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(54) Title: CIRCULARISABLE RNA SEQUENCES, DNA ENCONDING THE SEQUENCES, BACTERIAL STRAINS EXPRESSING THE SEQUENCE, COMPOSITIONS AND USES THEREOF

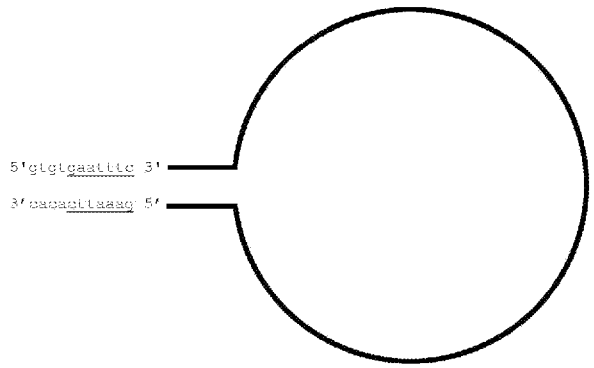

FIG. 1

(57) Abstract: There are disclosed circularisable RNA sequences comprising a first sequence, at least one target sequence, and a second sequence reverse complementary to the first sequence.

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CIRCULARISABLE RNA SEQUENCES, DNA ENCONDING THE SEQUENCES, BACTERIAL STRAINS EXPRESSING THE SEQUENCE, COMPOSITIONS AND USES THEREOF

Cross-Reference to Related Applications

This Patent Application claims priority from Italian Patent Application No. 102023000012357 filed on June 15, 2023, the entire disclosure of which is incorporated herein by reference.

Technical Field of the Invention

The present invention relates to circularisable RNA sequences comprising a first sequence, at least one target sequence, and a second sequence reverse complementary to the first sequence.

Prior Art

RNA therapeutics represents a fast-growing field. Main issues for their generalized applications are represented by their: i) instability, ii) immunogenicity iii) production costs and iv) need for appropriate delivery systems.

In order to overcome their instability and immunogenicity, linear RNA therapeutics are chemically or enzymatically modified by adding a number of possible chemical groups to various positions on the RNA bases.

The delivery issue, instead, is usually solved by complexation of the RNA in lipidic or polymeric

nanoparticles. Such complexation is also used to partially solve the instability and immunogenicity issues. Both chemical modifications and complexation with nanoparticles contribute to the high production costs of traditional RNA therapeutics.

In view of the above, there is a need for new therapeutic approaches which involve RNA therapeutics which are more stable, less immunogenic, have lower production costs and are effectively delivered to the organism.

Bacterial production of RNA therapeutics could minimize the costs and permit an easily scalable and simple methodology. Moreover, bacteria have the potential to be used directly as a delivery system, thus providing an alternative route to solve the issue of delivery.

Indeed, recombinant probiotic bacteria, defined as live biotherapeutics, are already used for the delivery in the gut of proteins or peptides of therapeutic interest. In principle recombinant probiotic bacteria expressing RNA therapeutics could be used as live biotherapeutics delivering RNA, rather than protein or peptides. However, delivery of RNA from live biotherapeutics is not possible with state-of-the-art technologies and has never been reported. This is due both to the immunogenicity of exogenous linear RNA, and to its instability.

There is therefore also the need to provide new

therapeutic approaches that allow the delivery of RNA from live biotherapeutics overcoming problems with immunogenicity and instability.

Bacterial circular RNAs have been discovered several decades ago. The first identified classes were group I introns (Chu, F. K., Maley, G. F., Maley, F. & Belfort, M. Intervening sequence in the thymidylate synthase gene of bacteriophage T4. Proc Natl Acad Sci U S A81, 3049-3053, doi:10.1073/pnas.81.10.3049 (1984)).

Later on, it was discovered that also group II introns circularize (Murray, H. L. et al. Excision of group II introns as circles. Mol Cell8, 201-211, doi:10.1016/s1097-2765(01)00300-8 (2001)).

More recently, other classes of circular RNA have been discovered in Archaea (Danan, M., Schwartz, S., Edelheit, S. & Sorek, R. Transcriptome-wide discovery of circular RNAs in Archaea. Nucleic Acids Res40, 3131-3142, doi:10.1093/nar/gkr1009 (2012)).

Research on bacterial circular RNAs has been disregarded afterwards, possibly due to the discovery of eukaryotic circular RNAs in 2012 and the shift of the focus to eukaryotic circular RNAs.

The first artificial circular RNA expressed in bacteria was reported in 1994 (Ford, E. & Ares, M. Synthesis of circular RNA in bacteria and yeast using RNA cyclase

ribozymes derived from a group I intron of phage T4. Proceedings of the National Academy of Sciences of the United States of America91, 3117-3121 (1994)). In this study, sequences derived from a group I intron were used to circularize the gene they belonged to. Circularization of foreign RNA in bacteria was not attempted.

