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(71) Applicants: **THE UNIVERSITY OF TOKYO** [JP/JP];
3-1, Hongo 7-chome, Bunkyo-ku, Tokyo, 1138654 (JP).
CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE [FR/FR]; 3, rue Michel Ange, 75794 Paris Cedex
16 (FR).

(72) Inventors: **GENOT, Anthony**; c/o The University of
Tokyo, 3-1, Hongo 7-chome, Bunkyo-ku, Tokyo, 1138654

(JP). **FUJII, Teruo**; c/o The University of Tokyo, 3-1,
Hongo 7-chome, Bunkyo-ku, Tokyo, 1138654 (JP). **BAC-
COUCHE, Alexandre**; c/o The University of Tokyo, 3-1,
Hongo 7-chome, Bunkyo-ku, Tokyo, 1138654 (JP).

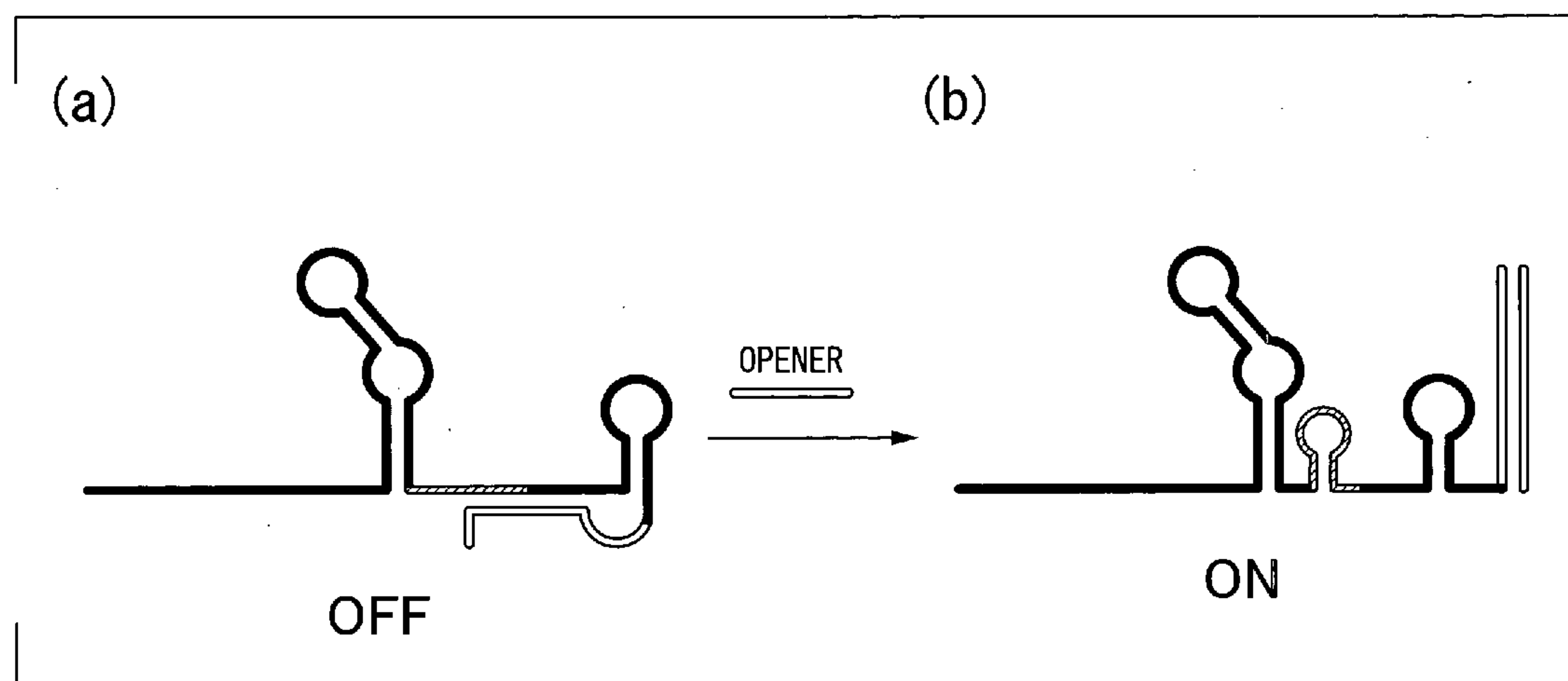
(74) Agent: **TANAI, Sumio** et al.; 1-9-2, Marunouchi, Chiyo-
da-ku, Tokyo, 1006620a (JP).

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FIG. 4



(57) Abstract: The present invention relates to a CRISPR-Cas system guide RNA consisting of one or more polynucleotide(s), wherein the guide RNA comprises: (A) the following elements (a) to (d), arranged in this order from 5' to 3': (a) a spacer which can hybridize to a target polynucleotide; (b) a first functional module comprising a crRNA:tracrRNA duplex; (c) a second functional module comprising a first switching domain; and optionally (d) a third functional module; and (B) a second switching domain, wherein at least a portion of the first switching domain is substantially complementary to at least a portion of the second switching domain, and when an opener is present, the opener disrupts the hybridization between the first switching domain and the second switching domain and the guide RNA forms at least one stem within the first switching domain, and thereby becomes capable of activating a Cas protein. The present invention provides conditional activation of CRISPR-Cas system using various kinds of triggers, for example an endogenous molecule.

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DESCRIPTION

TITLE OF INVENTION

GUIDE RNA FOR CONDITIONAL AND POST-TRANSCRIPTIONAL CONTROL OF CRISPR-CAS PROTEIN

TECHNICAL FIELD

[0001] The present invention relates to a novel guide RNA that can be specifically activated under a desired condition, as well as a polynucleotide encoding such a guide RNA, a composition and a kit comprising such a guide RNA, and a method of modifying a target polynucleotide using such a guide RNA.

BACKGROUND ART

[0002] A CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) associated (Cas) protein, Cas9, is an RNA-programmed endonuclease that enables the efficient, sequence-specific modification of target loci in a genome. Although the CRISPR-Cas9 system was originally found in bacteria, it is now widely applied in various organisms as a powerful tool for genome engineering. In addition to genome editing, it is now used for variety of purposes, such as to repress or activate gene expression, image DNA loci, generate targeted mutational diversity, and modify epigenetic markers. This broad applicability of the CRISPR-Cas9 system is based on its ability to target virtually any DNA sequence via complementary base pairing with a guide RNA, which forms a complex with Cas9 and directs it to the target polynucleotide.

[0003] As the applications of the CRISPR-Cas9 system become diverse, there has been an increased interest in developing strategies to regulate the CRISPR-Cas9 activity. Once Cas9 and a guide RNA are both present in a cell, the tandem will inevitably cleave its target, irrespective of the type, environment, internal state, or developmental stage of the cell. However, mistimed, misplaced or mismatched cleavage by Cas9 could be detrimental to the cell. Transcriptional control of Cas9 suffers from the classical drawbacks of genetic circuits (such as delayed response, poor predictability, restricted repertoire of actionable transcription factors, overloading of the host's machinery). Post-transcriptional strategies that modulate the activity of CRISPR-Cas9 with physical signals (such as light, temperature, magnetic fields) or biochemical signals (small molecule inducers, anti-CRISPR proteins) are limited by their low throughput, their lack of autonomy or multiplexing, their cytotoxicity, their low penetrability in deep tissues, as well as the difficulty of localizing or timing the delivery of the signal to specific cellular types or developmental stages.

[0004] An alternative is to regulate the activity of the guide RNA with nucleic acids as signals - which offers specific, multiplexed and predictable regulation. WO 2015/168404 discloses a gRNA to which additional nucleotide sequences have been added to the 3' and/or 5' ends of the gRNA such that complementary sequences hybridize to form a stem domain in the secondary structure that constrains the distance between the space and scaffold domains. WO 2017/223449 discloses a switchable guide RNA wherein the guide sequence (spacer) or the sequence upstream of the guide sequence is masked by the extended 5' or 3' end. WO 2018/231730 discloses a conditional guide RNA with an additional domain in the 5' terminus of the guide RNA and wherein the activated/inactivated state of the guide RNA is switched

when a polynucleotide is hybridized to the additional domain. Hanewich-Hollatz et al. (ACS Cent. Sci. 2019, 5, 7, 1241-1249) also discloses conditionally activating or inactivating a guide RNA by hybridization of an RNA trigger. However, most reported designs, including those mentioned above, cannot handle orthogonal RNA triggers and DNA targets because of the sequence constraints - thus excluding the most sought-after applications where either the guide or the target is endogenous. Also, long mRNAs which are used as the target in some of the systems could cause formation of secondary structures and impair the activation of the guide RNA.

SUMMARY OF INVENTION

[0005] The objective of the present invention is therefore to solve the above problems of the prior art and to provide a novel system of conditional activation of a CRISPR-Cas system using various kinds of triggers.

[0006] The above objective can be achieved by a CRISPR-Cas system guide RNA consisting of one or more polynucleotide(s), wherein the guide RNA comprises:

(A) the following elements (a) to (d), arranged in this order from 5' to 3':

- (a) a spacer which can hybridize to a target polynucleotide;
- (b) a first functional module comprising a crRNA:tracrRNA duplex;
- (c) a second functional module comprising a first switching domain; and
- optionally (d) a third functional module; and

(B) a second switching domain,

wherein at least a portion of the first switching domain is substantially complementary to at least a portion of the second switching domain, and when an opener is present, the opener disrupts the hybridization between the first switching domain and the second switching domain and the guide RNA forms at least one stem within the first switching domain, and thereby becomes capable of activating a Cas protein.

[0007] The second functional module may comprise or consist of the sequence represented by formula (I):



wherein:

N each independently represents any base selected from A, C, G, and U;

m is an integer ranging from 1 to 4, preferably 2;

p is an integer ranging from 1 to 4, preferably 2;

q is an integer ranging from 1 to 10, preferably from 3 to 7, more preferably 5;

r is an integer ranging from 1 to 10, preferably from 3 to 8, more preferably 6; and

(X)_m represents the sequence 5'- X¹...X^{m-1}X^m -3';

(Y)_m represents the sequence 5'- Y^mY^{m-1}...Y¹ -3';

the mth X (X^m) and the mth Y (Y^m) are capable of being hybridized to form a stem and

each of the pair of X^m/Y^m is independently selected from C/G, G/C, A/U, and U/A, and

wherein the first switching domain comprises at least region (X)_m or (Y)_m.

[0008] The second functional module may comprise or consist of the sequence represented by formula (II):



wherein:

N, p, q, and r have the meanings as defined above for formula (I); and the pairs X^1/Y^1 and X^2/Y^2 are each independently selected from C/G, G/C, A/U, and U/A, preferably both pairs X^1/Y^1 and X^2/Y^2 are G/C.

[0009] The second functional module may comprise or consist of the sequence represented by formula (III):



wherein:

N, X^1 , Y^1 , X^2 , and Y^2 have the meanings as defined above for formulae (I) and (II).

[0010] The second switching domain may be located at the 5'-terminus, at the 3'-terminus, or inside or between any of the elements (a) to (d).

[0011] The second switching domain may be substantially complementary to at least a portion of the opener, and the opener may disrupt the hybridization between the first switching domain and the second switching domain by hybridizing to the second switching domain, preferably by a toehold-mediated strand displacement.

[0012] The guide RNA may further comprise a third switching domain wherein at least a portion of the third switching domain is substantially complementary to at least a portion of a fourth switching domain, and when at least a portion of the third switching domain and at least a portion of the fourth switching domain are hybridized, the structure of the guide RNA may be altered such that the guide RNA does not maintain the function of activating a Cas protein.

[0013] The third switching domain may be located within the first functional module.

[0014] The fourth switching domain may be present as a part of the guide RNA and may be located at the 5'-terminus, at the 3'-terminus, inside or between any of the elements (a) to (d), or in the 5'-side or the 3'-side of the second switching domain, preferably in the 3'-side of the second switching domain, or the fourth switching domain may be present independently of the guide RNA.

[0015] The present invention also relates to a polynucleotide encoding at least one guide RNA according to the present invention, wherein preferably said polynucleotide may be comprised in a vector.

[0016] The present invention also relates to vector comprising at least one polynucleotide according to the present invention.

[0017] The present invention also relates to a composition comprising at least one guide RNA according to the present invention or at least one polynucleotide encoding thereof and at least one Cas protein or at least one polynucleotide encoding thereof, and optionally further comprising at least one opener or at least one polynucleotide encoding thereof.

[0018] The composition may be for treating, preventing, and/or detecting at least one disease or disorder in a subject.

[0019] The present invention also relates to a method of regulating the activation of a guide RNA according to the present invention, comprising the step of hybridizing at least a portion of the first switching domain and at least a portion of the second switching domain and/or disrupting the hybridization between the first switching domain and the second switching domain using a first opener, and optionally, when the guide RNA comprises a third switching domain, the step of hybridizing at least a portion of the third switching domain and at least a portion of a fourth switching domain and/or disrupting the hybridization between the third switching domain and the fourth switching domain using a second opener which may be the same as or different from the first opener.

[0020] The present invention also relates to a method of cleaving at least one target polynucleotide, modifying the sequence of at least one target polynucleotide, and/or altering the expression of at least one gene encoded by at least one target polynucleotide, comprising the step of contacting the target polynucleotide with at least one guide RNA according to the present invention, at least one opener, and at least one Cas protein.

[0021] The opener may be an RNA.

[0022] The opener may also be an endogenous RNA.

[0023] The opener may also be a micro RNA.

[0024] The Cas protein may be specifically activated in a certain type of cell, tissue, or individual; at a certain time point such as at a certain phase of the cell cycle; under a certain physiological condition such as in a patient with a specific disease; or in response to certain external stimuli such as temperature, light, force, and pH.

[0025] The present invention also relates to a kit comprising at least one guide RNA according to the present invention, or at least one polynucleotide encoding thereof, and optionally comprising at least one opener or at least one polynucleotide encoding thereof and/or at least one Cas protein or at least one polynucleotide encoding thereof.

[0026] The present invention is particularly suitable for condition-specific activation of a CRISPR-Cas system, for example, cell-specific or tissue-specific gene editing. Also, unlike conventional physical (such as light) or chemical stimuli, the present invention ensures multiplexing of the CRISPR-Cas system and allows use of multiple sets of guide RNA, Cas protein, and opener in the same environment. Furthermore, compared to the conventional methods of masking a region in a guide RNA, the structure of the guide RNA of the present invention is less complicated, which is advantageous in terms of the ease of preparation and reduced formation of secondary structures.

BRIEF DESCRIPTION OF DRAWINGS

[0027] Figure 1 illustrates schematic images of canonical structures of (A) single guide RNA and (B) dual guide RNA.

[0028] Figure 2 illustrates an example of a guide RNA for *Streptococcus pyogenes* Cas9 (SpCas9). “N” represents any base selected from A, C, G, and U. (ref. Briner *et al.*, Molecular Cell 56, 333-339, 2014)

[0029] Figure 3 illustrates consensus sequences and secondary structures of the guide RNAs from (A) *Streptococcus* and (B) *Lactobacillus* species. Black dots represent variable bases with at least three possible bases, and base positions not always present are circled. Circles between positions indicate base pairing present in only some family members. (ref. Briner *et al.*, Molecular Cell 56, 333-339, 2014)

[0030] Figure 4 illustrates the mechanism of the conditional activation of the guide RNA of the present invention. (a) When an opener is absent, at least a part of the first switching domain and a part of the second switching domain are hybridized, which prevents the first switching domain from forming a stem within the domain, and as such the guide RNA cannot maintain the canonical structure and is inactivated (“OFF” state). The unpaired 3’ terminal portion of the second switching domain is a “toehold” which enables toehold strand-mediated displacement by a single-stranded opener. (b) When an opener binds to the “toehold”, it disrupts the hybridization between the first switching domain and the second switching domain and the unbound (i.e. single-stranded) first switching domain then becomes able to form at least one stem structure within the domain, returning the guide RNA to its canonical structure and retrieving its function of activating the Cas protein (“ON” state).

[0031] Figure 5 illustrates an embodiment of activating the guide RNA of the present invention. (a) In this embodiment, the guide RNA and the opener are designed such that the opener hybridizes to the loop of a hairpin formed by hybridization between the first switching domain and the second switching domain. (b) When the opener binds to the loop, the hybridization between the first switching domain and the second switching domain is disrupted.

[0032] Figure 6 illustrates examples of the guide RNA of the present invention. “N” represents any base selected from A, C, G, and U.

[0033] Figure 7 illustrates schematic images of the fluorescence assays used in the examples of the application, (a) two-step assay and (b) one-pot assay. F: fluorophore, Q: quencher.

[0034] Figure 8 illustrates the results of the two-step fluorescence assay of Example 1. Each column used the same (or no) guide RNA and each line used the same (or no) opener. For each graph, the X axis represents time [min] and the Y axis represents RFU (relative fluorescence units) [arbitrary unit].

