ATF4-Renilla mRNA 5'UTR. The binding position of csRNA is indicated by a marker in the drawing.

Fig. 44 is a view showing optimization of the binding position of csRNA. (a) is a schematic view of mRNA and csRNA used for translation. Red circles in the drawing indicate the cap structure. (b) is a view showing luciferase luminescence measurement in HeLa cells. A relative value when the luminescence intensity of mRNA (cap(-)) or mRNA (cap(+)) was taken as 1 was shown. Error bars were standard errors of three experimental averages.

From these results, the translation promoting effect was improved by designing csRNA so that mRNA binds to a region that does not seem to form a double strand in the molecule (both mRNA cap(-) and mRNA cap(+)) showed a large increase in the amount of translation).

[0165]

13. Translation reaction in HeLa cell extract of csRNA into which chemical modification (OMe, LNA) was introduced (Fig. 45)

[0166]

(1) Synthesis of circular NanoLuc mRNA by in vitro transcription

In order to introduce a monophosphate group at the 5' end, an in vitro transcription reaction was performed with the following composition in which the ratio of GMP and GTP was set to 4 : 1. A transcription reaction solution (10 ng/μL template DNA, 1×T7 polymerase buffer (takara) (40 mM Tris-HCl (pH 8.0), 8 mM MgCl₂, 2 mM spermidine), 5 mM DTT, 2 mM ATP, 2 mM CTP, 2 mM UTP, 2 mM GTP, 10 mM GMP, 2.5 U/μL T7 RNA polymerase, scaled-up with MQ water) was prepared and incubated at 37°C for 2 hours. Recombinant DNase I (takara) was added to the reaction solution at 0.1 U/μL, and the mixture was incubated at 37°C for 15 minutes to decompose the template DNA. After the reaction, the protein

was removed by phenol/chloroform extraction, and the mixture was concentrated using Amicon Ultra (Merck Millipore, 0.5 mL, 10K). Thereafter, purification was performed by alcohol precipitation and reverse phase HPLC. Acetonitrile was removed with a
5 centrifugal evaporator, and then alcohol precipitation was performed. The nucleic acid aqueous solution was subjected to absorbance measurement at 260 nm to determine the concentration. The results are shown in Fig. 37.

[0167]

10 (2) Cyclization reaction of circular NanoLuc mRNA

1 mL of a reaction solution (0.5 μ M mRNA, 1.0 μ M splint DNA, T4 RNA ligase II, 1 $\times$ T4 RNA ligase II buffer, 10% PEG8000, scaled-up with MQ water) was prepared and incubated at 37°C for 1 hour. Recombinant DNase I (takara) was added to the reaction
15 solution at 0.1 U/ μ L, and the mixture was incubated at 37°C for 15 minutes to decompose the splint DNA. After the reaction, the protein was removed by phenol/chloroform extraction, and the mixture was concentrated using Amicon Ultra (0.5 mL, 10K) (Merck Millipore). Subsequently, alcohol precipitation was performed,
20 and progress of the cyclization reaction was confirmed by 5% denaturing PAGE. Thereafter, the circular mRNA was isolated and purified by denaturing PAGE. A gel containing a target band was cut out by UV shadowing, and the cut gel was crushed well. The gel piece was immersed in 2 mL of MQ water and permeated
25 overnight at 4°C to extract RNA. Centrifugation was performed at 4°C and 10000 rpm for 5 minutes, and the supernatant was recovered. 500 μ L of MQ water was added to the gel piece again, and the mixture was centrifuged in the same manner to recover the supernatant. All the recovered supernatants were filtered
30 (Millex-LCR, 0.45 μ m (Merck Millipore)) to remove the mixed gel piece. The filtrate was desalted and concentrated by ultrafiltration (Amicon Ultra (0.5 mL, 10K) (Merck Millipore)), and then alcohol precipitation was performed. The nucleic acid aqueous solution was subjected to absorbance measurement at 260
35 nm to determine the concentration. The results are shown in Fig.

38.