Subsequent studies (Wesselhoeft, R. A. et al. RNA Circularization Diminishes Immunogenicity and Can Extend Translation Duration In Vivo. Mol Cell74, 508-520 e504, doi:10.1016/j.molcel.2019.02.015 (2019); Chen, Y. G. et al. Sensing Self and Foreign Circular RNAs by Intron Identity. Mol Cell67, 228-238 e225, doi:10.1016/j.molcel.2017.05.022 (2017); Wesselhoeft, R. A., Kowalski, P. S. & Anderson, D. G. Engineering circular RNA for potent and stable translation in eukaryotic cells. Nat Commun9, 2629, doi:10.1038/s41467-018-05096-6 (2018)) still used the same method to circularize in vitro various foreign RNAs. Foreign RNA has never been shown to circularize in bacteria. Although the original method from Ford et al. (Ford, E. & Ares, M. Synthesis of circular RNA in bacteria and yeast using RNA cyclase ribozymes derived from a group I intron of phage T4. Proceedings of the National Academy of Sciences of the United States of America91, 3117-3121 (1994)), this application has never been shown and in any case complex sequences (large sequences deriving from fragments of introns) would have been required at the ends of the foreign RNA.

Summary of the Invention

It is an object of the present invention to provide RNA sequences which overcome the above said drawbacks and can be delivered safely and effectively to organisms.

This object is achieved by means of the circularisable RNA sequence as defined in claim 1.

Other objects of the present invention are to provide a DNA molecule encoding the circularisable RNA sequence as defined in claim 6, a bacterial strain expressing the circularisable RNA sequence as defined in claim 7, a composition as defined in claim 8 and uses as defined in claims 10 and 11.

Brief Description of the Drawings

Figure 1 shows a schematic representation of the stem-loop structure, where the stem is formed by the annealed first and second RNA sequences and the loop is formed by the target sequence of the circularisable RNA sequence according to an embodiment of the invention.

Figure 2 shows a schematic representation of the pUC19-m1 vector disclosed in Example 2.

Figure 3 shows the sequence segments of the pUC19-m1 vector disclosed in Example 2 (whole sequence corresponds to SEQ ID NO:5).

Figures 4A and 4B show photographs respectively of a culture of E.Coli DH5α bacteria transformed with the pUC19-

m1 and blue-white colony screening of transformed E.Coli DH5α bacteria.

Figure 5 shows the results of end-point reverse-transcription PCR (rtPCR) with divergent primers amplifying the circular RNA junction point. The low and high molecular weight bands in the figure were purified and sequenced by Sanger method, confirming the circularity of the RNA.

Figure 6 shows the results of RNAse R treatment of total RNA samples obtained by the blue colonies, followed by rtPCR and Sanger sequencing of the PCR product, confirming the circularity of the RNA coding for LacZα.

Detailed Description of the Invention

The circularisable RNA sequence according to the invention comprises, from 5′ to 3′ or from 3′ to 5′, a first sequence comprising from 7 to 30 nucleotides; at least one target sequence; and a second sequence reverse complementary to the first sequence. The RNA sequence is a spontaneously circularisable RNA sequence. "Spontaneously" circularisable means that the molecule circularises without the need of enzymatic ligation or other artificial circularisation methods such as click chemistry and the like.

The RNA molecule preferably consists of a first sequence comprising from 7 to 30 nucleotides; at least one target sequence; and a second sequence reverse complementary to the first sequence.

The first sequence preferably comprises from 9 to 15 nucleotides, more preferably from 10 to 12, even more preferably 11 nucleotides.

5 to 15 nucleotides of the first sequence preferably form a palindromic sequence, more preferably 6 to 8 nucleotides, even more preferably 7 nucleotides. Said sequence needs not be perfectly palindromic, e.g. it could be gaatttc.

By "target sequence" there is intended the sequence that needs to be expressed and/or translated into a polypeptide in the host organism. The target sequence may be a coding or a non-coding RNA, i.e. it may or may not necessarily give rise to a polypeptide. The target sequence is preferably 100 to 600 nucleotides in length, more preferably, 200 to 500. In addition, it can be related to the host bacteria in which it is introduced, or it may be a foreign RNA. Preferably, the target sequence encodes for a therapeutic agent that can be administered to an individual in need thereof either as "naked RNA" or as an expressed protein or even as a bacterial strain expressing the target sequence as one of the above.

The present invention also relates to a DNA sequence that encodes the above said circularisable RNA sequence.