[0035] Figure 9 illustrates the results of the one-pot fluorescence assay of Example 1. Each column used the same (or no) guide RNA added to the assay as a dsDNA and each line used the same (or no) opener. For each graph, the X axis represents time [min] and the Y axis represents RFU (relative fluorescence units) [arbitrary unit].

[0036] Figure 10 illustrates schematic images of regulating the activity of the guide RNA with two openers ((a) to (d)) and the results of the two-pot fluorescence assay of Example 2 ((e) and (f)). For the graphs in (e) and (f), the arrow indicates the time point when the Cas9 was injected to the pre-incubated mixture of the guide RNA, miRNAs, and the probe. The X

axis represents time [min] and the Y axis represents RFU (relative fluorescence units) [arbitrary unit].

[0037] Figures 11A and 11B illustrate schematic images of regulating the activity of the guide RNA with a polynucleotide encoding a fourth switching domain and an opener ((a) to (d) in Fig. 11A) and the results of the one-pot fluorescence assay of Example 3 ((a) and (b) in Fig. 11B). For the graphs in (a) and (b) in Fig. 11B, the X axis represents time [min] and the Y axis represents RFU (relative fluorescence units) [arbitrary unit].

DESCRIPTION OF EMBODIMENTS

[0038] After diligent research, the inventors surprisingly discovered that the activity of the guide RNA can be regulated by adding an additional domain, preferably a “tail” attached to the 3' terminus of the guide RNA, which reversibly inactivates the guide RNA when it is hybridized to at least a portion of the “nexus” region of the guide RNA, and that the inactive guide RNA may be activated by an “opener” that is capable of disrupting this hybridization.

Conditionally-activated guide RNA

[0039] In a first aspect, the present invention relates to a CRISPR-Cas system guide RNA consisting of one or more polynucleotide(s), wherein the guide RNA comprises the following elements (a) to (d), arranged in this order from 5' to 3': (a) a spacer which can hybridize to a target polynucleotide; (b) a first functional module comprising a crRNA:tracrRNA duplex; (c) a second functional module comprising a first switching domain; and optionally (d) a third functional module; and (B) a second switching domain, wherein at least a portion of the first switching domain is substantially complementary to at least a portion of the second switching domain, and when an opener is present, the opener disrupts the hybridization between the first switching domain and the second switching domain and the guide RNA forms at least one stem within the first switching domain, and thereby becomes capable of activating a Cas protein.

Guide RNA

[0040] The first aspect of the invention relates to a CRISPR-Cas system guide RNA. The term “guide RNA” as used herein refers to an RNA molecule or a complex of two or more RNA molecules which can recognize the target polynucleotide sequence and form a complex with a Cas protein and thereby direct the Cas protein to the target site.

[0041] According to the first aspect of the invention, the guide RNA comprises:

(A) the following elements (a) to (d), arranged in this order from 5' to 3':

- (a) a spacer which can hybridize to a target polynucleotide;
- (b) a first functional module comprising a crRNA:tracrRNA duplex;
- (c) a second functional module comprising a first switching domain; and
- optionally (d) a third functional module, and

(B) a second switching domain.

[0042] The guide RNA of the present invention may consist of a single polynucleotide, or may comprise two or more polynucleotides that may be linked to one another by any methods that are known in the art to join polynucleotides, such as hybridization. In one embodiment, a guide RNA comprising two or more polynucleotides may be designed by the following steps.

First, the sequence of a known wild-type or single guide RNA is divided into two or more fragments. The sequence may be split at any site as long as the division does not affect its inherent function of recognizing and binding to a target polynucleotide and activating a Cas protein. Then, one of a pair of complementary strands with any sequence is added to the end of one fragment, and the other one of the pair is added to the end of the other fragment such that the added strands can hybridize to link the two fragments together. As used herein, when two or more polynucleotides, fragments, elements, etc. are “arranged”, this means that the polynucleotides, fragments, elements, etc. are aligned such that each of the polynucleotides, fragments, elements, etc. are either linked by a covalent bond, or are hybridized via hydrogen bond. In the present application, unless otherwise specified, the arranged polynucleotides, fragments, elements are denoted in a direction from the 5' terminus to the 3' terminus.

[0043] In one embodiment, each of the elements (a) to (d) of the guide RNA may be covalently linked to one another via phosphodiester bonds. Alternatively, any two adjacent elements may be linked by hybridization of two complementary sequences. For example, elements (a) and (b) may be covalently linked (i.e. the two elements are transcribed together as a single polynucleotide) or they may be post-transcriptionally linked by adding one of the complementary strands to each of the elements (for example (a)-C and C'-(b) wherein sequences C and C' are complementary) and hybridizing the two elements ((a)-C:C'-(b)) (as used herein, the symbol “-” denotes a covalent bond and the symbol “:” denotes hybridization between the two bases via a hydrogen bond). As described above, the guide RNA may be split at any site, for example between the two adjacent elements and within the element.

[0044] In one embodiment, the guide RNA of the present invention may be a dual guide RNA or a single guide RNA. As used herein, the term “dual” guide RNA refers to a duplex consisting of two RNA molecules, namely CRISPR RNA (crRNA) and a *trans*-activating crRNA (tracrRNA). The term “single” or “chimeric” guide RNA as used herein refers to an artificial RNA consisting of one (single) RNA molecule mimicking the crRNA:tracrRNA duplex, and is typically generated by the truncation of the 3' terminal portion of the crRNA and the 5' terminal portion of the tracrRNA and connecting the two ends with a short loop structure, such as “GAAA”. In a preferred embodiment, the guide RNA of the present invention is a single guide RNA.

[0045] A guide RNA typically consists of the following modules, arranged in this order from 5' to 3': spacer which is responsible for targeting a polynucleotide; crRNA:tracrRNA duplex consisting of lower stem, bulge, and upper stem; nexus; bridge; and one or more hairpin(s) (Fig.1). According to the first aspect of the application, the first functional module corresponds to the crRNA:tracrRNA duplex, the second functional module corresponds to the nexus and the bridge, and the third functional module corresponds to the one or more hairpin(s).

[0046] The structure of the guide RNA is well-conserved among different bacterial species, and the present invention can utilize any of such guide RNAs, either in the form of a dual guide RNA or a single guide RNA, as a template to which necessary mutations may be introduced. Non-limiting examples of guide RNAs that can be used as a template of the guide RNA of the present invention include guide RNAs of the *Streptococcus* spp. such as *S. pyogenes*, *Lactobacillus* spp. such as *L. plantarum*, *Campylobacter* spp. such as *C. jejuni*, and *Staphylococcus* spp. such as *S. aureus*. For example, a generic sequence of a single guide RNA for *S. pyogenes* Cas9:

NNNNNNNNNNNNNNNNNNNNNGUUUAGAGCUAGAAAUAGCAAGUAAAAUAA

GGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU (SEQ ID NO: 1), whose secondary structure is shown in Fig. 2, may be used as a template for the guide RNA of the present invention. Also, sequences disclosed in Briner *et al.*, Molecular Cell 56, 333-339, 2014 (the entire contents of which are incorporated herein by reference) (Fig. 3) can be used as a template of the guide RNA of the present invention.

Spacer

[0047] According to the first aspect of the present invention, the guide RNA of the present invention comprises a spacer. As used herein, the term “spacer” refers to a region in the guide RNA that is responsible for recognizing the target polynucleotide and its sequence is designed to be substantially complementary to the sequence of the target polynucleotide. A Cas protein inherently recognizes the sequence called “protospacer adjacent motif (PAM)” and typically cleaves the sequence upstream of the PAM. Therefore, the sequence upstream of the PAM is usually selected and designated as a target polynucleotide. The length and the sequence of the PAM vary depending on the origin of the Cas protein. For example, in the case of *Streptococcus pyogenes* Cas9 (SpCas9), the PAM sequence is “5'-NGG” and for the two Cas9 proteins from *Streptococcus thermophilus*, CRISPR3 Cas9 and CRISPR1 Cas9, the PAMs are “5'-NGGNG” and “5'-NNAGAA”, respectively, wherein N represents any base selected from A, C, G, and T. The distance between the PAM and the cleavage site also differs depending on the bacterial species, but most Cas9 orthologs cleave 3 bases upstream of the PAM sequence.

[0048] In one embodiment, the spacer in the guide RNA of the present invention is from 15 nt to 25 nt, preferably from 17 nt to 23 nt, more preferably 19 nt to 21 nt in length, and most preferably 20 nt in length.

First functional module / crRNA:tracrRNA

[0049] According to the first aspect of the present invention, the first functional module comprises a crRNA:tracrRNA duplex. The term “crRNA:tracrRNA duplex” as used herein refers to a component of the guide RNA of the present invention in which crRNA and tracrRNA are hybridized, and encompasses both the complex of two separate RNAs as seen in a naturally-occurring dual guide RNA (Fig. 1(B)), and also the loop-connected structure as seen in an artificial single guide RNA (Fig. 1(A)).

Second functional module / nexus, bridge

[0050] According to the first aspect of the present invention, the guide RNA of the present invention comprises a first switching domain within the second functional module which comprises the nexus domain and the bridge domain. Preferably, the first switching domain contains at least a portion of the nexus domain. It is known that the nexus interacts with two regions from the two lobes of a Cas protein, i.e., the alpha-helical lobe, also known as the recognition (REC) lobe, and the nuclease (NUC) lobe. Also, when a guide RNA interacts with a Cas protein, the nexus is located at the interface of the REC lobe and the NUC lobe of the Cas protein. Therefore, without being bound to any particular theory, targeting the nexus domain is particularly effective in regulating the activity of a CRISPR-Cas system through the guide RNA because the nexus domain is considered to play an important role in the activation of the Cas protein by the guide RNA. In one embodiment, the first switching domain may also contain at least a portion of the bridge domain in addition to the nexus domain.

First and second switching domains

[0051] According to the first aspect of the present invention, the first switching domain and the second switching domain are designed such that (1) at least a portion of the first switching domain is substantially complementary to at least a portion of the second switching domain, and (2) when an opener is present, the hybridization between the first switching domain and the second switching domain is disrupted, and the guide RNA forms at least one stem within the first switching domain, and thereby becomes capable of activating a Cas protein.

[0052] As such, the guide RNA of the present invention may be prepared by genetically-modifying or introducing mutations to a known guide RNA, either in the duplex form or in the chimeric form. Such modifications may be made by the deletion, addition, and/or substitution of one or more nucleotide(s) in one or more domains of the guide RNA. Methods for introducing mutations into a polynucleotide, more specifically a guide RNA, are well-known in the art. As used herein, when a polynucleotide or polypeptide has been “mutated”, “genetically-modified”, or “genetically-engineered”, this means that at least one nucleotide or amino acid residue of said polynucleotide or polypeptide has been changed (i.e. deleted, added and/or substituted) as compared to the “original”, “wild type”, “naturally-occurring”, “native”, or “template” polynucleotide or polypeptide. In one embodiment, the guide RNA of the present invention is mutated at least in the first switching domain, in addition to the spacer which is custom-designed by its nature according to the sequence of the target polynucleotide and also the second switching domain which is an artificial sequence added to the guide RNA. In another embodiment, the guide RNA of the present invention may be modified also in other regions, such as in the crRNA:tracrRNA duplex or in the one or more hairpin(s).

[0053] Alternatively, it is also possible to provide the guide RNA of the present invention without mutating the sequence. In one embodiment, the guide RNA of the present invention is not mutated in the second functional module, preferably in the nexus region.

[0054] According to the first aspect of the present invention, at least a portion of the first switching domain is substantially complementary to at least a portion of the second switching domain. In one embodiment, the entire portion of the first switching domain is substantially complementary to at least a portion of the second switching domain. In another embodiment, at least a portion of the first switching domain is substantially complementary to the entire portion of the second switching domain. In yet another embodiment, the entire portion of the first switching domain is substantially complementary to the entire portion of the second switching domain. In this application, when two strands are “substantially complementary”, this means that the complementarity of the two strands is sufficient to form and maintain hybridization between the two strands, and, if the formation or disruption of hybridization triggers or keeps from triggering a certain downstream reaction or function, the complementarity is sufficient to trigger or keep from triggering such a reaction or function. For example, when at least a portion of the first switching domain is substantially complementary to at least a portion of the second switching domain, the complementarity is sufficient to inhibit the formation of a stem within the first switching domain, thereby suppressing the activity of the guide RNA of the present invention.

[0055] In one embodiment, the first switching domain is at least 5 nt, at least 6 nt, at least 7 nt, at least 8 nt, at least 9 nt, at least 10 nt, at least 11 nt, at least 12 nt, at least 13 nt, at least 14 nt, at least 15 nt, at least 16 nt, at least 17 nt, at least 18 nt, at least 19 nt, or at least 20 nt in

length. In another embodiment, the first switching domain is 50 nt or less, 40 nt or less, 30 nt or less, 20 nt or less, 19 nt or less, 18 nt or less, 17 nt or less, 16 nt or less, or 15 nt or less in length. In yet another embodiment, the first switching domain is from 5 nt to 30 nt, preferably from 10 nt to 20 nt in length.

[0056] In one embodiment, the portion of the second switching domain that is substantially complementary to the at least a portion of the first switching domain contains the 5' terminus and/or the 3' terminus of the second switching domain. In another embodiment, the portion of the second switching domain that is substantially complementary to the at least a portion of the first switching domain does not contain either end of the second switching domain. Preferably, the portion of the second switching domain that is substantially complementary to the at least a portion of the first switching domain only contains the 5' terminus of the second switching domain.

[0057] The second switching domain used in the CRISPR-Cas system of the present invention may be present either as an individual polynucleotide that is independent of the other elements of the guide RNA, i.e. the elements (a) to (d) above, or in the polynucleotide containing one or more of the other elements of the guide RNA. Preferably, the second switching domain is present in the polynucleotide containing one or more of the other elements of the guide RNA. If the second switching domain is present in the polynucleotide containing one or more of the other elements of the guide RNA, it may be located at any part of the polynucleotide, for example at the 5'-terminus (i.e. upstream of the spacer), at the 3'-terminus (i.e. downstream of the one or more hairpin(s)), or inside the elements (a) to (d) above (i.e. anywhere between the spacer and the one or more hairpins) as long as the addition (or insertion) of the second switching domain does not affect the inherent function of the guide RNA, i.e. recognizing and binding to a target polynucleotide and recruiting a Cas protein. Preferably, the second switching domain is located at the 3'-terminus of the elements (a) to (d) above.

[0058] In one embodiment, the second switching domain is at least 10 nt, at least 11 nt, at least 12 nt, at least 13 nt, at least 14 nt, at least 15 nt, at least 16 nt, at least 17 nt, at least 18 nt, at least 19 nt, at least 20 nt, at least 21 nt, at least 22 nt, at least 23 nt, at least 24 nt, or at least 25 nt in length. In another embodiment, the second switching domain is 50 nt or less, 40 nt or less, 30 nt or less, 29 nt or less, 28 nt or less, 27 nt or less, 26 nt or less, 25 nt or less, 24 nt or less, 23 nt or less, 22 nt or less, 21 nt or less, 20 nt or less, 19 nt or less, 18 nt or less, 17 nt or less, 16 nt or less, or 15 nt or less in length. In yet another embodiment, the second switching domain is from 10 nt to 50 nt, preferably from 20 nt to 30 nt in length.

[0059] In one embodiment, the portion of the first switching domain that is substantially complementary to at least a portion of the second switching domain is at least 3 nt, at least 4 nt, at least 5 nt, at least 6 nt, at least 7 nt, at least 8 nt, at least 9 nt, at least 10 nt, at least 11 nt, at least 12 nt, at least 13 nt, at least 14 nt, or at least 15 nt in length. In another embodiment, the portion of the first switching domain that is substantially complementary to at least a portion of the second switching domain is 20 nt or less, 19 nt or less, 18 nt or less, 17 nt or less, 16 nt or less, 15 nt or less, 14 nt or less, 13 nt or less, 12 nt or less, 11 nt or less, 10 nt or less, 9 nt or less, 8 nt or less, 7 nt or less, 6 nt or less, or 5 nt or less in length. In yet another embodiment, the portion of the first switching domain that is substantially complementary to at least a portion of the second switching domain is from 3 nt to 20 nt, preferably from 5 nt to 15 nt in length.

Opener

[0060] According to the first aspect of the present invention, an opener, when it is with the CRISPR-Cas system, disrupts the hybridization between the first switching domain and the second switching domain, and the guide RNA forms at least one stem within the first switching domain, and thereby becomes capable of activating a Cas protein. In the present application, when an opener is referred to as being “present” or “introduced”, or “with a CRISPR-Cas system”, it means that the opener is able to be in contact with a guide RNA and optionally with a Cas protein of said CRISPR-Cas system.