[0168]

(3) Synthesis of csRNA (Nluc-csRNA_polyA, Nluc-csRNA_polyA_OME, Nluc-csRNA_polyA_LNA) with respect to circular Nluc mRNA

5 (a) RNA synthesis

RNA synthesis was performed on a 0.2 μ mol scale, and an NTS M-2-MX DNA/RNA synthesizer or an NRS-4A10R7 DNA/RNA synthesizer manufactured by NIHON TECHNO SERVICE CO., LTD. was used. As the phosphoramidite used for RNA solid phase synthesis, rA(5'-DMT-2'-TOM-ribo-Adenosine (n-acetyl) OP), rC(5'-DMT-2'-TOM-ribo-Cytidine (n-acetyl) OP), rG(5'-DMT-2'-TOM-ribo-Guanosine (n-acetyl) OP), and U(5'-DMT-2'-TOM-ribo-Uridine OP) (ChemGenes) were used. As the phosphorylation reagent, 5' phosphate-ON Reagent (ChemGenes) was used. As the 2'-OME RNA amidite, 2'-OME-C-CE-phosphoramidite and 15 2'-OME-G(iBu)-CE-phosphoramidite (ChemGenes) were used. As the LNA amidite, MBN-Ganosine(N,N-DMF)-phosphoramidite (ChemGenes) and MBN-5'-Methyl-Cytidine(N-bz)-phosphoramidite (ChemGenes) were used. As the CPG, 3'-TOM-ribo Cytidine (n-acetyl) 2'-lcaa CPG 1000Å, 3'-TOM-ribo Adenosine (n-acetyl) 2'-lcaa CPG 1000Å 20 (ChemGenes), and 3'-phosphate ON lcaa CPG 1000A were used. After the synthesis, the CPG was transferred to a screw tube with 1.5 mL loop (SARSTEDT AG & Co. KG), 1.0 mL of 40% aq. methylamine/28% aq. ammonia (1 : 1) was added, and the mixture was incubated at 65°C for 20 minutes. The mixture was filtered using a Millex LH 25 0.45 μ m filter, washed with sterile water (500 μ L), and dried and solidified on a centrifugal evaporator. 1.0 mL of 1 M TBAF in THF was added and dissolved, and the mixture was allowed to stand still at room temperature overnight. 1.0 mL of a 1 M Tris-HCl buffer (pH 7.5) was added, and the organic solvent was distilled 30 off with a centrifugal evaporator. Desalination was performed using a NAP-25 column (GE Healthcare). Purification was performed by reverse phase HPLC. HPLC (HITACHI) conditions; Column: hydrosphere C18 column (4.6 x 250 mm; YMC), Mobile phase: A) 50 mM TEAA buffer (pH 7.0), 5% acetonitrile, B) acetonitrile, 35 Gradient: 0 to 20 min B conc. 0% to 20%, Flow rate: 1.0 mL/min

Analysis was performed by 15% denatured polyacrylamide gel electrophoresis (PAGE) (acrylamide : bisacrylamide = 19 : 1 (wt/wt), 7.5 M urea). For PAGE, a TBE buffer (89 mM Tris-borate, 2 mM EDTA, pH 8.3) was used. Analysis by LC/MS was performed.

5 LC/MS (agilent) conditions; ACQUITY UPLC BEH C18 Column, 0.3 mL/min, A; 8.6 mM TEA/100 mM HFIP solution, B; MeOH, 0 to 30%/20 min. The results are shown in Fig. 39.