The present invention also relates to a bacterial strain expressing the above said circularisable RNA sequence or DNA

sequence.

The bacterial strain is preferably a probiotic bacteria, in particular if the RNA sequence is to be administered as a medicament to an individual.

The present invention also relates to a composition comprising the above said circularisable RNA sequence, DNA sequence or bacterial strain. One example of composition of the invention includes circular RNA purified from producing bacteria, and formulated with excipients for its storage and delivery. Possible excipients include, but are not limited to, polymers, lipids, and/or nanoparticles. Another example of composition includes the vector DNA producing a gene flanked by the inverted repeat (IR) and the live biotherapeutic strain transformed with such vector DNA, and thus able to produce the desired circular RNA.

The above said circularisable RNA sequence, DNA sequence, bacterial strain or composition may be used as a medicament. In particular, the target sequence may encode a therapeutic polypeptide and the circularisable RNA sequence, the DNA sequence, the bacterial strain or the composition may be administered to the gut of an individual.

In different words, the invention describes pairs of short RNA sequences that show the ability to circularise, in bacteria, an RNA target that is located between them. In particular, the RNA between such sequences can be either

coding or non-coding, either related to the bacteria or foreign. The sequences have the structure of inverted repeats (IR). By "inverted repeats", there is intended that one of the sequences is the reverse complement of the other, so that the two sequences anneal, originating a stem-loop structure, where the stem is formed by the annealed IR and the loop is formed by the RNA to be circularised. The functionality of the IRs is independent of the expression vector. Indeed, it has been shown that the functionality is retained in two totally different sequence backgrounds. These are: 1) the original sequence of Lactobacillus acidophilus, from which one example of such IRs was originally identified, and modified pUC19 plasmids, which express the IRs flanking a foreign, unrelated, RNA. Moreover, the functionality of such IRs is independent of the expressing organisms, as the IRs function equally well in artificial systems where Escherichia coli is transformed with modified pUC19 plasmids described below and in the natural host L.acidophilus.

Examples

Example 1

In particular, in one example, such functional IRs are found at the 5' and the 3' of the RNAse P-b subunit (rnpB) gene in Lactobacillus acidophilus. The sequences are 11 nucleotides long and embed a simil-palindromic portion. The

sequences of such IRs are 5'gtgtgaatttc 3' (SEQ ID NO:1) and 5'gaaattcacac 3' (SEQ ID NO:2), where the simil-palindromic portion is underlined. These sequences are responsible for the circularization of the rnpB non-coding RNAin L. acidophilus.

Example 2

In an embodiment of the invention, the IRs were placed at the 5' and 3' end of a circularly permuted (inactive) LacZα gene in pUC19 plasmid, thus producing a first modified pUC19 vector (pUC19-m1) expressing the construct "IR-circularly permuted LacZα-IR" (Figures 2 and 3).

A circularly permuted LacZα was designed by splitting in two portions the original LacZα gene construct and reversing the order of the portions, so that the second portion is positioned 5' to the first portion. Thereby, the obtained DNA construct encodes an inactive RNA, where the 5' portion of the RNA is positioned at its 3' end, and viceversa. The start signal (Shine-Dalgarno sequence) is at the 5' end of the first portion of the circularly permuted LacZα. Similarly, the stop codon is at the 3' end of the second portion of the circularly permuted LacZα.

Example 3

An Escherichia coli DH5α bacteria strain was transformed with pUC19-m1 and grown on media containing IPTG and X-gal, to perform a blue-white colony screening.

IRs showed the ability to circularize LacZα, restoring the functionality of the permuted LacZα as a circular RNA, which correctly produced the LacZα protein subunit. Indeed, in the circular transcript, the correct order of the two portions of LacZα is restored, so that the first portion is at the 5' end of the second portion.

This is demonstrated by the production of blue colonies (Fig. 4A) that grow into blue cultures (Fig. 4B) in a blue-white colony screening of transformed E.Coli DH5α bacteria.

Example 4

The formation of circRNA expressing LacZα from pUC19-m1 has been demonstrated by:

- end-point reverse-transcription PCR (rtPCR) with divergent primers amplifying the circRNA junction point (Fig. 5). The appearance of specific PCR bands of the expected size (lower bands, circled in Fig. 5), which are absent both and in miniprep DNA and in the genomic DNA extracted from transformed bacteria (Fig. 5) confirms the specific formation of the functional transcript. In addition, the appearance of an additional band of higher molecular weight in the RNA sample (upper band, circled in Fig. 5) suggests the amplification of tandem copies of the target RNA, originated by rolling circle reverse transcription of the circRNA.

- Sanger Sequencing of both the low and high molecular

weigth PCR bands confirmed the formation of the circRNA junction.