[0061] As used herein, the term “opener” refers to any substance which is capable of activating the guide RNA of the present invention by disrupting the hybridization between the first switching domain and the second switching domain. Such substances include, but are not limited to, nucleic acids such as DNA and RNA, peptides, proteins, and other small molecules. When interpreted in a broad sense, the term “opener” may also include non-material factors such as physical stimuli, for example light, pressure, and sound. The opener may be endogenous or exogenous, and may be naturally-occurring or of synthetic origin. As used herein, the term “endogenous” or “cognate” substance refers to a substance that is not added from outside of the system or a substance that is inherently present in the site of reaction (e.g. in the cell, tissue, or organism of interest), and the term “exogenous” or “foreign” is used to explain a substance that is not inherent in the environment. For example, pathogenic RNA or DNA in an infected cell, or RNA that is delivered from one cell to another by exosome may be referred to as “exogenous”. In one embodiment, the opener may be an enzyme, for example an enzyme that processes polynucleotides such as a restriction enzyme and nicking enzyme, or a ligation enzyme. In another embodiment, the first switching domain and/or the second switching domain may be designed such that it contains an aptamer sequence which is capable of specifically binding to a target substance, and said target substance may serve as the opener. In a preferred embodiment, the opener is an RNA, which may be an endogenous RNA such as miRNA.

[0062] When an opener is absent, at least a portion of the first switching domain and a portion of the second switching domain are hybridized, which prevents the first switching domain from forming a stem within the domain. As such, the guide RNA cannot maintain the canonical structure and is inactivated (“OFF” or “inactivated” state, Fig. 4 (a)). When an opener is introduced, it disrupts the hybridization between the first switching domain and the second switching domain. The unbound (i.e. single-stranded) first switching domain then forms at least one stem structure within the domain, and the guide RNA of the present invention can return to its canonical structure, retrieving its function of activating a Cas protein (“ON” or “activated” state, Fig. 4 (b)). As used herein, when the guide RNA “activates” a Cas protein, it means that the guide RNA recognizes a target sequence and recruits a Cas protein to the target sequence.

[0063] The mechanism by which the opener disrupts the hybridization between the first switching domain and the second switching domain is not limited and any known method in the art may be used to disrupt the hybridization. For example, in one embodiment, the opener contains or consists of a sequence that is substantially complementary to the second switching domain, and the opener disrupts the hybridization between the first switching domain and the second switching domain by hybridizing to the second switching domain. Alternatively, the opener may be able to process or degrade a polynucleotide (e.g. by cleaving, excising, or introducing a nick into a polynucleotide), and the hybridization between the first switching

domain and the second switching domain may be disrupted by processing or degrading one or both domains.

[0064] In one embodiment, the hybridization of the opener to the second switching domain may be performed by a toehold-mediated strand displacement. Toehold-mediated strand displacement is known in the art and is described for example in Siu and Chen, *Nature Chemical Biology* 15, 217-220 (2019), and Guo et al., *Quant Biol* (2017) 5: 25, the entire contents of which are incorporated herein by reference. Typically, toehold-mediated strand displacement involves a double-stranded polynucleotide containing an overhanging region so-called “toehold”, and a single-stranded polynucleotide which is at least partially complementary to the toehold. When the single-stranded polynucleotide hybridizes to the toehold region, a process called branch migration occurs, which allows the displacement of the initially-hybridized strand. In one embodiment, the toehold is located at either end of the second switching domain, preferably at the 3' terminus of the second switching domain.

[0065] Also, the system may be designed such that the opener hybridizes to the loop of a hairpin formed by the hybridization between the first switching domain and the second switching domain (Fig. 5 (a)). In principle, the opener binds to the loop when the free energy obtained by the binding of the opener to the loop exceeds the loss of free energy from the breaking of the hybridization between the first switching domain and the second switching domain, thereby disrupting the hybridization between the first switching domain and the second switching domain (Fig. 5 (b)). Therefore, the length of the hybridization between the opener and the loop needs be long enough in relation to the length of the hybridization between the first switching domain and the second switching domain in order to ensure the binding of the opener to the loop. In one embodiment, the hybridization between the opener and the loop is 5 nucleotides or more in length, preferably 10 nucleotides or more in length, such as 10, 11, 12, 13, 14, or 15 nucleotides in length, or more than 15 nucleotides in length. In another embodiment, the length of the hybridization between the opener and the loop is at least 70%, preferably at least 75%, more preferably at least 80%, and even more preferably at least 85%, and most preferably at least 90% of the length of the hybridization between the first switching domain and the second switching domain.

[0066] As mentioned above, the first switching domain of the present invention, when not hybridized with the second switching domain, can form at least one stem within the domain. The term “stem” as used herein refers to a partial double-stranded structure formed within a single-stranded RNA. In one embodiment, the stem formed in the first switching domain contains at least one pair of paired bases, preferably two or more pairs of paired bases. The base pair(s) formed in the first switching domain may be either G-C or A-U. In one embodiment, the stem formed in the first switching domain contains at least one G-C pair, preferably two G-C pairs, more preferably two consecutive G-C pairs. In a preferred embodiment, the stem formed in the first switching domain mimics the nexus of the original guide RNA.

Third functional module / hairpin(s)

[0067] According to the first aspect of the present invention, the guide RNA may optionally comprise a third functional module in the downstream (the 3'-side) of the second functional module. In other words, the guide RNA does not necessarily need a third functional module to have its function in recognizing and binding to a target polynucleotide. When the guide RNA comprises a third functional module, the third functional module may comprise one or

more hairpins, for example one hairpin or two hairpins. The term “hairpin” as used herein refers to a secondary structure of polynucleotides consisting of a stem and a loop. The number of the hairpin(s) and the structure of each hairpin differ depending on the species from which the guide RNA is derived. In one embodiment, the sequence and the structure of the third functional module may be the same as those of the naturally-occurring guide RNAs. In another embodiment, the third functional module may be modified such that the number of the hairpin(s) is increased or reduced, or the structure of each hairpin is changed, as long as the function of the guide RNA is maintained. For example, in the case of SpCas9, the second hairpin (the one in the 5' terminal side) may be omitted from the guide RNA. In yet another embodiment, the third functional module does not comprise any hairpin.

[0068] As an illustrative example, the guide RNA of the present invention may be designed according to the following steps. First, a suitable opener is selected in consideration of the desired condition where the CRISPR-Cas system is to be activated. Second, the second switching domain is designed such that it is substantially complementary to at least a portion of the selected opener. Third, the first switching domain is designed such that at least a portion thereof is substantially complementary to at least a portion of the second switching domain, preferably a portion comprising the 5' terminus of the second switching domain, and also such that at least one stem is formed within the first switching domain when it is not hybridized with the second switching domain.

Specific embodiment of the second functional module

[0069] In one embodiment, the second functional module may comprise or consist of the sequence represented by formula (I):



wherein:

N each independently represents any base selected from A, C, G, and U;

m is an integer ranging from 1 to 4, preferably 2;

p is an integer ranging from 1 to 4, preferably 2;

q is an integer ranging from 1 to 10, preferably from 3 to 7, more preferably 5;

r is an integer ranging from 1 to 10, preferably from 3 to 8, more preferably 6; and

(X)_m represents the sequence 5'- X¹...X^{m-1}X^m -3';

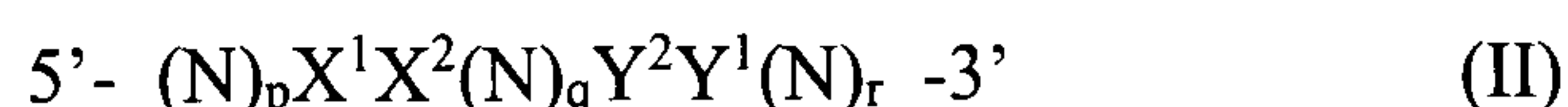
(Y)_m represents the sequence 5'- Y^mY^{m-1}...Y¹ -3';

the mth X (X^m) and the mth Y (Y^m) are capable of being hybridized to form a stem and

each of the pair of X^m/Y^m is independently selected from C/G, G/C, A/U, and U/A.

[0070] In one embodiment, the first switching domain may comprise at least region (X)_m or (Y)_m. Also, the region (X)_m may comprise one or more hybridization between the two bases within the region.

[0071] In a preferred embodiment, the second functional module may comprise or consist of the sequence represented by formula (II):



wherein:

N, p, q, and r have the meanings as defined above for formula (I); and

the pairs X^1/Y^1 and X^2/Y^2 are each independently selected from C/G, G/C, A/U, and U/A, preferably both pairs X^1/Y^1 and X^2/Y^2 are G/C.

[0072] In a more preferred embodiment, the second functional module may comprise or consist of the sequence represented by formula (III):



wherein:

N, X^1 , Y^1 , X^2 , and Y^2 have the meanings as defined above for formulae (I) and (II).

[0073] In one embodiment, the first functional module may comprise or consist of the sequence represented by 5'- GUUUUAGAGCGUAGAAAUAGCAAGUUAAAAU -3' (SEQ ID NO: 2). In another embodiment, when the guide RNA comprises a third functional module, the sequence of the third functional module may comprise or consist of the sequence represented by 5'- AACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU -3' (SEQ ID NO: 3) or 5'- AACUUGAAAAAGUGG -3' (SEQ ID NO: 4).

[0074] In one embodiment, the second functional module comprising a first switching domain comprises or consists of the sequence selected from the group consisting of SEQ ID NOs: 11 to 15.

[0075] In one embodiment, the second switching domain comprises or consists of the sequence selected from the group consisting of SEQ ID NOs: 16 to 20.

[0076] In one embodiment, the guide RNA comprises or consists of the polynucleotide encoding the sequence selected from the group consisting of SEQ ID NOs: 21 to 25. Examples of the guide RNA of the present invention are shown in Fig. 6.

Further switching domains for regulating the activity of a guide RNA

[0077] As a variation of the first aspect, the guide RNA of the present invention may comprise a further domain (a third switching domain) for regulating the activation of the guide RNA. In this variation, the structure of the guide RNA is altered by the hybridization of at least a portion of the third switching domain with at least a portion of a fourth switching domain that is substantially complementary to the portion of the third switching domain. As a result, the guide RNA is inactivated, i.e. it does not maintain the function of activating a Cas protein.

[0078] The third switching domain may be located anywhere in the guide RNA as long as the guide RNA is inactivated by the hybridization between the third and the fourth switching domains. In one embodiment, the third switching domain is located within the first functional module and the structure of the crRNA:tracrRNA duplex is altered by the hybridization between the third and the fourth switching domains.

[0079] In one embodiment, the fourth switching domain is present as a part of the guide RNA. More specifically, the fourth switching domain may be located at the 5'-terminus or at the 3'-terminus of the guide RNA, or inside or between any of the elements (a) to (d), or, if the second switching domain is present as a part of the guide RNA, in the 5'-side or the 3'-side of the second switching domain. Preferably, the fourth switching domain is located in the 3'-

side of the second switching domain. In another embodiment, the fourth switching domain is present independently of the guide RNA.

[0080] In one embodiment, when the second switching domain and/or the fourth switching domain are present in the guide RNA, the guide RNA may also comprise one or more linker sequences adjacent to the switching domain(s), in order to operably connect the switching domain(s) to the rest of the guide RNA sequence. The sequence and length of such a linker may be readily determined by a person skilled in the art. In one embodiment, when the second switching domain and the fourth switching domain are connected next to each other in a guide RNA, the guide RNA may comprise a linker of at least 5 nt, at least 10 nt, or at least 15 nt in length. In another embodiment, the linker may be 30 nt or less, 25 nt or less, or 20 or less in length. In yet another embodiment, the linker may be in the range from 5 nt to 30 nt, from 10 nt to 25 nt, or from 15 nt to 20 nt in length.

[0081] The sequences of the third and the fourth switching domains that would function in the guide RNA of the present invention may be readily determined by a person skilled in the art using any method known in the art. For example, the third and the fourth switching domains may be designed according to the method described above for the first and the second switching domains.

[0082] Similar to the first and second switching domains, the hybridization between the third and the fourth switching domains may be disrupted by an opener. The opener for disrupting the hybridization between the third and the fourth switching domains may be the same as or different from the opener for disrupting the hybridization between the first and the second switching domains, and may be selected or prepared in the same manner as described above.

Polynucleotide encoding the guide RNA

[0083] In a second aspect, the present invention relates to a polynucleotide encoding at least one guide RNA of the invention. When the guide RNA consists of two or more polynucleotides (RNAs), they may be encoded in the same polynucleotide (DNA) or in different polynucleotides (DNAs).

[0084] In one embodiment, the polynucleotide is comprised in a vector. Accordingly, as a variation of the second aspect, the present invention also relates to a vector comprising at least one polynucleotide of the invention. The vector may be any type of expression vector, for example a viral expression vector, such as poxvirus, adenovirus, adeno-associated virus (AAV), herpesvirus, and lentivirus; or a plasmid or DNA or nucleic acid molecule vector. In one embodiment, the vector also comprises one or more module(s) or element(s) operably linked to a polynucleotide encoding the guide RNA of the present invention that can drive the expression of the polynucleotide including, but are not limited to, a promoter such as T7 promoter, a terminator, and other tags and sequences that are suitable for desired use of the vector such as nuclear localization signals (NLSs). These module(s) or element(s), together with the polynucleotide encoding the guide RNA of the present invention, may form an "expression cassette".

[0085] In one embodiment, the vector may comprise one expression cassette for the expression of a full guide RNA sequence. In another embodiment, the vector may comprise more than one expression cassette, each comprising a polynucleotide encoding at least a portion of a guide RNA, and a full guide RNA may be formed by assembling the expression

products from different expression cassettes. For example, a first strand comprising a spacer can be expressed from one expression cassette and a second strand comprising the rest of the guide RNA can be expressed from another expression cassette, and the two strands can be assembled by hybridization. Alternatively, in yet another embodiment, more than one vector may be used to assemble one guide RNA, wherein each of the vectors comprises at least one expression cassette encoding at least a portion of the guide RNA. The construct of such a vector or vectors can be flexibly designed by routine procedures of a person skilled in the art.

[0086] In one embodiment, the vector may additionally comprise at least one polynucleotide encoding at least one Cas protein, and/or at least one polynucleotide encoding at least one opener in the same vector. In this regard, the present invention also relates to the combination of two or more vectors each comprising at least one polynucleotide encoding at least one guide RNA, at least one polynucleotide encoding at least one Cas protein, and/or at least one polynucleotide encoding at least one opener.

[0087] In one embodiment, the polynucleotide or the vector of the present invention comprises or consists of a sequence selected from the group consisting of SEQ ID NOs: 5 to 9.

Composition

[0088] In a third aspect, the present invention relates to a composition comprising at least one guide RNA of the present invention or at least one polynucleotide encoding thereof and at least one Cas protein or at least one polynucleotide encoding thereof. In one embodiment, the composition may further comprises at least one opener or at least one polynucleotide encoding thereof.

Cas protein

[0089] As used herein, the term "Cas protein" refers to any CRISPR-associated protein that is responsible for recognition and optionally cleavage of the target polynucleotide. The Cas protein may be a single protein or may be in the form of a complex with other component(s). In one embodiment, the Cas protein used in the present invention is selected from Type I, Type II, and Type III Cas proteins including, but not limited to, Cascade complex, Cas3 protein, Cas9 protein, Csm6 (Gootenberg et al., Science, 360(6387): 439-444, 2018), Csm complex, and Cmr complex, or other Cas proteins such as Cas12a, Cas12b (Strecker et al., Nature Communications, volume 10, Article number: 212, 2019), Cas13 (Cox et al. Science, 358(6366): 1019-1027, 2017), and CasX and CasY (Burstein et al., Nature, volume 542, pages 237-241, 2017) (the entire contents of each document are incorporated herein by reference). In a preferred embodiment, the Cas protein is a Cas9 protein. The Cas protein used in the present invention does not have to be from the same origin as the origin of the target polynucleotide, and may be of any bacterial origin. For example, when a Cas9 is used as the Cas protein of the present invention, the Cas9 may be selected from Cas 9 proteins from *Streptococcus* spp., *Lactobacillus* spp., *Campylobacter* spp., and *Staphylococcus* spp. In one embodiment, the Cas protein used in the present invention is a Cas9 protein selected from the group consisting of: *Streptococcus pyogenes* Cas9 (SpCas9), *Staphylococcus aureus* Cas9 (SaCas9), *Lactobacillus plantarum* Cas9 (LpCas9), and *Campylobacter jejuni* Cas9 (CjCas9). Preferably, the Cas protein used in the present invention is SpCas9.

[0090] In one embodiment, the Cas protein used in the present invention may contain one or more mutations. In the present application, substitution mutations in an amino acid sequence

may be expressed using a one-letter code of the original amino acid, a position number, and then a one-letter code of the substituted amino acid. For example, when the aspartic acid (D) at position 10 is substituted with asparagine (N), this mutation is expressed as “D10N”, and this is synonymous with “the substitution of Asp at position 10 with Asn”.