LC-MS

Nluc-sRNA_polyA; calcd. 16259.1, obs. 16261.4

10 Nluc-sRNA_polyA_OME; calcd. 16287.1, obs. 16289.5

Nluc-sRNA_polyA_LNA; calcd. 16373.2, obs. 16375.2

[0169]

(4) Capping reaction of phosphorylated RNA

The mixture was mixed in a 1.5 mL tube so as to have a
15 final concentration of 20 μ M RNA and 10 mM CaCl_2 , respectively, and the mixture was freeze-dried and solidified and then dissolved in DMSO. Im (m7GDP) $\cdot$ Na (Hongene Biotech) dissolved in DMSO was added thereto so as to have a final concentration of 10 mM and 2-nitroimidazole was added thereto so as to have a final
20 concentration of 10 mM. This reaction solution was incubated at 55°C for 3 hours. The reaction solution was diluted two-fold with sterile water, and then isopropanol precipitation was performed. HPLC purification was performed. HPLC conditions; Column: hydrosphere C18 column (4.6 x 250 mm; YMC), Mobile phase: A) 50
25 mM TEAA buffer (pH 7.0), 5% acetonitrile, B) acetonitrile, Gradient: 0 to 20 min B conc. 0% to 20%, Flow rate: 1.0 mL/min

Analysis by LC/MS was performed. LC/MS (agilent) conditions; ACQUITY UPLC BEH C18 Column, 0.3 mL/min, A; 8.6 mM TEA/100 mM HFIP solution, B; MeOH, 0 to 30%/20 min

30 The HPLC-purified capped RNA was precipitated using isopropanol and sodium acetate (pH 5.2).

LC-MS

Nluc-csRNA_polyA; calcd. 16699.3, obs. 16701.0

Nluc-csRNA_polyA_OME; calcd. 16727.4, obs. 16728.7

35 Nluc-csRNA_polyA_LNA; calcd. 16813.5, obs. 16814.9

[0170]

(5) Chemical synthesis of RNA

RNA was synthesized with an automated nucleic acid synthesizer using Universal UnyLinker Support 2000Å CPG (ChemGenes). The 5' end was phosphorylated using 5'-Phosphate-ON Reagent (ChemGenes). After the synthesis, the CPG was transferred to a screw tube with 1.5 mL loop (SARSTEDT AG & Co. KG), 1 mL of 40% aq. methylamine/28% aq. ammonia (1 : 1) was added, and the mixture was incubated at 65°C for 30 minutes. After cooling on ice, the mixture was filtered using a Millex LH 0.45 µm filter, washed with 1 mL of MQ water, and dried and solidified on a centrifugal evaporator. 1 mL of 1 M TBAF in THF was added, and the mixture was allowed to stand still at room temperature overnight. 1 mL of a 1 M Tris-HCl buffer (pH 7.5) was added, and the organic solvent was distilled off with a centrifugal evaporator. Desalination was performed using a NAP-25 column (GE Healthcare), and then purification was performed by alcohol precipitation and reverse phase HPLC. After acetonitrile was removed with a centrifugal evaporator, alcohol precipitation was performed, and the concentration of the nucleic acid aqueous solution was determined from absorbance measurement at 260 nm. The results are shown in Fig. 40.

[Table 6]

LC-MS

<u>Nluc-sRNA natural</u>	<u>Nluc-sRNA LNA 10/20 3' type</u>	<u>Nluc-sRNA LNA 5/20 3' type</u>
Calcd. 8052.8091	Calcd. 8243.0541	Calcd. 8154.9451
Obs. 8051.69	Obs. 8241.99	Obs. 8153.64
<u>Nluc-sRNA LNA 10/20 type</u>	<u>Nluc-sRNA LNA 5/20 type</u>	<u>Nluc-sRNA LNA 6/20 type</u>
Calcd. 8243.0541	Calcd. 8126.8911	Calcd. 8152.9291
Obs. 8242.05	Obs. 8125.75	Obs. 8151.74

[0171]