- RNAse R treatment of total RNA samples obtained by the blue colonies, followed by rtPCR and Sanger sequencing of the PCR product confirms the circularity of the RNA coding for LacZα (Fig. 6). RNAse R destroys linear RNAs, leaving circular RNAs intact. Digestion with RNAse R prior to rtPCR and Sanger Sequencing thus allows to distinguish real circRNA from potential false positives, linear RNA.

These experiments show that both IRs are needed for activity, as a pUC19-m1 lacking the 3′ IR is not able to produce a functional LacZα, leading to white colonies. In addition, pairing of the two IR is crucial for activity, as disruption of pairing by just 3 mutations in one of the two IR abolishes the ability to produce a functional LacZα, leading to white colonies (SEQ ID NO:3 -> 5′ GTATGGATCTC 3′).

The correct orientation of the IR is also important for activity. Indeed, a pUC19-m1 carrying both IR in reverse orientation is not able to produce a functional LacZα, leading to white colonies.

The sequence of the IRs is, instead, not crucial, as the introduction of up to 7 conservative mutations in the 11 bases with random nucleotides is tolerated and retains the ability to produce a functional LacZα, leading to blue

colonies (SEQ ID NO:4 -> 5′ GAGATCCATAC 3′). By "conservative mutations" there is intended mutations of both 5′ and 3′ IR sequences, which preserve the pairing among them.

As LacZα is an example of a foreign RNA, in a different genomic context (pUC19 plasmid) with respect to the original one (L.acidophilus genome) and in a different organism (E.coli) with respect to the original one (L.acidophilus), it can be inferred that the IRs are able to circularise virtually any foreign RNA, independently of the plasmid backbone and independently of the bacterial strain.

Thus, this invention can be used to produce any circular RNA molecule in bacteria, with a simple and easily scalable procedure.

Advantages

The use of circular RNAs naturally solves the problem of instability of RNA therapeutics, due to the resistance of circular RNAs to degradation by nucleases.

In addition, circular RNAs are less immunogenic than linear RNAs. Thus, circular RNAs may be used without chemical modifications.

In addition, chemical or enzymatic production of circular RNAs is costly. According to the invention, circular RNAs are produced by bacterial strains, solving cost issues and achieving an easily scalable and simple methodology.

Finally, bacteria have the potential to be used directly

as a delivery system, thus providing an alternative route to solve the issue of delivery.

According to the invention, the bacterial strain expressing the circularisable RNA sequence is a probiotic bacteria and can be used for the delivery in the gut of proteins or peptides of therapeutic interest. These recombinant probiotic bacteria expressing circRNA therapeutics can therefore be used as live biotherapeutics delivering RNA, rather than protein or peptides.

As mentioned in the introductory paragraphs, delivery of RNA from live biotherapeutics is currently not possible due to the immunogenicity of exogenous linear RNA, and to its instability. The production of circular RNAs in bacteria as disclosed in the present invention enables instead to deliver live biotherapeutics because exogenous circRNAs are much less immunogenic and much more stable than their linear counterparts.

In summary, the advantages of the present invention are:

- that any RNA can be circularized;

- that the first sequence and second sequence (reverse complementary to the first sequence) of the circularizable RNA sequence are very short and manageable sequences, easy to insert anywhere needed;

- independence on the genomic context;

- independence on the producing bacterial strain;

- circular RNA therapeutics potentially do not need modifications:

- circular RNA therapeutics do not need complexation with nanoparticles for the delivery;

- circular RNAs are also stable in the extracellular environment and can be used as “naked RNA”;

- the producing bacteria can potentially be used for the delivery of the circular RNA, analogously to protein/peptides producing live biotherapeutics.

CLAIMS

1. A spontaneously circularisable RNA sequence comprising from 5′ to 3′ or from 3′ to 5′:

- a first sequence comprising from 7 to 30 nucleotides;
- at least one target sequence to be expressed and/or translated into a polypeptide in a host organism; and
- a second sequence reverse complementary to the first sequence.

2. The spontaneously circularisable RNA sequence according to claim 1, wherein the first sequence comprises from 9 to 15 nucleotides.

3. The spontaneously circularisable RNA sequence according to claim 1 or 2, wherein 5 to 15 nucleotides of the first sequence form a palindromic sequence.

4. The spontaneously circularisable RNA sequence according to any of the preceding claims, wherein the first sequence is 5′ of the target sequence and the second sequence is 3′ of the target sequence.

5. The spontaneously circularisable RNA sequence according to any of the preceding claims, wherein the spontaneously circularisable RNA sequence consists of the first sequence, the at least one target sequence and the second sequence.

6. A DNA sequence encoding the spontaneously circularisable RNA sequence according to any of the preceding

claims.