[0091] In one embodiment, the Cas protein used in the present invention is an endonuclease that cleaves both strands of the double-stranded target polynucleotide. In another embodiment, the Cas protein used in the present invention is a nickase which cleaves only one strand of the double-stranded target polynucleotide. Such a nickase may be produced by genetically-engineering naturally-occurring Cas nucleases. For example, it is known that *Streptococcus pyogenes* Cas9 (SpCas9) is converted to a nickase by the substitution of Asp at position 10 of the RuvC I catalytic domain to Ala (D10A). Other non-limiting examples of the mutations that convert a Cas9 to a nickase are E762A, H840A, N854A, N863A, and D986A of SpCas9. Cas9 orthologs from other species may be also converted to a nickase by introducing the above mutations at corresponding positions in said orthologs. In yet another embodiment, the Cas protein used in the present invention may be genetically-engineered such that it does not cleave any strand of the target polynucleotide although it maintains the ability of being directed to the target polynucleotide by the guide RNA. Such an engineered Cas protein may be used for various purposes including, but not limited to, the detection of the target polynucleotide or the activation of the gene encoded by the target polynucleotide or by a polynucleotide adjacent to the target polynucleotide.

[0092] In another embodiment, the Cas protein used in the present invention may be a split Cas protein, such as a split Cas9 in which the nuclease lobe and the alpha-helical lobe are expressed as separate polypeptides but which maintains the activity of full-length Cas9 when the two lobes are recruited into a ternary complex by a guide RNA. Such a split Cas protein is known in the art and is described for example in Wright et al., PNAS March 10, 2015 112 (10) 2984-2989, the entire contents of which are incorporated herein by reference. Since the nexus is located at the interface of the nuclease lobe and the alpha-helical lobe of the Cas protein, the two lobes would rarely come together when the nexus is hidden by the hybridization between the first switching domain and the second switching domain of the present invention. Therefore, without being bound to any particular theory, it may be possible to even more effectively control the activation of a Cas protein if the guide RNA of the present invention is used in combination with such a split Cas protein.

[0093] In one embodiment, the composition of the present invention is a pharmaceutical composition comprising at least one excipient, preferably a pharmaceutically acceptable excipient. In one embodiment, the guide RNA, the polynucleotide, and the composition of the present invention may be for therapeutic, prophylactic, and/or diagnostic use. In one embodiment, the guide RNA, the polynucleotide, and the composition of the present invention may be used for treating, preventing, and/or detecting at least one condition in a subject, such as at least one disease or disorder. The subject may be any living organisms, either eukaryotic or prokaryotic. Specific examples of such a subject include, but are not limited to, animals, for example mammals such as humans and non-human primates (e.g. macaques), rodents (e.g. mice and rats), rabbits, dogs, horses, cows, sheep, and goats; birds, reptiles, amphibians, and fish; plants; and microorganisms such as fungi and bacteria.

[0094] The pharmaceutical composition of this embodiment may be administered orally in the form of tablets, coated tablets, pills, powders, granules, capsules, liquids, suspensions,

emulsions, etc., or parenterally in the form of injectable solutions, suppositories, or as an external agent for skin.

[0095] In this embodiment, any substances that are routinely used in the preparation of pharmaceutical compositions may be used as the pharmaceutically acceptable excipients or carriers of the present invention. Such excipients or carriers include, for example, binders such as gelatin, corn starch, tragacanth gum, and gum arabic; starch and crystalline cellulose; swelling agents such as alginic acid; injectable solvents such as water, ethanol, and glycerol; and adhesives such as rubber-based or silicone-based pressure sensitive adhesives. The pharmaceutically acceptable excipient or carrier may be used solely or as a combination of two or more excipients or carriers.

[0096] The composition according to this aspect may further contain any additives known in the art. As such additives, lubricants such as calcium stearate and magnesium stearate; sweeteners such as sucrose, lactose, saccharin, and maltitol; flavours such as peppermint; stabilizers such as benzyl alcohol and phenol; buffers such as phosphate and acetate buffers; solubilizers such as benzyl benzoate and benzyl alcohol; antioxidants; and preservatives may be used. The additives may be used solely or as a combination of two or more additives.

Method

Method of activating the guide RNA

[0097] In a fourth aspect, the present invention relates to a method of regulating the activation of a guide RNA of the present invention, comprising the step of hybridizing at least a portion of the first switching domain and at least a portion of the second switching domain and/or disrupting the hybridization between the first switching domain and the second switching domain using a first opener. In addition, when the guide RNA comprises the third switching domain as described above, the method may also comprise the step of hybridizing at least a portion of the third switching domain and at least a portion of the fourth switching domain and/or disrupting the hybridization between the third switching domain and the fourth switching domain using a second opener, and said second opener may be the same as or different from the first opener.

[0098] In this aspect, the method of regulating the activation of a guide RNA includes both activating a guide RNA which is initially in an inactivated (OFF) state, and inactivating a guide RNA which is initially in an activated (ON) state. The step of hybridization between the first switching domain and the second switching domain or disruption of said hybridization may be performed before, after, or simultaneously with the step of hybridization between the third switching domain and the fourth switching domain or disruption of said hybridization.

[0099] In one embodiment, the method may be performed by regulating the hybridization of one, two, three, or more pairs of switching domains that are substantially complementary and in which at least one switching domain of each pair is located within the guide RNA. For example, the method may further comprise an additional step of hybridizing another pair of switching domains (e.g. a fifth and sixth switching domains) that are substantially complementary and/or disrupting said hybridization using a third opener which may be the same as or different from the first and/or second openers. Designing such additional pair(s) of

switching domains and opener(s) may be performed in the same manner as described above for the first and second switching domains and the opener.

Multiplexing and logic gate

[0100] As a variation of the fourth aspect, the present invention relates to a method of regulating the activation of at least one guide RNA under multiplexed conditions. In one embodiment, the method of the present invention may be used to simultaneously target more than one polynucleotide in the same environment, by regulating the activation of more than one guide RNA, each directed to different target polynucleotides. In another embodiment, the method of the present invention may be also used to target a single polynucleotide by regulating the activation of more than one guide RNA, each directed to the same target polynucleotide. In yet another embodiment, the method of the present invention may be also used to target more than one polynucleotide by regulating the activation of more than one guide RNA, each directed to different target polynucleotides, with a single opener. In yet another embodiment, the method of the present invention may be also used to target a single polynucleotide by regulating a single guide RNA with more than one opener.

[0101] In addition, as a variation of the fourth aspect, the present invention also relates to a method of regulating the activation of a guide RNA in a highly-controlled manner, using various logic gates. For example, when the activation of a guide RNA is regulated with two openers, they may be designed such that the guide RNA is activated only when both openers are present (AND gate); when at least one of the openers is present (OR gate); only when one of the openers is present (XOR gate); only when one specific opener X is present and the other specific opener Y is absent (X AND NOT Y); either when both openers are present or when both opener are absent (XNOR gate); or under any condition except when both openers are present (NAND gate). In one specific embodiment, when the activation of a guide RNA comprising the first, second, third, and the fourth switching domains is to be regulated by two openers, they may be designed such that a first opener is capable of disrupting the hybridization between the first and the second switching domains and a second opener is capable of disrupting the hybridization between the third and the fourth switching domains, and the guide RNA is activated only when both the first and second openers are present (AND gate) (Fig. 10). In another specific embodiment, when a guide RNA comprises first, second, and the third switching domains and a fourth switching domain is present independently of the guide RNA and an opener is capable of disrupting the hybridization between the first and the second switching domains, the guide RNA is activated only when the opener is present and the fourth switching domain is absent (Figs. 11A and 11B). In this embodiment, the fourth switching domain serves as a NOT gate.

Gene-editing method

[0102] In a fifth aspect, the present invention relates to a method of cleaving at least one target polynucleotide, modifying the sequence of at least one target polynucleotide, and/or altering the expression of at least one gene encoded by at least one target polynucleotide, comprising the step of contacting the target polynucleotide with at least one guide RNA of the present invention, at least one opener, and at least one Cas protein. In one embodiment, the method of the fifth aspect may comprise the step of regulating the activation of a guide RNA using the method of the fourth aspect described above.

[0103] As is the case with the conventional guide RNAs, the guide RNA of the present invention may be used in all the possible applications of the CRISPR-Cas system. For example, in one embodiment, the guide RNA of the present invention may be used with a canonical Cas9 nuclease to cleave both strands of the target polynucleotide (double-strand break). The double-strand break in the target polynucleotide is then repaired by the mechanism called non-homologous end joining (NHEJ) and during this process one or more mutations may be introduced into the site of the double-strand break. Alternatively, one or more donor polynucleotides (also known as a donor vector, DNA donor, or template) may be used to introduce desired mutations into the target polynucleotide by homologous recombination (HR).

[0104] In another embodiment, the guide RNA of the present invention may be used with a nickase which cleaves only one strand of the double-stranded target polynucleotide. Since the repair of a single-strand break (nick) involves HR but not NHEJ, genome editing using a nickase enables more accurate and precise base-wise modification of the target polynucleotide (base-editing), such as modification of SNP (single nucleotide polymorphism), compared to the genome editing using a nuclease.

[0105] In yet another embodiment, the guide RNA of the present invention may be also used with a mutated Cas protein which does not cleave any strand of the target polynucleotide while it maintains the ability of recognizing and binding to the target polynucleotide. Such a nuclease-deficient Cas protein (dCas) may be used in a wide variety of applications including, but not limited to, tagging, activating, inactivating, chemically-modifying, pulling-down (precipitating) or visualising the target polynucleotide, wherein necessary substances such as enzymes, tags, transcription factors, fluorescent dyes and labels, etc., are delivered to the target polynucleotide by the CRISPR-dCas complex.

[0106] The method of the present invention may be performed in any environment such as *in vitro*, *in vivo*, or *ex vivo*. In one embodiment, the method of the present invention may be performed *in vitro* or *ex vivo*. In another embodiment, the method of the present invention may be performed *in vivo*, for example in bacteria, fungi, plants, and animals, more specifically mammals such as mice and humans. In yet another embodiment, the method of the present invention may be performed in cells including prokaryotic cells and eukaryotic cells, in viruses, or in a solution containing naked genomes or plasmids.

[0107] When the target polynucleotide is located in cells, tissues, or in living organisms, one or more the components of the CRISPR-Cas system, i.e., the Cas protein, the guide RNA, and the opener, may be delivered to the site of reaction by any conventional delivery methods. In one embodiment, the guide RNA of the present invention may be delivered to the site of reaction in the form of an RNA. Alternatively, the guide RNA of the present invention may be delivered in the form of a plasmid or vector comprising a polynucleotide encoding the guide RNA. Likewise, the Cas protein may be delivered either as a protein or as a vector comprising the polynucleotide encoding this protein.

[0108] The opener used in the present invention may be any substances that are capable of disrupting the hybridization between the first switching domain and the second switching domain. Such substances include, but are not limited to, nucleic acids such as DNA and RNA, peptides, proteins, and other small molecules. In one embodiment, the opener used in the present invention is an RNA. The opener may be endogenous or exogenous, and may be naturally-occurring or of synthetic origin. In one embodiment, the opener may be an enzyme,

for example an enzyme that processes polynucleotides such as a restriction enzyme and nicking enzyme, a ligation enzyme. In another embodiment, the opener may be a target substance of an aptamer sequence, and such an aptamer sequence is used either as the first switching domain or as the second switching domain. Any known substance that can be recognized by an aptamer sequence may be used as the opener of the present invention. Such substances include, but are not limited to, nucleic acids such as ribozymes and viral RNA, proteins, for example coagulation factors such as thrombin and fibrinogen, interferon (IFN), vascular endothelial growth factor (VEGF), and antigens such as prostate specific antigen (PSA), small molecule compounds such as porphyrin and dopamine, carbon nanotubes, and atoms such as Pb.

[0109] In one embodiment, the opener used in the present invention is an endogenous molecule, such as an endogenous RNA. In one embodiment, the opener used in the present invention is an endogenous RNA that is specifically or preferentially expressed in a certain type of cell, tissue, or individual, at a certain time point such as at a certain phase of the cell cycle, and/or under a certain physiological condition such as in a patient with a specific disease. As used herein, when a gene, protein, etc. is referred to as being “specifically” expressed under a certain condition, this means that the gene, protein, etc. is substantially not expressed under other conditions. When a gene, protein, etc. is referred to as being “preferentially” expressed under a certain condition, this means that the gene, protein, etc. may be also expressed under other conditions but it is expressed substantially in higher level under said condition compared to the expression under other conditions.

[0110] In one embodiment, the opener used in the present invention is a microRNA (miRNA). A MicroRNA is a small non-coding RNA normally containing from 20 to 25 nucleotides that is found in various organisms including plants and animals. In one embodiment, the opener used in the present invention is an RNA which is from 5 nt to 50 nt, preferably from 10 nt to 40 nt, more preferably 15 nt to 25 nt in length. Non-limiting examples of the opener that may be used in the present invention are microRNAs that are specifically or preferentially expressed in certain types of cells, such as endothelial cells, or certain types of tissues, such as skin or pancreatic tissues, certain time points such as a certain phase of the cell cycle or a certain developmental stage, and certain physiological conditions including diseases such as psoriasis and cancer, and trauma.

[0111] In one embodiment, in the method of the present invention, the Cas protein is activated specifically under the targeted conditions. Such targeted conditions include, but are not limited to, certain types of cells, such as endothelial cells, or certain types of tissues, such as skin or pancreatic tissues, certain time points such as a certain phase of the cell cycle or a certain developmental stage, and certain physiological conditions including diseases such as psoriasis and cancer, and trauma. Also, activation of the Cas protein may be triggered by external stimuli such as or temperature, light including visible light, UV, and IR, physical forces such as pressure, and pH. Such conditionally-controlled activation of the Cas protein may be achieved by the appropriate selection of the opener according to the desired condition.

[0112] In one specific embodiment, the method of the present invention may be used to protect a subject against pathogens such as bacteria and viruses and the opener may be DNA or RNA of such pathogens. For example, when a subject is infected with a pathogen, the RNA or DNA of said pathogen activates the CRISPR-Cas system of the present invention and the activated CRISPR-Cas system in turn kills or inhibits growth of said pathogen by any

method known in the art, for example by directly cleaving the DNA or RNA of the pathogen or inducing an immune response.

Therapeutic method

[0113] As a variation of the fifth aspect, the present invention also relates to a method of treating at least one condition in a subject, including treating at least one disease or disorder in a subject, comprising a step of administering to said subject at least one guide RNA of the present invention or at least one polynucleotide encoding thereof and at least one Cas protein or at least one polynucleotide encoding thereof, or alternatively a composition comprising one or more of these active ingredients, each in a therapeutically effective amount. In one embodiment, the guide RNA or a polynucleotide encoding thereof and the Cas protein or a polynucleotide encoding thereof may be administered simultaneously or at different time points to the subject. When they are administered simultaneously, they may be administered as the same composition or as separate compositions. In one embodiment, at least one opener or at least one polynucleotide encoding thereof may be also administered exogenously to the subject. Alternatively, in another embodiment, at least one endogenous RNA such as a microRNA may be used as the opener.

[0114] In this embodiment, the method of administering the guide RNA, Cas protein, and/or opener is not limited and may be appropriately determined by a person skilled in the art according to the disease or symptoms to be treated, general condition, body weight, age, sex, etc., or the subject. For example, tablets, coated tablets, pills, powders, granules, capsules, liquids, suspensions, emulsions, etc., may be orally administered. Also, injectable solutions may be intravenously administered solely or in combination with known infusions such as glucose and amino acids, and may be also administered intraarterially, intramuscularly, intradermally, subcutaneously, or intraperitoneally, as necessary.

[0115] As used herein, the term “therapeutically effective amount” refers to an amount that is sufficient to obtain desired therapeutic effects, such as to prevent, treat, improve, reverse, ameliorate, and/or alleviate the target condition in the subject. Such an amount may be appropriately determined by a person skilled in the art in accordance with the disease or symptoms to be treated, general condition, body weight, age, sex, etc., of the subject. In one embodiment, a therapeutically effective amount of the composition of the present invention may be from 1 µg (microgram) to 10 g, for example 0.01 to 2000 mg, such as 0.001 to 200 mg of active ingredients per day.

[0116] In a more specific embodiment of the fifth aspect, the present invention also relates to a therapeutic method of modifying a target polynucleotide in a tissue-specific manner, using a therapeutically effective amount of the guide RNA of the present invention and optionally a Cas protein and/or an opener.

[0117] It is well known in the art that systemic treatment with anticancer agents, including gene therapy agents, could cause severe side effects because these anticancer agents target genes that are responsible for proliferation of not only the cancer cells but also normal cells. By using the guide RNA of the present invention which specifically activates the CRISPR-Cas system in the tissue suffering from disease or disorder such as cancer, it would be possible to reduce such side effects.

Kit

[0118] In a sixth aspect, the present invention relates to a kit comprising at least one guide RNA of the present invention or at least one polynucleotide encoding thereof, and optionally comprising at least one opener or at least one polynucleotide encoding thereof and/or at least one Cas protein or at least one polynucleotide encoding thereof.