(6) Chemical capping of RNA

An aqueous RNA solution and an aqueous CaCl₂ solution were placed in a 1.5 mL tube, and then freeze-dried and solidified. A capping reagent dissolved in DMSO and 2-nitroimidazole were added thereto, and the mixture was scaled-up with DMSO. The composition

of the final capping reaction solution is as follows (20 μ M RNA, 10 mM CaCl_2 , 10 mM capping reagent, 10 mM 2-nitroimidazole, scaled-up with DMSO). The reaction solution was incubated at 55°C for 3 hours, and then purification was performed by alcohol precipitation and reverse phase HPLC. Acetonitrile was removed with a centrifugal evaporator and then concentrated by freeze drying, and the concentration of the nucleic acid aqueous solution was determined from absorbance measurement at 260 nm. Thereafter, analysis by denaturing PAGE was performed. 10 pmol of RNA was mixed with 2 $\times$ formamide loading solution and loaded onto the gel after heating at 80°C for 5 minutes. The gel was electrophoresed at 25 mA for 2 hours, stained by SYBR Green II, and then photographed by ChemiDoc. The capping efficiency was calculated from the band intensity. The results are shown in Figs. 41 and 42.

[Table 7]

LC-MS

<u>Nluc-csRNA natural</u>	<u>Nluc-csRNA LNA 10/20_3' type</u>	<u>Nluc-csRNA LNA 5/20_3' type</u>
Calcd. 8490.0077	Calcd. 8680.2527	Calcd. 8592.1437
Obs. 8491.40	Obs. 8680.93	Obs. 8592.84
<u>Nluc-csRNA LNA 10/20 type</u>	<u>Nluc-csRNA LNA 5/20 type</u>	<u>Nluc-csRNA LNA 6/20 type</u>
Calcd. 8680.2527	Calcd. 8564.0897	Calcd. 8590.1277
Obs. 8681.02	Obs. 8564.98	Obs. 8590.83

[0172]

An annealing solution (0.2 μ M circular NanoLuc mRNA, 0.4 μ M csRNA (SEQ ID NOS: 56 to 58), 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA (pH 8.0)) was prepared and heated at 80°C for 5 minutes. Thereafter, annealing of mRNA and csRNA was performed by gradually cooling until the temperature reached room temperature. 4.85 μ L of Mixture-2 w/o DTT, 1.648 μ L of 50 mM DTT, and 8.7 μ L of a HeLa cell extract were mixed and incubated at room temperature for 10 minutes, and then 1.8 μ L of Mixture-3 and 1 μ L of an annealing solution were added. After incubation at 32°C for 4 hours, 1 μ L of the reaction solution was mixed with 20 μ L of Nano-Glo (registered trademark) Luciferase Assay Substrate (Promega) diluted with 1 $\times$ Glo Lysis Buffer (Promega), and

luminescence measurement was performed.

[0173]

Fig. 45(a) shows the sequence of csRNA into which chemical modification was introduced. The complementary strand region with mRNA is indicated by underline. N_m, 2'OMe; bold letters, LNA. The sequences were all described in the 5' → 3' direction. (b) is a view showing luciferase luminescence measurement in a HeLa cell extract. A relative value when the luminescence intensity of circ. Nluc mRNA was taken as 1 was shown. Error bars were standard errors of three experimental averages.

From these results, in the case of annealing (w/annealing), cap-structure-dependent translation promotion was observed in any csRNA. The effect was increased by introducing LNA. On the other hand, when annealing was not performed (w/o annealing), the translation promoting effect of Nluc-csRNA_polyA_LNA was high, and it was found that an excellent effect was exhibited even without annealing by containing LNA.

[0174]

14. Translation reaction in HeLa cells of csRNA and circular mRNA into which LNA modification was introduced (Fig. 46)

[0175]

(1) Annealing and luminescence measurement

An annealing solution (0.1 μM circular NanoLuc mRNA, 1 μM csRNA (SEQ ID NOS: 59 to 64), 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA (pH 8.0)) was prepared and heated at 80°C for 5 minutes. Thereafter, annealing of mRNA and csRNA was performed by gradually cooling until the temperature reached room temperature. HeLa cells suspended in 100 μL of 10% FBS-containing D-MEM (Wako) at 1.5×10⁴ cells/well were seeded in a 96-well plate the day before transfection. The next day, the medium was removed and transfected with annealed mRNA by using Lipofectamine (registered trademark) MessengerMAX (registered trademark) (Thermo Fisher SCIENTIFIC) (Lipofectamine (registered trademark) MessengerMAX (registered trademark) 0.3 μL, annealing solution 1 μL, scaled-up to 100 μL with Opti-MEM (registered trademark) (Thermo Fisher