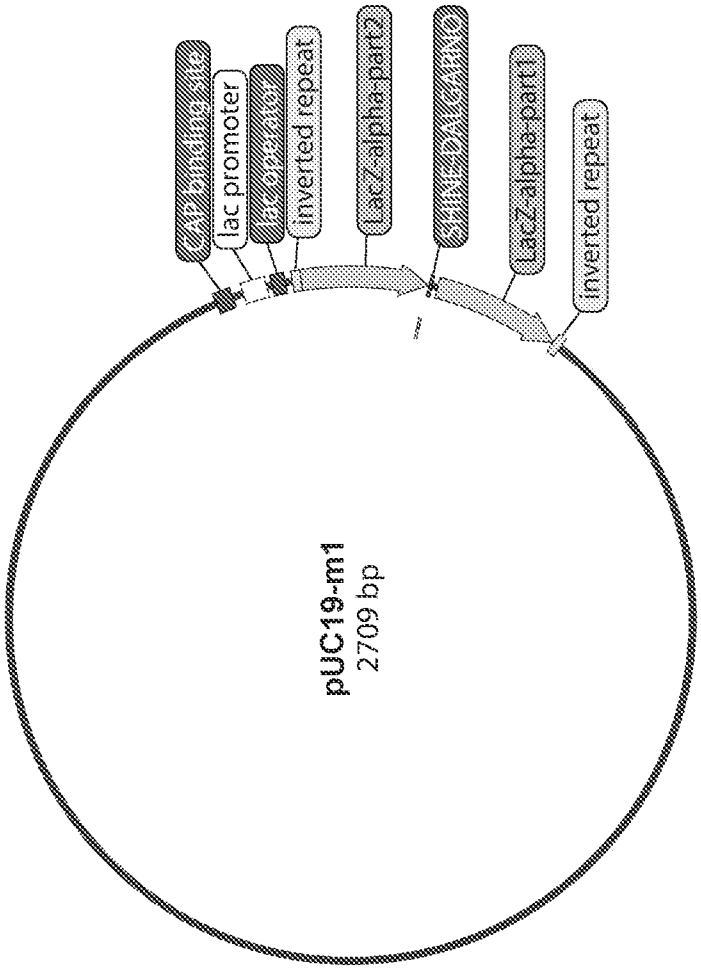
7. A bacterial strain expressing the spontaneously circularisable RNA sequence according to any of claims 1 to 5 or the DNA sequence according to claim 6.

8. The bacterial strain according to claim 7, wherein the bacterial strain is a probiotic bacteria.

9. A composition comprising a spontaneously circularisable RNA sequence according to any of claims 1 to 5, a DNA sequence according to claim 6, or a bacterial strain according to claim 7 or 8.

10. A spontaneously circularisable RNA sequence according to any of claims 1 to 4, a DNA sequence according to claim 6, a bacterial strain according to claim 7 or 8, or a composition according to claim 9 for use as a medicament.

11. The spontaneously circularisable RNA sequence, the DNA sequence, the bacterial strain, or the composition for use according to claim 10, wherein the target sequence encodes for a therapeutic polypeptide and the spontaneously circularisable RNA sequence, the DNA sequence, the bacterial strain or the composition is administered to the gut of an individual.


Ampicillin ori
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LacZ alpha part2
Stuffer DNA/MCS
LacZ alpha part1
inverted repeat
pUC19-m1
2709 bp

FIG. 2

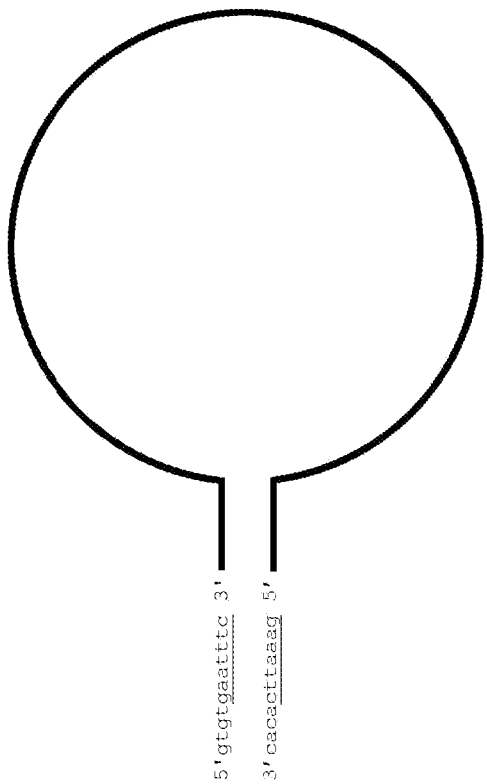
5'gtgtgaattc 3'
3'cacacttaaag 5'