[0119] In one embodiment, the kit may also contain other reagents that are necessary for the preservation of the guide RNA, Cas protein, and/or opener in the kit, or for the activation of the guide RNA, Cas protein, and/or opener, and/or manufacturer's instructions for using the kit.

Additional embodiments

[0120] In one aspect, the present invention also relates to an agent or a medicament comprising a guide RNA of the present invention or at least one polynucleotide encoding thereof and at least one Cas protein or at least one polynucleotide encoding thereof, and optionally at least one opener or at least one polynucleotide encoding thereof.

[0121] In one aspect, the present invention also relates to a combination of the guide RNA of the present invention with a Cas protein and/or an opener, for example a combination medicament comprising at least one guide RNA of the present invention or at least one polynucleotide encoding thereof and at least one Cas protein or at least one polynucleotide encoding thereof, and optionally further comprising at least one opener or at least one polynucleotide encoding thereof. In one aspect, the present invention also relates to a combination treatment regimen comprising a step of administering to a subject at least one guide RNA of the present invention or at least one polynucleotide encoding thereof and at least one Cas protein or at least one polynucleotide encoding thereof, either simultaneously or at different points in time. According to said aspect, at least one opener may also be administered to the subject, or alternatively at least one endogenous RNA may be used as the opener.

[0122] In one aspect, the present invention also relates to a use of at least one guide RNA of the present invention or at least one polynucleotide encoding thereof, at least one Cas protein or at least one polynucleotide encoding thereof, and/or at least one opener or at least one polynucleotide encoding thereof for the manufacture of a medicament.

[0123] In one aspect, the present invention also relates to a method for generating a CRISPR-Cas system guide RNA that is specifically activated under a target condition, wherein said guide RNA consists of one or more polynucleotide(s) and comprises:

(A) the following elements (a) to (d), arranged in this order from 5' to 3':

- (a) a spacer which can hybridize to a target polynucleotide;
- (b) a first functional module comprising a crRNA:tracrRNA duplex;
- (c) a second functional module comprising a first switching domain; and
- optionally (d) a third functional module; and

(B) a second switching domain,

wherein at least a portion of the first switching domain is substantially complementary to at least a portion of the second switching domain, and when an opener is present in the target condition, the opener disrupts the hybridization between the first switching domain and the second switching domain and the guide RNA forms at least one stem within the first

switching domain, and thereby becomes capable of activating a Cas protein, and said method includes steps of:

- (i) selecting at least one opener, preferably an RNA that is specifically present or expressed in said target condition and obtaining sequence information of said opener;
- (ii) constructing the second switching domain such that it is substantially complementary to at least a portion of the selected opener; and
- (iii) constructing the first switching domain such that at least a portion thereof is substantially complementary to at least a portion of the second switching domain, preferably a portion comprising the 5' terminus of the second switching domain, and also such that at least one stem is formed within the first switching domain when it is not hybridized to the second switching domain.

[0124] In this aspect, the target condition may be certain types of cells, such as endothelial cells, or certain types of tissues, such as skin or pancreatic tissues, certain time points such as a certain phase of the cell cycle or a certain developmental stage, and certain physiological conditions including diseases such as psoriasis and cancer, and trauma.

Examples

[0125] The following examples are non-limiting and other variants contemplated by one of ordinary skill in the art are included within the scope of this disclosure.

(Example 1: Activation of a guide RNA with an opener in the multiplexed condition)

Materials and Methods

[0126] In Example 1, guide RNAs with different sequences in the first and second switching domains were prepared to confirm whether they would be specifically activated by the corresponding opener. In this example, a guide RNA comprising a first switching domain in the second functional module (the nexus) and a second switching domain at the 3'-terminus was prepared. Five constructs were prepared, and for each of the guide RNA the hybridization between the first and second switching domains is designed to be specifically disrupted by one opener (micro RNA).

Fluorescence assays

[0127] In this experiment, the inventors adopted the cell-free fluorescence assay described in Mekler *et al.*, Nucleic Acids Research, 2016, Vol. 44, No. 6 (the entire contents of which are incorporated herein by reference), instead of the conventional plasmid cleavage assay. This assay is based on measuring emission from fluorescently labeled derivatives of target DNA. The target DNA derivative is a fragment of about 40 nt comprising a protospacer, a functional PAM, and the sequence of about 15 bp downstream of the PAM, and is named "fluorescent beacon" or "probe". The PAM-distal end of the beacon target and non-target strands are labeled with a fluorescent label and fluorescence quencher, respectively. When the guide RNA is able to activate the Cas9 protein, it directs the invasion of the fluorescent beacon by the Cas9 protein, which results in the separation of the fluorescent from the quencher and the emission of the fluorescence (Fig. 7).

[0128] In the two-step assay, a transcribed and purified guide RNA is added to a solution containing a Cas9 protein and a fluorescence beacon (probe) (Fig. 7 (a)). On the other hand,

in the one-pot assay, instead of isolating/purifying the guide RNA after its production, it is produced *in situ* by adding a DNA template, a T7 RNA polymerase and rNTPs in the solution (Fig. 7 (b)). The inventors used both assays to confirm the controlled targeting of DNA by the guide RNA of the present invention.

[0129] All experiments were performed at 34°C in a CFX96 Touch™ (BioRAD) thermal cycler, and activity was monitored upon fluorescence level shifts.

In-vitro transcription of guide RNA

[0130] Transcription was initiated upon addition of 4 nM of dsDNA template (gblocks® Gene Fragment, IDT) to a solution of 5 U/μL of T7 RNA polymerase (NEB, M0251), 1 U.μL⁻¹ of murine RNase Inhibitor (NEB M0314), 500 μM of rNTPs (NEB, N0450) and 1x of SYBR™ Green II RNA Gel Stain (Invitrogen) in the reaction buffer containing 40 mM Tris-HCl, 6 mM MgCl₂, 1 mM 1,4-Dithiothreitol, 2 mM spermidine with a final volume of 20 μL and incubated for 6 to 8 hours. The inventors used low yield conditions to reduce the proportion of transcription by-products. After transcription, RNA was isolated by precipitation in isopropanol and resuspended in 10 μL nuclease free water (NEB, B1500) before quantification by BioDrop μLITE (Biodrop). Transcripts were then stored at -25°C.

[0131] The constructs of the DNA templates encoding the guide RNAs used in Example 1 are shown below in Table 1 and are represented by SEQ ID NOs: 5 to 9.

Table 1: Sequences of DNA templates encoding the guide RNAs used in Example 1
*The underlined sequence corresponds to a T7 promoter. Sequences upstream of the promoter are for padding.

Name	Sequence	SEQ ID NO:
Guide RNA for SgmiR-31	CACTACACTACTAACACACCACCAAATAATACGACTCACTATAG GCTAAAGAGGAAGAGGACAGTTTTAGAGCTAGAAATAGCAAGT TAAAATAAGCCTAGGGCATAGCTAACTTGAAAAAGTGGAGCTA TGCCAGCATCTTGCCT	5
Guide RNA for SgmiR-98	CACTACACTACTAACACACCACCAAATAATACGACTCACTATAG GCTAAAGAGGAAGAGGACAGTTTTAGAGCTAGAAATAGCAAGT TAAAATAAGTCTAGTACTTTCCCAACTTGAAAAAGTGGGGGAA AGTAGTAAGTTGTATAG	6
Guide RNA for SgmiR-451	CACTACACTACTAACACACCACCAAATAATACGACTCACTATAG GCTAAAGAGGAAGAGGACAGTTTTAGAGCTAGAAATAGCAAGT TAAAATAAGTCTAGTACTGAGTTAACTTGAAAAAGTGGAAGTC AGTAATGGTAACGGTTT	7
Guide RNA for SgmiR-3945	CACTACACTACTAACACACCACCAAATAATACGACTCACTATAG GCTAAAGAGGAAGAGGACAGTTTTAGAGCTAGAAATAGCAAGT TAAAATAAACCTAGGGTTGATATAACTTGAAAAAGTGGATATC AACCCTCTCCTATGCCCT	8
Guide RNA for SgmiR-21	CACTACACTACTAACACACCACCAAATAATACGACTCACTATAG GCTAAAGAGGAAGAGGACAGTTTTAGAGCTAGAAATAGCAAGT TAAAATAATCCTAGTGATGTTGAACTTGAAAAAGTGGTCAAC ATCAGTCTGATAAGCTA	9

[0132] The constructs of the guide RNAs used in Example 1 are shown below in Tables 2 to 4 and are represented by SEQ ID NOs: 2, 4, and 10 to 25.

Table 2: Sequences of the spacer and first and third functional modules used in Example 1

Name	Sequence	SEQ ID NO:
Spacer	GGCUAAAGAGGAAGAGGACA	10
First Functional Module	GUUUUAGAGCUAGAAAUAGCAAGUUAAAAU	2
Third Functional Module	AACUUGAAAAAGUGG	4

Table 3: Sequences of the second functional modules and second switching domains used in Example 1

Corresponding Opener	Second Functional Module comprising First Switching Domain (Bold : the portion hybridizing to the Second Switching Domain)	SEQ ID NO:	Second Switching Domain (Bold : the portion hybridizing to the First Switching Domain, <u>Underlined</u> : the toehold)	SEQ ID NO:
SgmiR-31	AAGCCUAG GGG CAUAGCU	11	AGCUAUGCCAGCAUCUUGCCU	16
SgmiR-98	AAGUCUAGUACUUUCCC	12	GGG AAAGUAGUAAGUUGUAUAG	17
SgmiR-451	AAGUCUAGUACUGAGUU	13	AACUCAGUAAUGGUAACGGUUU	18
SgmiR-3945	AAACCUAG GGG UUGAUAU	14	AUAUCAACCCUCUCCUAUGCCCU	19
SgmiR-21	AAUCCUAGUGAUGUUGA	15	UCAACAUCAGUCUGAUAAGCUA	20

Table 4: Sequences of the guide RNAs used in Example 1

Corresponding Opener	Sequence	SEQ ID NO:
SgmiR-31	GGCUAAAGAGGAAGAGGACAGUUUUAGAGCUAGAAAUAGCAAGU UAAAAUAAGCCUAGGGCAUAGCUAACUUGAAAAAGUGGAGCUAU GCCAGCAUCUUGCCU	21
SgmiR-98	GGCUAAAGAGGAAGAGGACAGUUUUAGAGCUAGAAAUAGCAAGU UAAAAUAAGUCUAGUACUUUCCCAACUUGAAAAAGUGGGGGAAA GUAGUAAGUUGUAUAG	22
SgmiR-451	GGCUAAAGAGGAAGAGGACAGUUUUAGAGCUAGAAAUAGCAAGU UAAAAUAAGUCUAGUACUGAGUUAACUUGAAAAAGUGGAACUCA GUA AUGGUAACGGUUU	23
SgmiR-3945	GGCUAAAGAGGAAGAGGACAGUUUUAGAGCUAGAAAUAGCAAGU UAAAAUAAACCUAGGGUUGAUUAACUUGAAAAAGUGGAUAUCA ACCCUCUCCUAUGCCCU	24
SgmiR-21	GGCUAAAGAGGAAGAGGACAGUUUUAGAGCUAGAAAUAGCAAGU UAAAAUAAUCCUAGUGAUGUUGAAACUUGAAAAAGUGGUCAACA UCAGUCUGAUAAGCUA	25

MicroRNAs

[0133] In this experiment, microRNAs (miRNAs) were selected as the “opener” which activates the guide RNA of the present invention. Overexpression of specific miRNAs has been reported in many tissues and pathologies -which make them ideal biomarkers to infer the state or type of the cell in which the guide RNA of the present invention operates. After manual screening, the inventors chose human miRNAs that are highly enriched in psoriasis skin cells (miR-31, SEQ ID NO: 26), hypertrophic scarring tissues (miR-98, SEQ ID NO: 27), pancreatic cancer tissues (miR-451, SEQ ID NO: 28), neutrophils following traumatic injury (miR-3945, SEQ ID NO: 29), and in endothelial cells (miR-21, SEQ ID NO: 30). The sequences of these microRNAs are shown in Table 5. Cognate miRNAs were chemically synthesized from IDT (standard desalting), re-suspended in 10 mM Tris-HCl (pH 8), 1 mM EDTA and stored at -25°C prior to use. A final concentration of 500 nM was used in each sample.

Table 5: Sequences of the microRNAs used as the opener in Example 1

microRNA	sequence	SEQ ID NO:
miR-31	AGGCAAGAUGCUGGCAUAGCU	26
miR-98	CUAUACAACUUACUACUUUCCC	27
miR-451	AAACCGUUACCAUACUGAGUU	28
miR-3945	AGGGCAUAGGAGAGGGUUGAUAU	29
miR-21	UAGCUUAUCAGACUGAUGUUGA	30

Two-step assay

[0134] A guide RNA (at a final concentration of 200 nM) was injected in a pre-incubating solution of 200 nM Cas9 (*S. pyogenes*, NEB, M0386), 120 nM fluorescent beacon (dsDNA labelled with Atto647N and BBQ-650 quencher), and 1 U. μ L⁻¹ murine RNase Inhibitor (NEB M0314) in the reaction buffer containing 50 mM Tris-HCl (pH 7.5), 5.5 mM MgSO₄, 75 mM NaCl, 100 μ g. μ L⁻¹ BSA (NEB, B9000S). The final volume of the solution was 10 μ L.

One-pot assay

[0135] 4 nM of dsDNA template (gblocks[®] Gene Fragment, IDT) was added to a solution containing 5 U/ μ L of T7 RNA polymerase (NEB, M0251), 200 nM Cas9 (*S. pyogenes*, NEB, M0386), 120 nM fluorescent beacon (dsDNA labelled with Atto647N and BBQ-650 quencher), 1 U. μ L⁻¹ of murine RNase Inhibitor (NEB M0314), 500 μ M of rNTPs (NEB, N0450) and 1x of SYBR[™] Green II RNA Gel Stain (Invitrogen) in the reaction buffer containing 50 mM Tris-HCl (pH 7.5), 5.5 mM MgSO₄, 75 mM NaCl, 100 μ g.mL⁻¹ BSA (NEB, B9000S). The final volume of the solution was 10 μ L.

[0136] In both assays, all the combinations of the microRNA (opener) and the guide RNA were tested. In one sample, one of the five types of guide RNA was mixed with one of the five types of microRNA. Samples containing no guide RNA and/or no microRNA were also prepared and used as controls.

Results

[0137] The results for the two-step assay are shown in Fig. 8. As can be seen, fluorescence did not increase when no guide RNA was present (the far-left column) or when no opener was present (the top line). Fluorescence did not increase when the sequence of the opener did not match the sequence of the second switching domain (for example, the combination of the guide RNA designed for miR31 with miR98 as the opener). On the other hand, when the guide RNA designed for a specific microRNA was combined with that microRNA (for example, the combination of the guide RNA designed for miR31 with miR31 as the opener), a large increase in fluorescence was observed, indicating that the CRISPR-Cas9 complex-directed invasion of the fluorescent beacon took place.

[0138] The same constructs were tested also in the one-pot assay and the results are shown in Fig. 9. Similar to the two-step assay, condition-specific activation of guide RNA was observed also in the one-pot assay.

[0139] These results demonstrate that the guide RNA of the present invention can be specifically activated by the corresponding microRNA that is derived from human endogenous sequences, suggesting the possibility of conditional control of CRISPR-Cas

system using an endogenous molecule as a regulating factor. Since the tested microRNAs are specific to psoriasis skin cells (miR-31), hypertrophic scarring tissues (miR-98), pancreatic cancer tissues (miR-451), neutrophils following traumatic injury (miR-3945), and in endothelial cells (miR-21), it is expected the CRISPR-Cas system would be especially effective in these conditions.

(Example 2: Regulating the activation of a guide RNA with two openers)

Materials and Methods

[0140] In this example, a guide RNA comprising a first switching domain in the second functional module (the nexus), a third switching domain in the first functional module (the crRNA:tracrRNA duplex), and second and fourth switching domains at the 3'-terminus was prepared. Two openers that are capable of disrupting the hybridization between the first/second switching domains and between the third/fourth switching domains, respectively, were used to regulate the activation of said guide RNA (Figure 10 (a)-(d)). The two-pot assay was used as described above to detect the activation of the Cas9 protein. The experimental condition was the same as Example 1 except that two openers (miRNAs) were added to a sample, instead of one, at a concentration of 500 nM each.

[0141] The constructs of the DNA templates encoding the guide RNAs used in Example 2 are shown below in Table 6 and are represented by SEQ ID NOs: 31 and 32. As can be seen from Table 6, two constructs of guide RNAs (Example 2 (A) and Example 2 (B)) were prepared in this example. The sequence in the first functional module that is complementary to fourth switching domain is shorter in Example 2 (A) (9 nt) than in Example 2 (B) (13 nt), but the rest of the constructs are identical.

Table 6: Sequences of the DNA templates encoding the guide RNAs used in Example 2
*The underlined sequence corresponds to a T7 promoter. Sequences upstream of the promoter are for padding.