SCIENTIFIC)). After incubation in an environment of 37°C and 5% CO₂ for 3 hours, the medium was removed and 100 µL of 10% FBS-containing D-MEM (Wako) was added. After incubation again in an environment of 37°C and 5% CO₂ for 3 hours, the medium was removed, and 20 µL of 1×Glo Lysis Buffer (Promega) was added thereto for lysis. 10 µL of the lysate was mixed with 20 µL of Nano-Glo (registered trademark) Luciferase Assay Substrate (Promega) diluted with 1×Glo Lysis Buffer (Promega), and luminescence measurement was performed.

10 [0176]

Fig. 46(a) shows the sequence of csRNA used for translation. The complementary strand region with mRNA is indicated by underline. Bold letters, LNA. The sequences were all described in the 5' → 3' direction. (b) shows luciferase luminescence measurement in HeLa cells. A relative value when the luminescence intensity of mRNA (cap(-)) or mRNA (cap(+)) was taken as 1 was shown. Error bars were standard errors of three experimental averages.

From these results, full improvement in activity is observed in Nluc-csRNA_LNA_6/20 type as compared with natural csRNA.

Sequence Listing

International Application under International Patent Cooperation

25 Treaty

CLAIMS

[Claim 1]

A capped RNA comprising:

a translated region beginning with a start codon and
5 encoding a protein; and
an upstream region upstream of the start codon, a branch
part having a cap structure being provided in the upstream
region.

10 [Claim 2]

The capped RNA according to claim 1, wherein the branch
part has at least one structure of (a) to (c) below:

(a) a capped probe having a sequence complementary to a
sequence of a part or all of the upstream region and having the
15 cap structure is hybridized to the upstream region;

(b) a branched chain branching from a main chain
constituting the RNA is provided in a part of the upstream region
and the cap structure is provided in the branched chain; or

(c) the RNA is a linear RNA and has a branch structure in
20 which the 5' end of a main chain of the upstream region is
branched into two or more branches, the cap structure being
provided in each branch.

[Claim 3]

25 The capped RNA according to claim 2, wherein
in the (a), the RNA is a circular RNA, and
a capped probe having a sequence complementary to a
sequence of a part or all of the upstream region of the circular
RNA and having a cap structure is hybridized to the upstream
30 region of the circular RNA.

[Claim 4]

The capped RNA according to claim 2, wherein
in the (a), the RNA is a linear RNA, and
35 a capped probe having a sequence complementary to a

The capped RNA according to claim 2, wherein
in the (b), the RNA is a circular RNA, and

a branched chain branching from a main chain constituting

A is provided in a part of the upstream region of the

The capped RNA according to claim 2, wherein
in the (b), the RNA is a linear RNA, and

a branched chain branching from a main chain constituting

A is provided in a part of the upstream region of the

The capped RNA according to claim 2, which has a structure represented by Formula (3) below in the (b):