FIG. 1

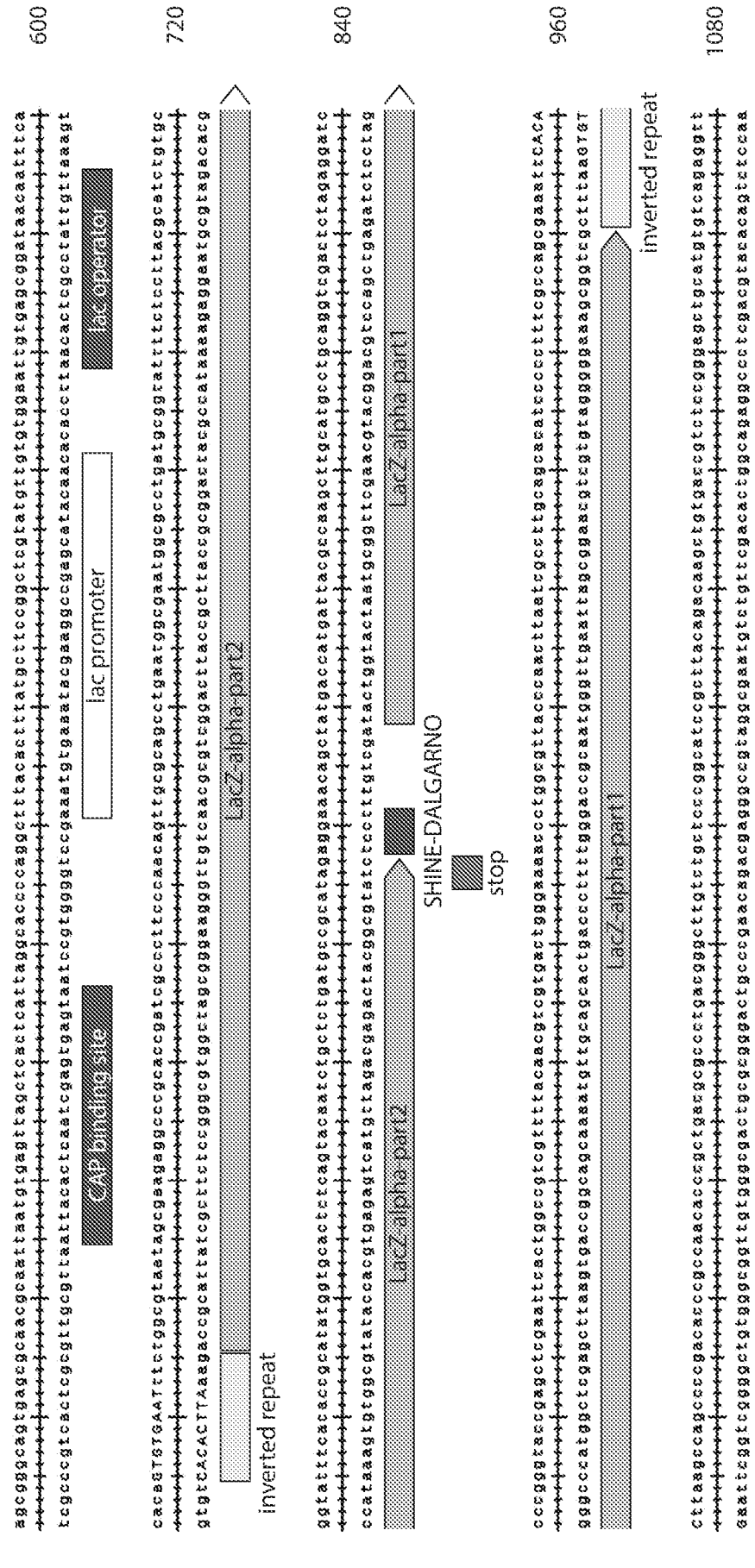
600
720
840
960
1080
CAP binding site
lac promoter
lac operator
LacZ alpha part2
inverted repeat
LacZ alpha part2
SHINE-DALGARNO
stop
LacZ alpha part1
LacZ alpha part1
inverted repeat
inverted repeat