Name	Sequence	SEQ ID NO:
Guide RNA for Example 2 (A)	CACTACACTACTAACACACCACCAAATAATACGACTCACTATAG GCTAAAGAGGAAGAGGACAGTTTTAGAGCTAGGCATAGCTTAG CAAGTTAAAATAATCCTAGTGATGTTGAAACTTGAAAAAGTGG TCAACATCAGTCTGATAAGCTAGGAAAGAGGATGAATAGCTAT GCCAGCATCTTGCCT	31
Guide RNA for Example 2 (B)	CACTACACTACTAACACACCACCAAATAATACGACTCACTATAG GCTAAAGAGGAAGAGGACAGTTTTAGAGCTATGCTGGCATAGC TTAGCAAGTTAAAATAATCCTAGTGATGTTGAAACTTGAAAAA GTGGTCAACATCAGTCTGATAAGCTAGGAAAGAGGATGAATAG CTATGCCAGCATCTTGCCT	32

[0142] The constructs of the guide RNAs used in the examples are shown below in Tables 7 and 8 and are represented by SEQ ID NOs: 4, 10, 15, 16, 20, and 33 to 35.

Table 7: Sequences of the spacer, the second and third functional modules, the linker between the second and the fourth switching domains, and the fourth switching domain used in the examples

Name	Sequence	SEQ ID NO:
Spacer	GGCUAAAGAGGAAGAGGACA	10
Second Functional Module comprising First Switching Domain (Bold : the portion hybridizing to the Second Switching Domain)	AAUCCUAGUGAUGUUGA	15
Third Functional Module	AACUUGAAAAAGUGG	4
Second Switching Domain (Bold : the portion hybridizing to the First Switching Domain)	UCAACAUCAGUCUGAUAAGCUA	20
Linker	GGAAAGAGGAUGAAU	35
Fourth Switching Domain (<u>Underlined</u> : the portion hybridizing to the Third Switching Domain for Example 2 (A); Bold : the same portion for Example 2 (B))	<u>AGCUAUGCC</u> AGCAUCUUGCCU	16

Table 8: Sequences of the first functional module comprising a third switching domain used in the examples

Construct	First Functional Module comprising Third Switching Domain (Bold : the portion hybridizing to the Fourth Switching Domain)	SEQ ID NO:
Example 2(A)	GUUUUAGAGCUAG GGCAUAG CUUAGCAAGUUA AA AAU	33
Example 2(B)	GUUUUAGAGCUAUG CUGGGCAUAG CUUAGCAAGUUA AA AAU	34

[0143] Two of the microRNAs used in Example 1, namely miR21 (SEQ ID NO: 30) and miR31 (SEQ ID NO: 26) were used as the openers in Example 2.

Results

[0144] The results of detected fluorescence are shown in Fig. 10 (e) and (f). As can be seen, fluorescence did not increase or increased only in a limited range when no opener was present (Fig. 10 (a)) or when only one of the openers miR21 and miR31 was present (Fig. 10 (b) and (d)). On the other hand, a significant increase in fluorescence was observed when both miR21 and miR31 were present (Fig. 10 (c)). The results obtained for Example 2(A) with a shorter third switching domain (9 nt) (Fig. 10 (e)) were similar to the results for Example 2(B) with a longer third switching domain (13 nt) (Fig. 10(f)), suggesting that hybridization of 9 nt in the crRNA:tracrRNA duplex is sufficient to regulate the activity of the guide RNA. Overall, these results indicate that it is possible to regulate the activation of the guide RNA of the present invention in a highly-controlled manner using two openers each targeting different domains of the guide RNA.

(Example 3: Regulating the activation of a guide RNA with an opener and the fourth switching domain)

Materials and Methods

[0145] In Example 3, it was confirmed whether it would be possible to use a NOT gate to regulate the activation of the guide RNA of the present invention. In this example, a guide RNA comprising a first switching domain in the second functional module (the nexus), a third switching domain in the first functional module (the crRNA:tracrRNA duplex), and a second switching domain at the 3'-terminus was prepared. A polynucleotide encoding a fourth switching domain (referred to as a "NOT" RNA) and an opener for disrupting the hybridization between the first and switching domains (referred to as an "AND" RNA) were

used to regulate the activation of said guide RNA (Figure 11A (a)-(d)). The one-pot assay was used as described above to detect the activation of the Cas9 protein. The experimental condition was the same as Example 1 except that 2 μ M of NOT RNA and 500 nM of AND RNA were added instead of 500 nM of the opener microRNA.

[0146] The constructs of the DNA templates encoding the guide RNAs used in Example 3 are shown below in Table 9 and are represented by SEQ ID NOs: 36 and 37. As can be seen from Table 9, two constructs of guide RNAs (Example 3(A) and Example 3(B)) were prepared in this example. In the guide RNA of Example 3 (A), the third switching domain is designed to hybridize to miR31 and the hybridization between the first and second switching domain is designed to be disrupted by miR21, therefore in Example 3(A) miR31 serves as the NOT RNA and miR21 serves as the AND RNA. The guide RNA of Example 3(B) was prepared by switching miR31 and miR21 of Example 3(A), i.e. the third switching domain is designed to hybridize to miR21 and the hybridization between the first and second switching domain is designed to be disrupted by miR31, therefore in Example 3(B) miR21 serves as the NOT RNA and miR31 serves as the AND RNA. The rest of the constructs are identical.

Table 9: Sequences of the DNA templates encoding the guide RNAs used in Example 3
*The underlined sequence corresponds to a T7 promoter. Sequences upstream of the promoter are for padding.

Name	Sequence	SEQ ID NO:
Guide RNA for Example 3(A)	CACTACACTACTAACACACCACCAAATAATACGACTCACTATAG GCTAAAGAGGAAGAGGACAGTTTTAGAGCTATCAACATCAGTC TGATAAGCTATAGCAAGTTAAAATAAGCCTAGGGCATAGCTAA CTTGAAAAAGTGGAGCTATGCCAGCATCTTGCCT	36
Guide RNA for Example 3(B)	CACTACACTACTAACACACCACCAAATAATACGACTCACTATAG GCTAAAGAGGAAGAGGACAGTTTTAGAGCTAAGCTATGCCAGC ATCTTGCCTTAGCAAGTTAAAATAATCCTAGTGATGTTGAAACT TGAAAAAGTGGTCAACATCAGTCTGATAAGCTA	37

Results

[0147] The results of detected fluorescence are shown in Fig. 11B (a) and (b). As can be seen from Fig. 11B (a), fluorescence did not increase or increased only in a limited range when neither the AND nor NOT RNA was present (Fig. 11A (a)), when both the AND and NOT RNAs were present (Fig. 11A (c)), and when only the NOT RNA is present (Fig. 11A (d)). On the other hand, a significant increase in fluorescence was observed when only the AND RNA was present (Fig. 11A (b)). The results were similar even when the NOT RNA and the AND RNA were switched (Fig. 11B (b)), suggesting that the effect is not dependent on particular sequences in the switching domains. Overall, these results indicate that it is possible to regulate the activation of the guide RNA of the present invention in a highly-controlled manner by combining the AND and NOT logic gates, and the regulation does not rely on the sequence of the switching domains.

CLAIMS

1. A CRISPR-Cas system guide RNA consisting of one or more polynucleotide(s), wherein the guide RNA comprises:
 - (A) the following elements (a) to (d), arranged in this order from 5' to 3':
 - (a) a spacer which can hybridize to a target polynucleotide;
 - (b) a first functional module comprising a crRNA:tracrRNA duplex;
 - (c) a second functional module comprising a first switching domain; and
 - optionally (d) a third functional module; and
 - (B) a second switching domain,
 wherein at least a portion of the first switching domain is substantially complementary to at least a portion of the second switching domain, and when an opener is present, the opener disrupts the hybridization between the first switching domain and the second switching domain and the guide RNA forms at least one stem within the first switching domain, and thereby becomes capable of activating a Cas protein.
2. The guide RNA according to Claim 1, wherein the second functional module comprises or consists of the sequence represented by formula (I):



wherein:

N each independently represents any base selected from A, C, G, and U;

m is an integer ranging from 1 to 4, preferably 2;

p is an integer ranging from 1 to 4, preferably 2;

q is an integer ranging from 1 to 10, preferably from 3 to 7, more preferably 5;

r is an integer ranging from 1 to 10, preferably from 3 to 8, more preferably 6; and

(X)_m represents the sequence 5'-X¹...X^{m-1}X^m-3';

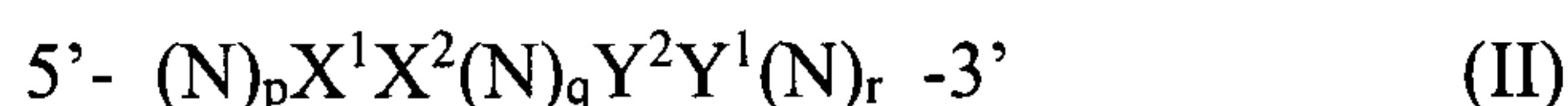
(Y)_m represents the sequence 5'-Y^mY^{m-1}...Y¹-3';

the mth X (X^m) and the mth Y (Y^m) are capable of being hybridized to form a stem and

each of the pair of X^m/Y^m is independently selected from C/G, G/C, A/U, and U/A, and

wherein the first switching domain comprises at least region (X)_m or (Y)_m.

3. The guide RNA according to Claim 2, wherein the second functional module comprises or consists of the sequence represented by formula (II):



wherein:

N, p, q, and r have the meanings as defined for formula (I) in Claim 2; and

the pairs X¹/Y¹ and X²/Y² are each independently selected from C/G, G/C, A/U, and U/A, preferably both pairs X¹/Y¹ and X²/Y² are G/C.

4. The guide RNA according to Claim 2 or 3, wherein the second functional module comprises or consists of the sequence represented by formula (III):



wherein:

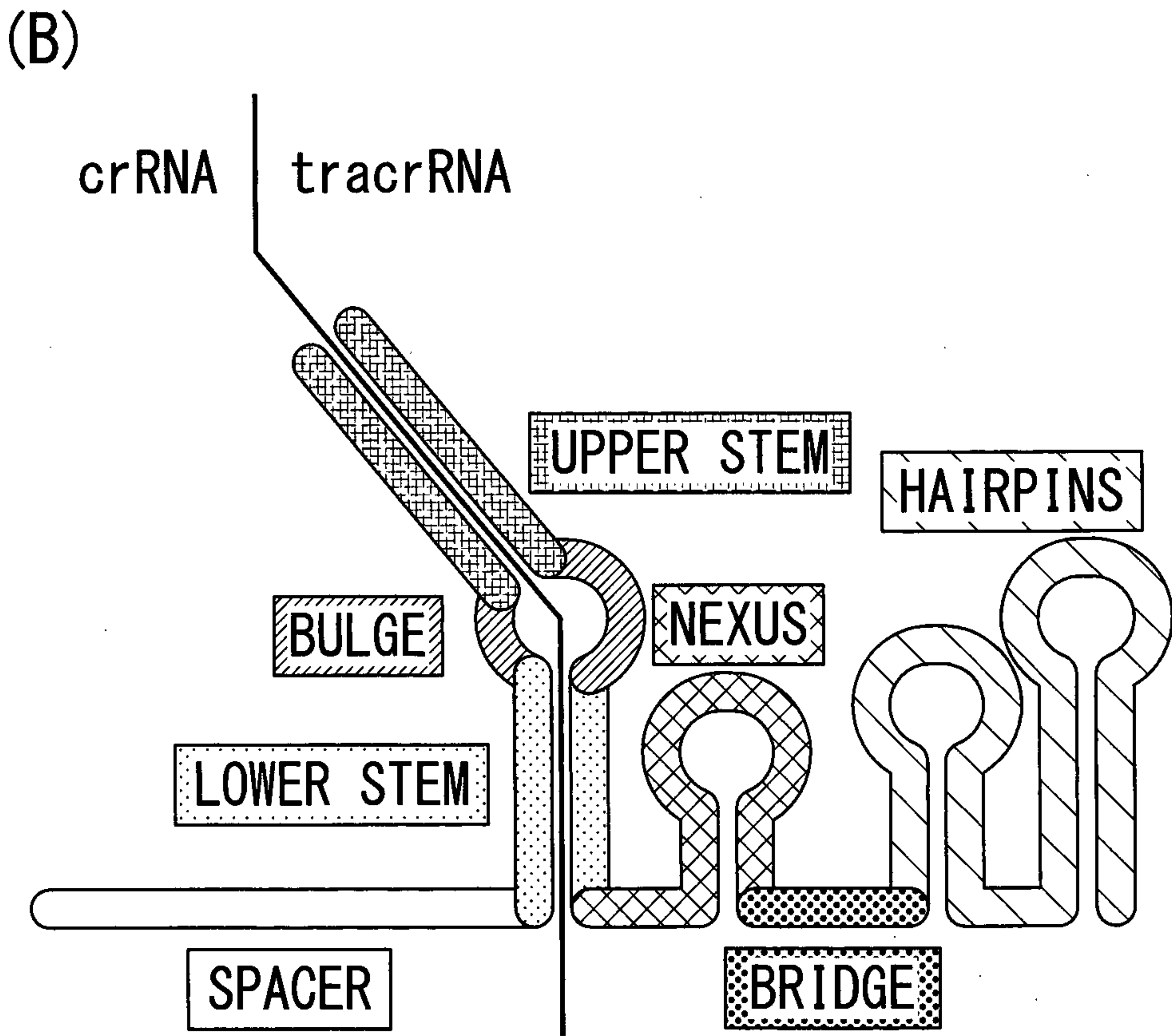
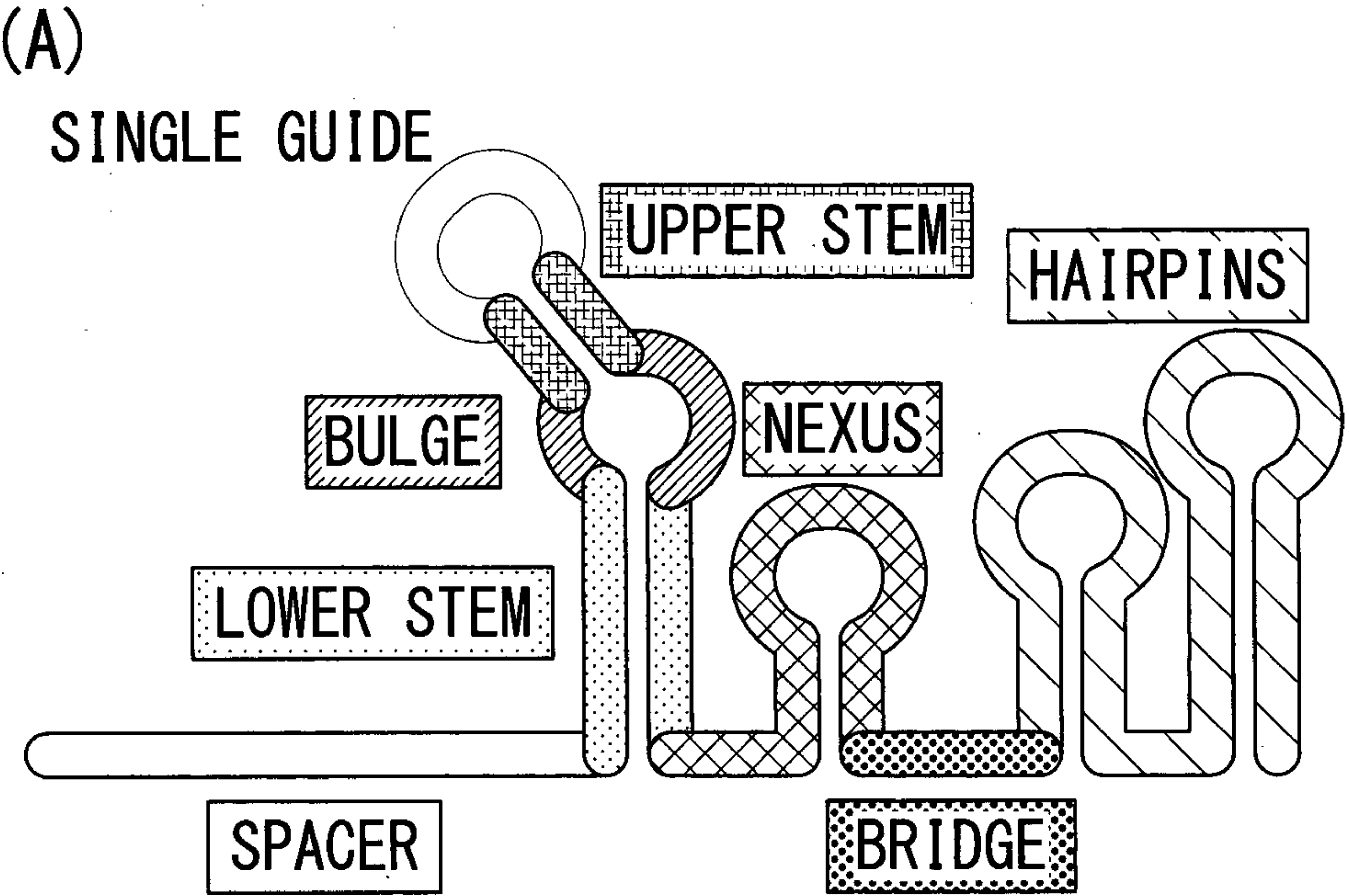
N, X¹, Y¹, X², and Y² have the meanings as defined for formulae (I) and (II) in Claims 2 and 3.