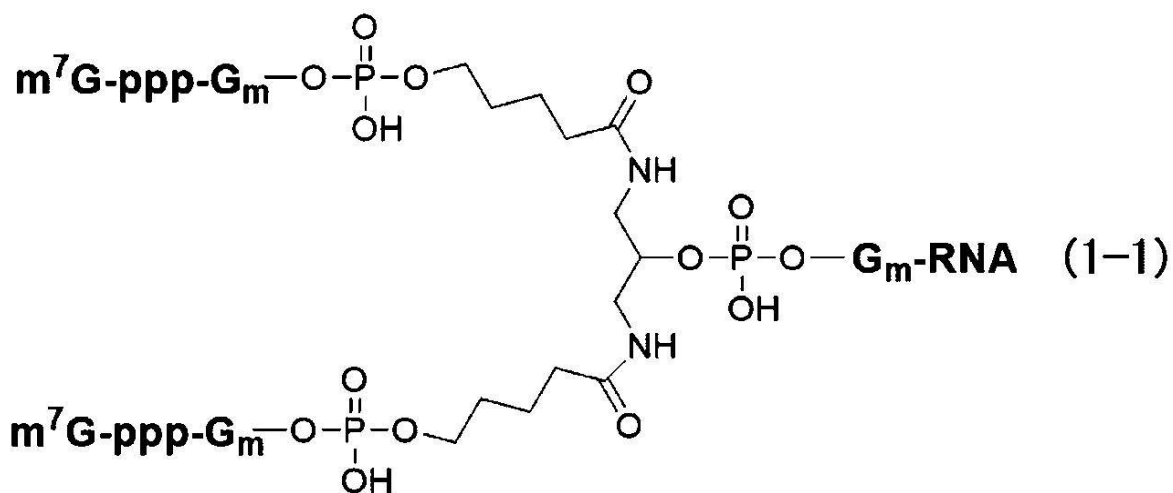
$$\begin{array}{c}
 \text{m}^7\text{G}-\text{ppp}-\text{Nuc}-\text{O}-\text{P}(=\text{O})(\text{OH})-\text{O}-(\text{CH}_2)_m-\text{C}(=\text{O})-\text{NH} \\
 | \\
 (\text{CH}_2)_m \\
 | \\
 \text{O}-\text{P}(=\text{O})(\text{OH})-\text{RNA2} \\
 | \\
 (\text{CH}_2)_m \\
 | \\
 \text{RNA1}-\text{O}-(\text{CH}_2)_m-\text{C}(=\text{O})-\text{NH}
 \end{array} \quad (3)$$

where m⁷G represents a cap, and p represents a phosphate group; Nuc represents a polynucleotide in which one or more natural or

The capped RNA according to claim 2, which has a structure represented by Formula (1) or Formula (2) below in the (c):

$$\begin{array}{c}
 m^7G\text{-ppp-Gm-O-P(=O)(OH)-O-(CH}_2)_m\text{-C(=O)-NH-} \\
 | \\
 \text{(CH}_2)_m\text{-O-P(=O)(OH)-Gm-RNA} \\
 | \\
 \text{(CH}_2)_m\text{-NH-} \\
 | \\
 m^7G\text{-ppp-Gm-O-P(=O)(OH)-O-(CH}_2)_m\text{-C(=O)-NH-}
 \end{array}
 \quad (1)$$
$$\begin{array}{c}
 m^7G\text{-}ppp\text{-}Gm\text{-}O\text{-}\overset{\overset{O}{\parallel}}{\underset{\underset{HO}{|}}{P}}\text{-}O\text{-}(CH_2)_n\text{-}O\text{-} \\
 m^7G\text{-}ppp\text{-}Gm\text{-}O\text{-}\overset{\overset{O}{\parallel}}{\underset{\underset{HO}{|}}{P}}\text{-}O\text{-}(CH_2)_n\text{-}O\text{-} \\
 m^7G\text{-}ppp\text{-}Gm\text{-}O\text{-}\overset{\overset{O}{\parallel}}{\underset{\underset{HO}{|}}{P}}\text{-}O\text{-}(CH_2)_n\text{-}O\text{-}
 \end{array}
 \left[\begin{array}{c} \\ \\ \\ \end{array} \right]
 (CH_2)_n\text{-}O\text{-}\overset{\overset{O}{\parallel}}{\underset{\underset{HO}{|}}{P}}\text{-}Gm\text{-}RNA \quad (2)$$