FIG. 3

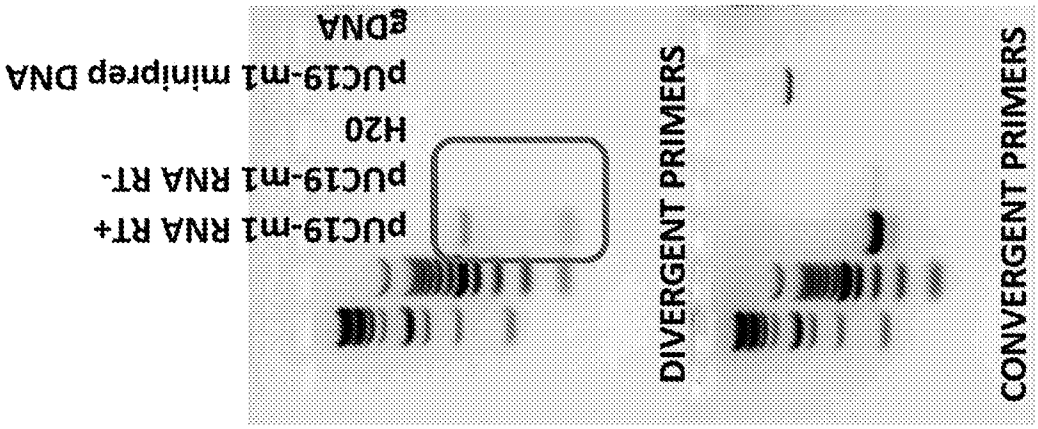
DIVERGENT PRIMERS
CONVERGENT PRIMERS
pUC19-m1 RNA RT+
pUC19-m1 RNA RT-
H20
pUC19-m1 miniprep DNA
gDNA

FIG. 5

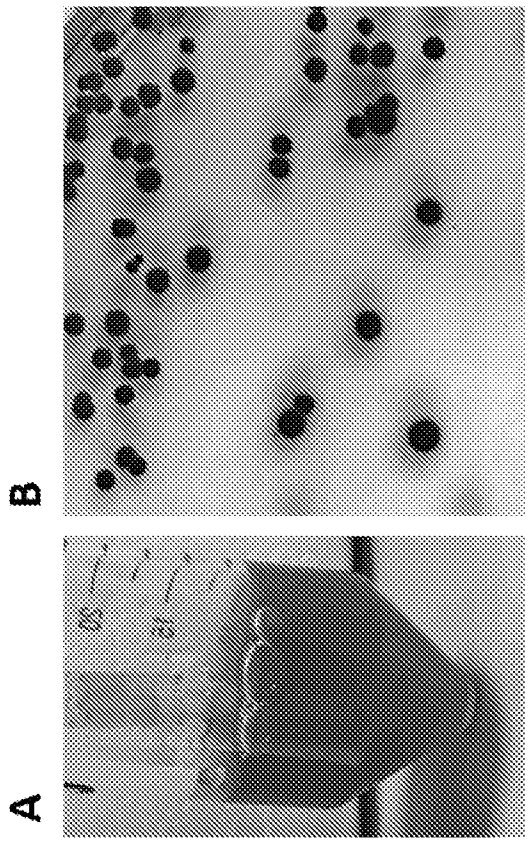
A
B
pUC19-m1

FIG. 4

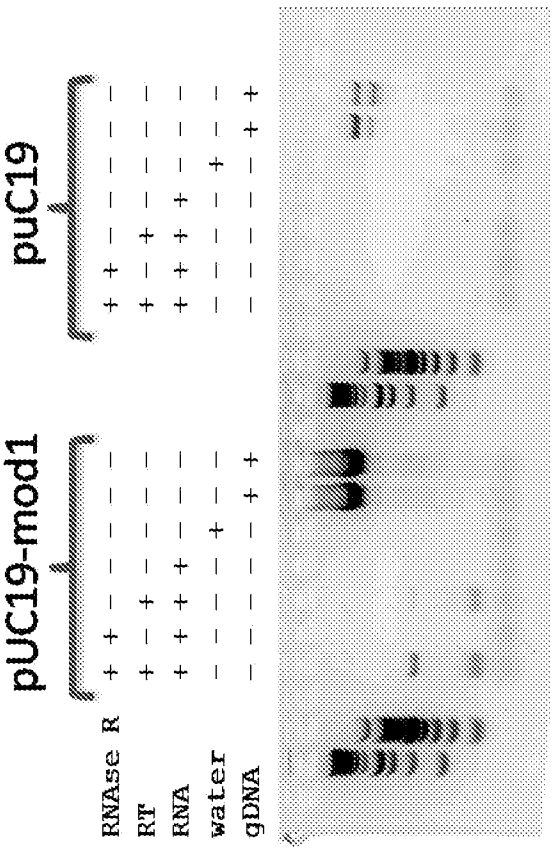

FIG. 6

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2024/055739

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12N9/22 C12N15/11 C12N15/63 C12N15/66 C12N15/70
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N C40B

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

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

EPO-Internal, EMBASE, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2022/256578 A2 (BEAM THERAPEUTICS INC [US]) 8 December 2022 (2022-12-08)	1,2,4-7, 9-11
Y	p.2 l.1-10, 12, p.2 l.20 - p3 l.4, p.5 l.1-14, Fig.13G, p.67 l.8	3,8

X	CA 3 181 628 A1 (WILLIAMS MARTIN [AR]; SYTE BIO INC [US]) 16 May 2023 (2023-05-16)	1,2,4-7, 9,10
Y	the whole document & EP 4 219 723 A1 (WILLIAMS MARTIN [AR]; SYTE BIO INC [US]) 2 August 2023 (2023-08-02) par.53-62, 101, par.84 - 92	3,8

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[x] Further documents are listed in the continuation of Box C.