5. The guide RNA according to any one of Claims 1 to 4, wherein the second switching domain is located at the 5'-terminus, at the 3'-terminus, or inside or between any of the elements (a) to (d).
6. The guide RNA according to any one of Claims 1 to 5, wherein the second switching domain is substantially complementary to at least a portion of the opener, and the opener disrupts the hybridization between the first switching domain and the second switching domain by hybridizing to the second switching domain, preferably by a toehold-mediated strand displacement.
7. The guide RNA according to any one of Claims 1 to 6, further comprising a third switching domain wherein at least a portion of the third switching domain is substantially complementary to at least a portion of a fourth switching domain, and when at least a portion of the third switching domain and at least a portion of the fourth switching domain are hybridized, the structure of the guide RNA is altered such that the guide RNA does not maintain the function of activating a Cas protein.
8. The guide RNA according to Claim 7, wherein the third switching domain is located within the first functional module.
9. The guide RNA according to Claim 7 or 8, wherein the fourth switching domain is present as a part of the guide RNA and is located at the 5'-terminus, at the 3'-terminus, inside or between any of the elements (a) to (d), or in the 5'-side or the 3'-side of the second switching domain, preferably in the 3'-side of the second switching domain, or the fourth switching domain is present independently of the guide RNA.
10. A polynucleotide encoding at least one guide RNA according to any one of Claims 1 to 9, wherein preferably said polynucleotide is comprised in a vector.
11. A vector comprising at least one polynucleotide according to Claim 10.
12. A composition comprising at least one guide RNA according to any one of Claims 1 to 9 or at least one polynucleotide encoding thereof and at least one Cas protein or at least one polynucleotide encoding thereof, and optionally further comprising at least one opener or at least one polynucleotide encoding thereof.
13. The composition according to Claim 12, for treating, preventing, and/or detecting at least one disease or disorder in a subject.
14. A method of regulating the activation of a guide RNA according to any of Claims 1 to 9, comprising the step of hybridizing at least a portion of the first switching domain and at least a portion of the second switching domain and/or disrupting the hybridization between the first switching domain and the second switching domain using a first opener, and optionally, when the guide RNA comprises a third switching domain, the step of hybridizing at least a portion of the third switching domain and at least a portion of a fourth switching domain and/or disrupting the hybridization between the third switching domain and the fourth

switching domain using a second opener which may be the same as or different from the first opener.

15. A method of cleaving at least one target polynucleotide, modifying the sequence of at least one target polynucleotide, and/or altering the expression of at least one gene encoded by at least one target polynucleotide, comprising the step of contacting the target polynucleotide with at least one guide RNA according to any one of Claims 1 to 9, at least one opener, and at least one Cas protein.
16. The method according to Claim 15, wherein the opener is an RNA.
17. The method according to Claim 15, wherein the opener is an endogenous RNA.
18. The method according to Claim 15, wherein the opener is a microRNA.
19. The method according to any one of Claims 15 to 18, wherein the Cas protein is specifically activated in a certain type of cell, tissue, or individual; at a certain time point such as at a certain phase of the cell cycle; under a certain physiological condition such as in a patient with a specific disease; or in response to certain external stimuli such as temperature, light, force, and pH.
20. A kit comprising at least one guide RNA according to any one of Claims 1 to 9, or at least one polynucleotide encoding thereof, and optionally comprising at least one opener or at least one polynucleotide encoding thereof and/or at least one Cas protein or at least one polynucleotide encoding thereof.