The capped RNA according to claim 8, wherein the Formula
10 (1) has a structure represented by Formula (1-1) below, and the
Formula (2) has a structure represented by Formula (2-1) below.
[Chemical Formula 4]


$$\begin{array}{c}
 \text{m}^7\text{G-ppp-G}_m\text{-O-P(=O)(OH)-O-CH}_2\text{CH}_2\text{O-} \\
 \text{m}^7\text{G-ppp-G}_m\text{-O-P(=O)(OH)-O-CH}_2\text{CH}_2\text{O-} \\
 \text{m}^7\text{G-ppp-G}_m\text{-O-P(=O)(OH)-O-CH}_2\text{CH}_2\text{O-}
 \end{array}
 \begin{array}{c}
 \text{CH}_2 \\
 \text{CH}_2 \\
 \text{CH}_2
 \end{array}
 \begin{array}{c}
 \text{O-CH}_2\text{CH}_2\text{O-P(=O)(OH)-O-G}_m\text{-RNA} \\
 \text{O-CH}_2\text{CH}_2\text{O-P(=O)(OH)-O-G}_m\text{-RNA} \\
 \text{O-CH}_2\text{CH}_2\text{O-P(=O)(OH)-O-G}_m\text{-RNA}
 \end{array}
 \quad (2-1)$$

The capped RNA according to claim 1, wherein the RNA has a

plurality of translated regions.

[Claim 11]

A method for producing the capped RNA according to claim 1,
5 the method comprising at least one of steps (a) to (c) below:

(a) hybridizing a capped probe having a sequence
complementary to a sequence of a part or all of the upstream
region and having a cap structure to the upstream region; or

(b) synthesizing the RNA having a branched chain branching
10 from a main chain constituting the RNA in a part of the upstream
region, and then binding the cap structure to the branched chain;
or

(c) with the RNA being a linear RNA, synthesizing RNA
having a branch structure in which the 5' end of a main chain of
15 the upstream region is branched into two or more branches, and
then binding the cap structure to each branch.

[Claim 12]

An apparatus for producing a protein, comprising:
20 the capped RNA according to claim 1; and
an expression system of a eukaryotic cell capable of
expressing the protein encoded by the translated region using the
capped RNA as a template.

25 [Claim 13]

A method for producing a protein, comprising adding the
capped RNA according to claim 1 to an expression system of a
eukaryotic cell capable of expressing the protein encoded by the
translated region using the capped RNA as a template to express
30 the protein.

[Claim 14]

A detection device of a target RNA having a cap structure
at the 5' end of RNA, the detection device comprising:
35 a detection probe containing a circular RNA having a

translated region beginning with a start codon and encoding a labeled protein and an upstream region upstream of the start codon, the upstream region having a sequence complementary to a sequence of a part or all of the target RNA to be detected;

5 a means for hybridizing the target RNA to the upstream region of the circular RNA by adding the circular RNA to a sample containing the target RNA to generate a capped RNA;

 an expression system of a eukaryotic cell capable of expressing the labeled protein by adding the capped RNA; and

10 a means for detecting the labeled protein expressed in the expression system.

[Claim 15]

A detection method of a target RNA having a cap structure
15 at the 5' end of RNA, the detection method comprising:

 a step of preparing a detection probe containing a circular RNA having a translated region beginning with a start codon and encoding a labeled protein and an upstream region upstream of the start codon, the upstream region having a sequence complementary
20 to a sequence of a part or all of the target RNA to be detected;

 a step of hybridizing the target RNA to the upstream region of the circular RNA by adding the circular RNA to a sample containing the target RNA to generate a capped RNA;

 a step of adding the capped RNA to an expression system of
25 a eukaryotic cell capable of expressing the labeled protein to express the labeled protein; and

 a step of detecting the labeled protein expressed in the expression system.

30 [Claim 16]

A capped RNA having a cap structure in an untranslated region of RNA having a translated region beginning with a start codon and encoding a protein and the untranslated region upstream of the start codon, the capped RNA comprising at least one
35 structure of (a) to (c) below:

(a) a capped probe having a sequence complementary to a sequence of a part or all of the untranslated region and having a cap structure is hybridized to the untranslated region;

(b) a branched chain branching from a main chain
5 constituting the RNA is provided in a part of the untranslated region and the cap structure is provided in the branched chain;
or

(c) the RNA is a linear RNA and has a branch structure in which the 5' end of a main chain of the untranslated region is
10 branched into two or more branches, the cap structure being provided in each branch.