[x] See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 September 2024	30/09/2024

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Bonello, Steve

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Ford Ethan ET AL: "Synthesis of circular RNA in bacteria and yeast using RNA cyclase ribozymes derived from a group I intron of phage T4 (spdng/RNA /Eschenclhi coli/Skecmmyces ceremisia/td gene)", , 22 December 1993 (1993-12-22), XP093109234, DOI: 10.1073/pnas.91.8.3117 Retrieved from the Internet: URL:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC43526/pdf/pnas01130-0248.pdf [retrieved on 2023-12-05] cited in the application p.3117 col.2 par.2, p.3118 Fig.1, p.3119 col.1 par.1, p.3129 col.1 par.2, p.33121 col.1 par.1,2 -----	3,8
Y	WESSELHOEFT R. ALEXANDER ET AL: "Engineering circular RNA for potent and stable translation in eukaryotic cells", NATURE COMMUNICATIONS , vol. 9, no. 1 6 July 2018 (2018-07-06), XP093109188, UK ISSN: 2041-1723, DOI: 10.1038/s41467-018-05096-6 Retrieved from the Internet: URL:https://www.nature.com/articles/s41467-018-05096-6 [retrieved on 2023-12-05] p.2 col.1 par.2,3, col.2 par.1, Fig.1, p.4 col.1 par.2, p.8 col.2 par.2 -----	1-11
Y	WESSELHOEFT R ALEXANDER ET AL: "RNA Circularization Diminishes Immunogenicity and Can Extend Translation DurationIn Vivo", MOLECULAR CELL, vol. 74, no. 3, 2 May 2019 (2019-05-02), page 508, XP085676575, ISSN: 1097-2765, DOI: 10.1016/J.MOLCEL.2019.02.015 cited in the application p.508 col.2 par.2, p.509 Figure, p.510 col.1 par.2 ----- -/--	1-11

1

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Chen Chuyun ET AL: "A flexible, efficient, and scalable platform to produce circular RNAs as new therapeutics", bioRxiv, 1 June 2022 (2022-06-01), XP055962269, DOI: 10.1101/2022.05.31.494115 Retrieved from the Internet: URL:https://www.biorxiv.org/content/10.1101/2022.05.31.494115v2.full.pdf [retrieved on 2022-09-19] p.5 par.1	1-11
Y	CHEN XINJIE ET AL: "Circular RNA: Biosynthesis in vitro", FRONTIERS IN BIOENGINEERING AND BIOTECHNOLOGY, vol. 9, 30 November 2021 (2021-11-30), XP055962206, DOI: 10.3389/fbioe.2021.787881 p.4 col.1 par.2, p.6 col.1 par.1,2 p.5 col.2 par.2, p.7 Fig.4	1-11
Y	WO 2023/015192 A1 (10X GENOMICS INC [US]) 9 February 2023 (2023-02-09) par.5, 6, 10-12, 174, 117	1-11
T	So Umekage ET AL: "In Vivo Circular RNA Expression by the Permuted Intron-Exon Method" In: "Innovations in Biotechnology", 17 February 2012 (2012-02-17), InTech, XP055295215, ISBN: 978-953-51-0096-6 DOI: 10.5772/28220, p.75 par.2	1

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2024/055739

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

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed.

   b. [ ] furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

      [ ] accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. [ ] With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IB2024/055739

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2022256578 A2	08-12-2022	AU 2022285744 A1	14-12-2023
		CA 3221008 A1	08-12-2022
		EP 4347830 A2	10-04-2024
		WO 2022256578 A2	08-12-2022
CA 3181628 A1	16-05-2023	AU 2022271382 A1	01-06-2023
		CA 3181628 A1	16-05-2023
		EP 4219723 A1	02-08-2023
		JP 2023073997 A	26-05-2023
		US 2023235337 A1	27-07-2023
WO 2023015192 A1	09-02-2023	CN 118043476 A	14-05-2024
		EP 4381095 A1	12-06-2024
		US 2023057571 A1	23-02-2023
		WO 2023015192 A1	09-02-2023