FIG. 1



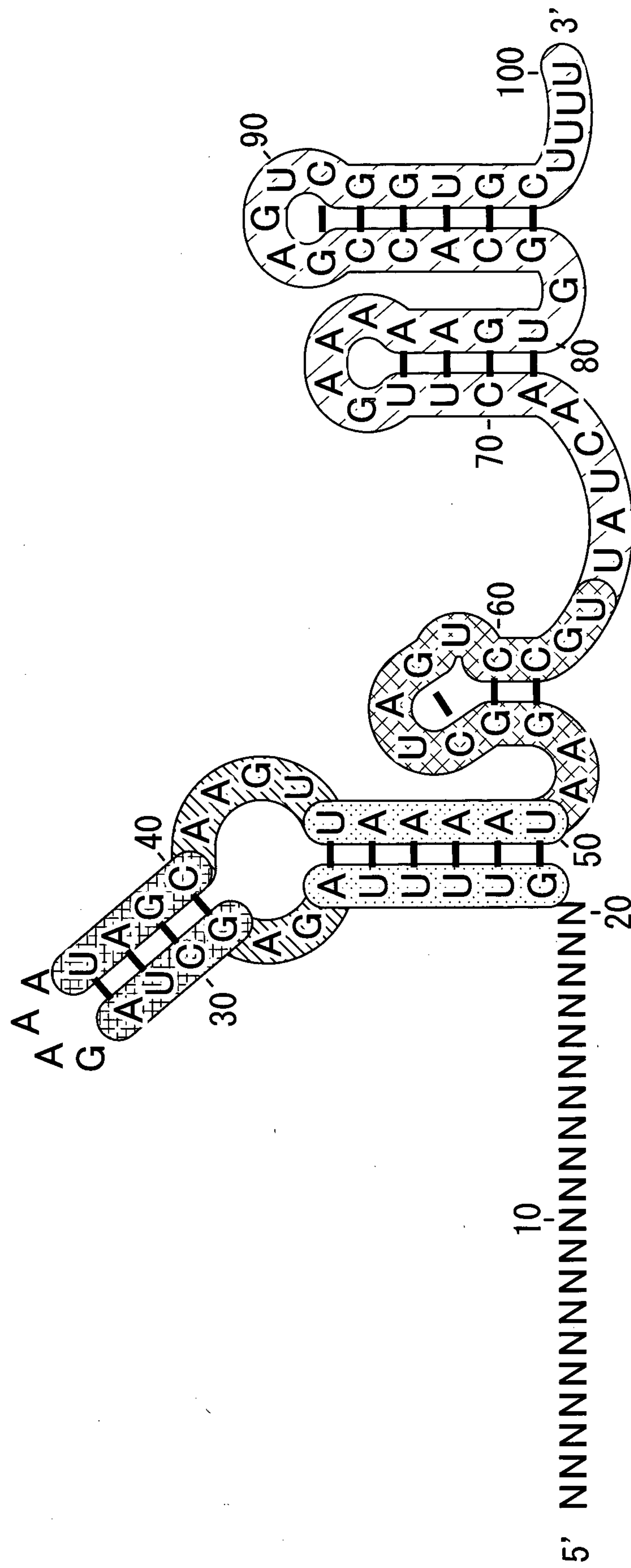


FIG. 2

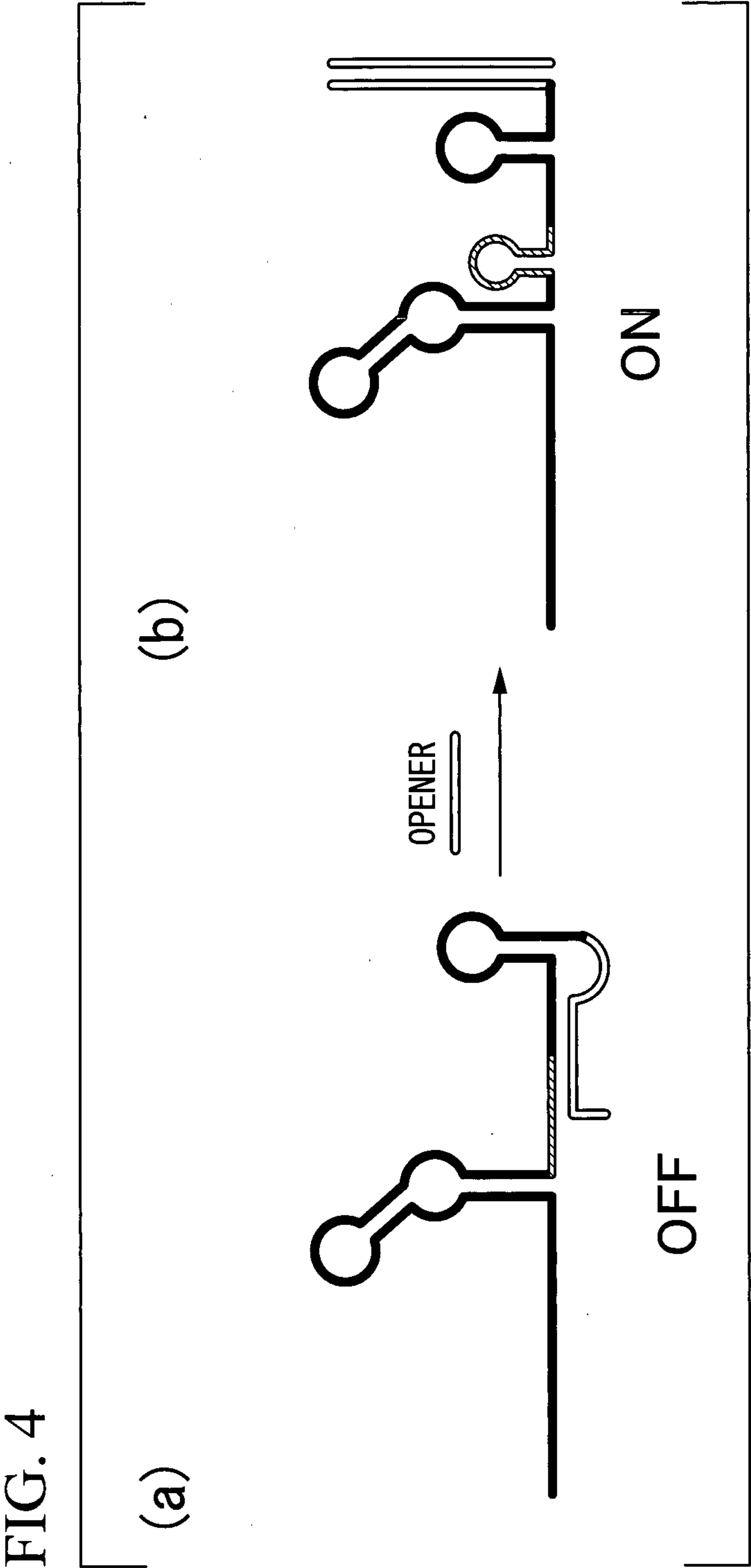


FIG. 5

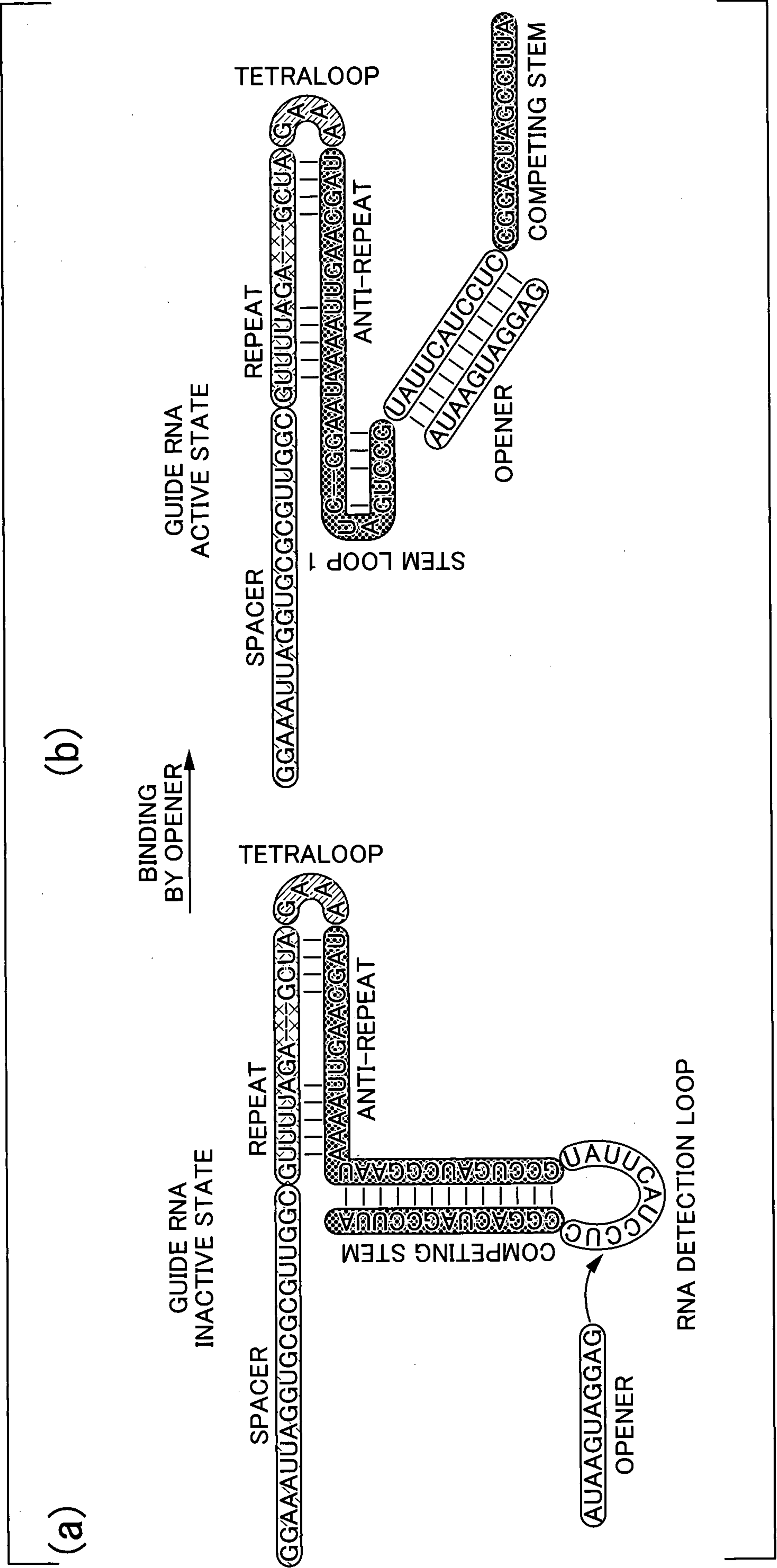


FIG. 6

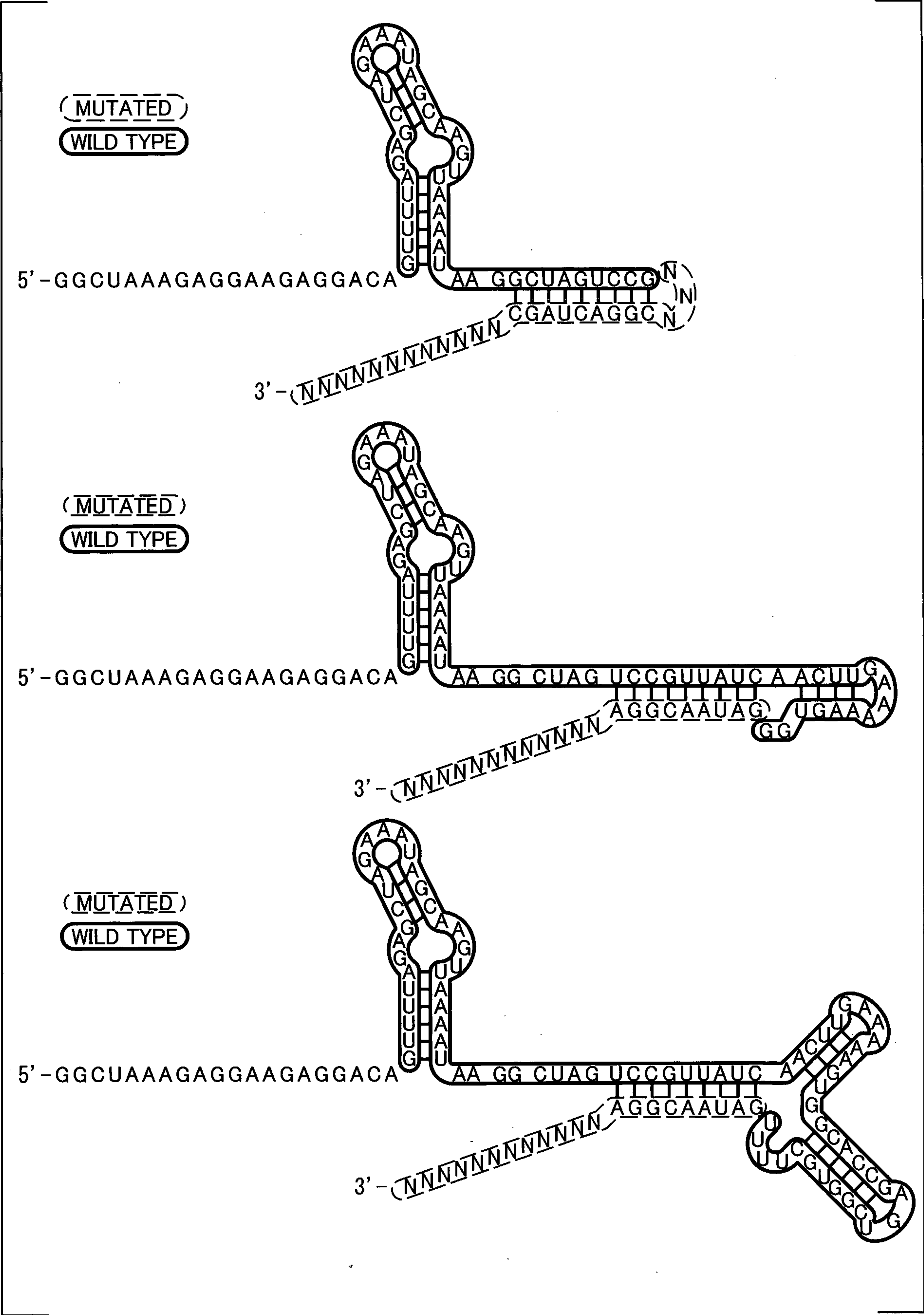


FIG. 7

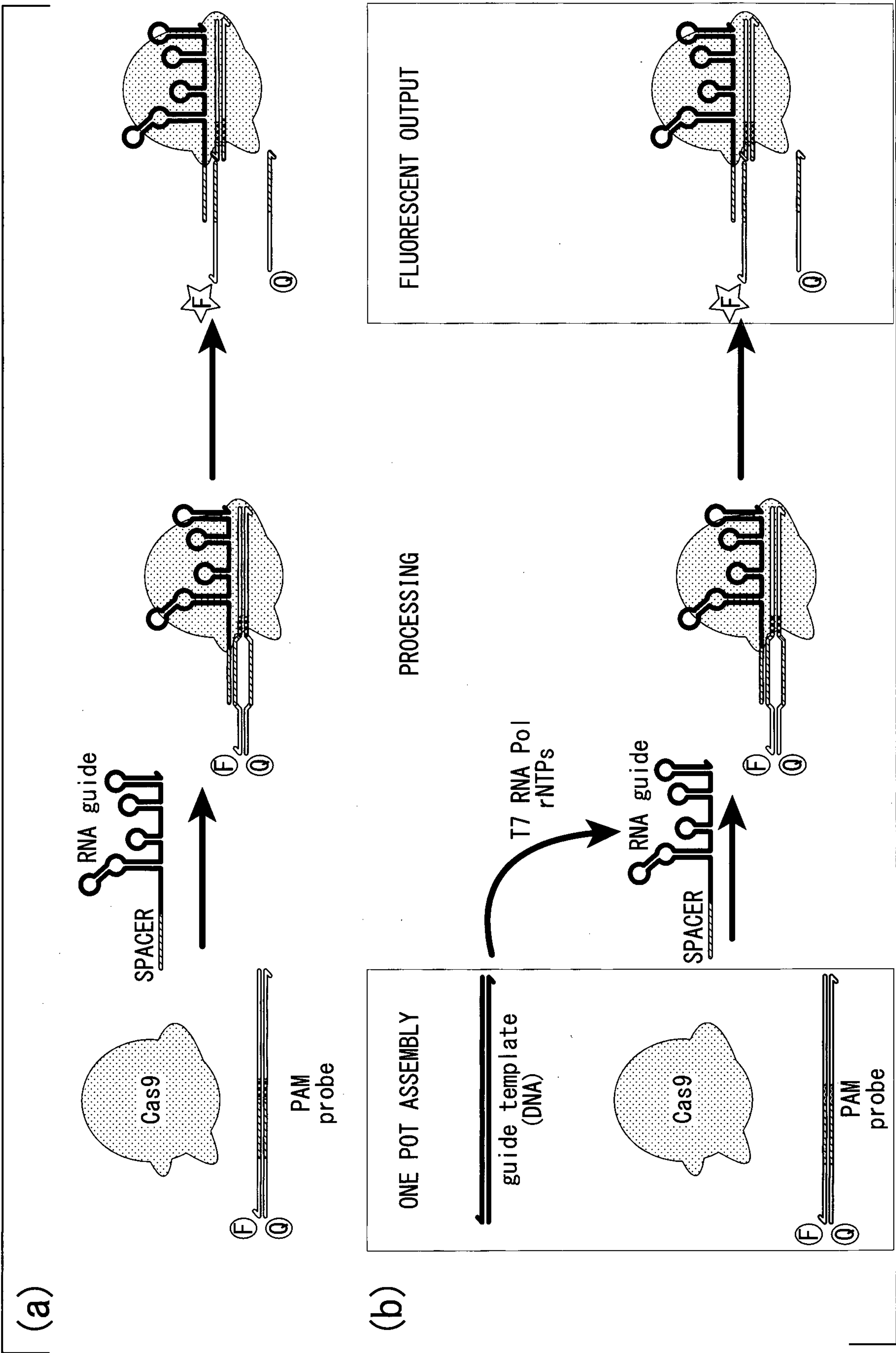
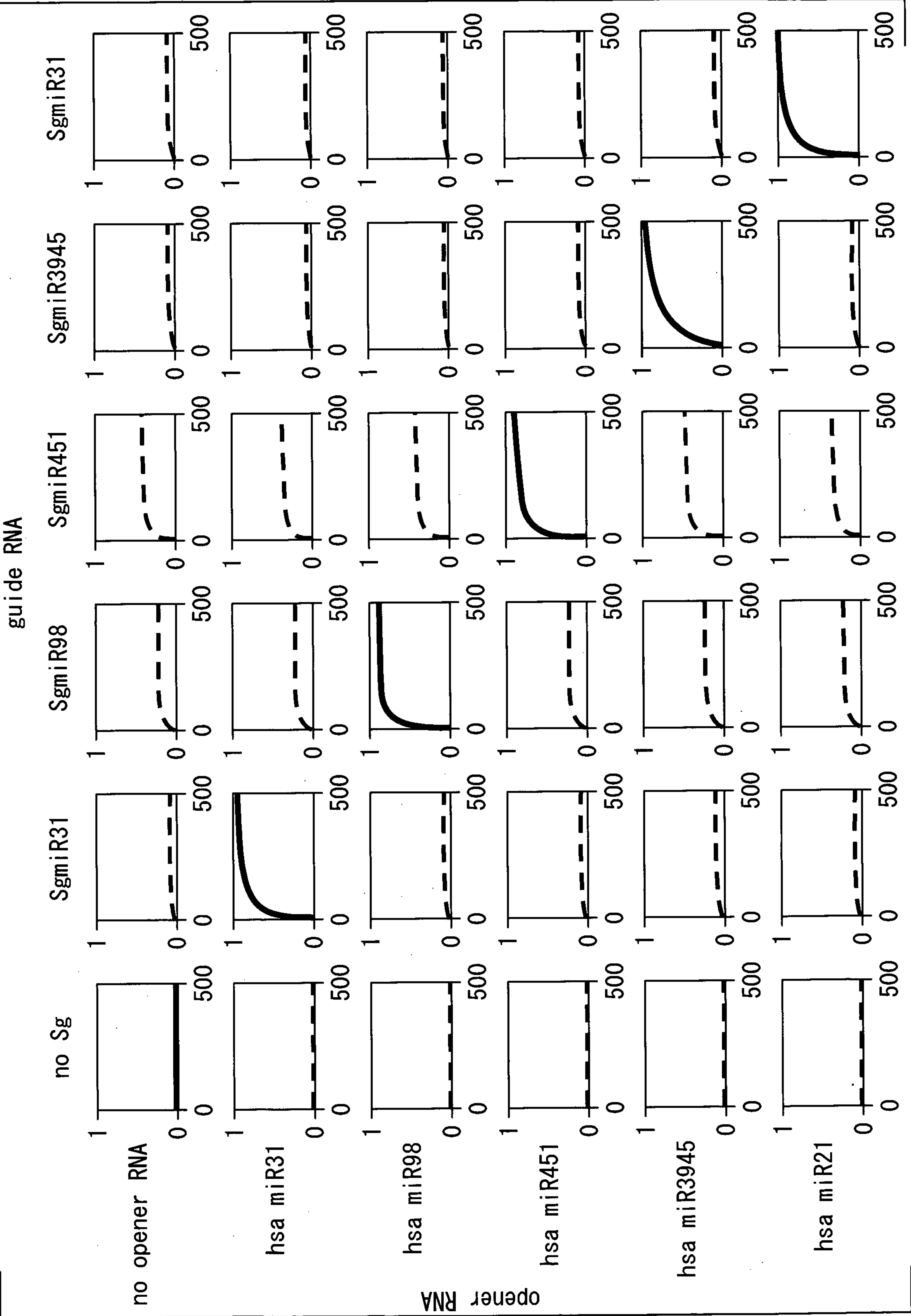


FIG. 8



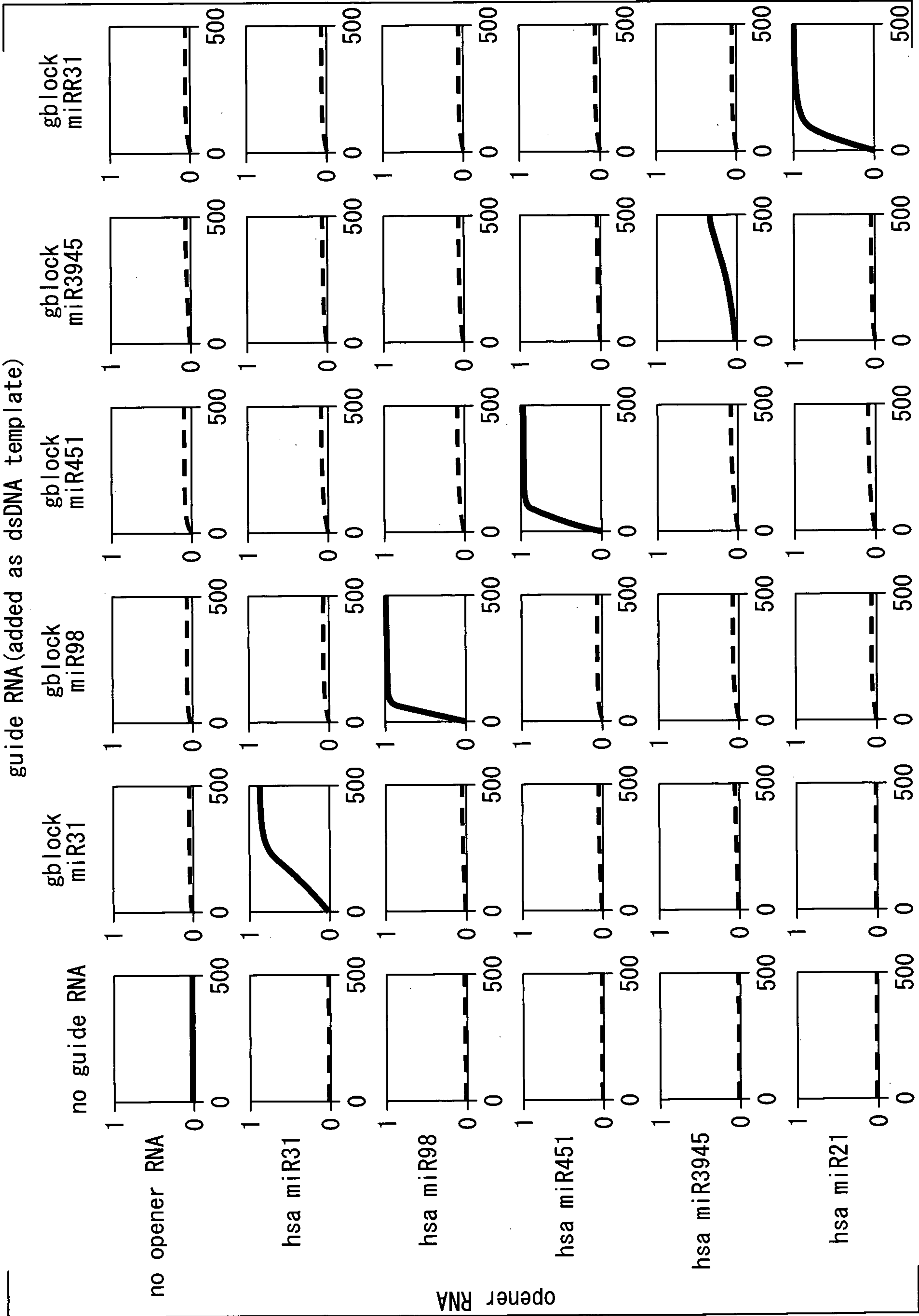


FIG. 10

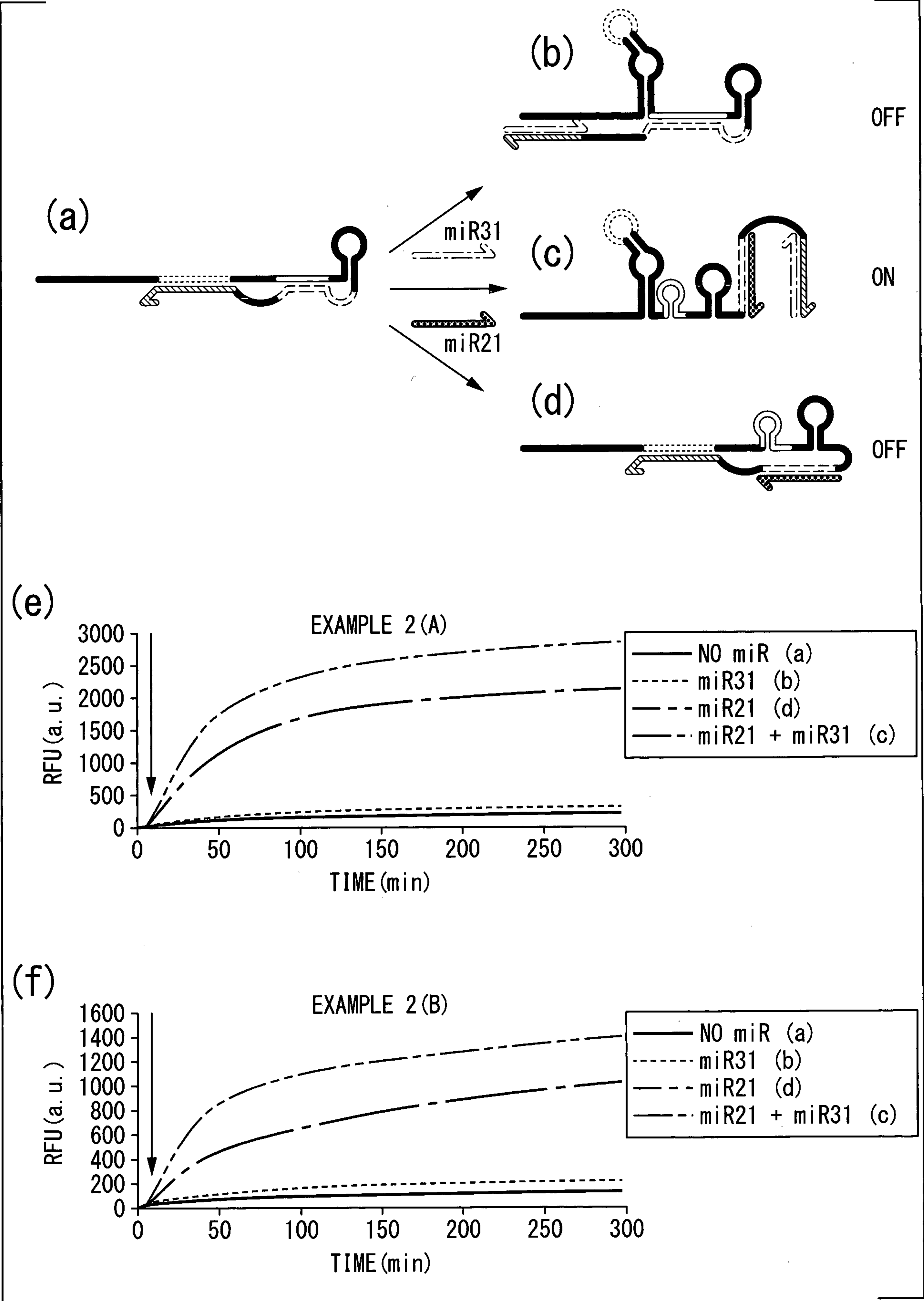
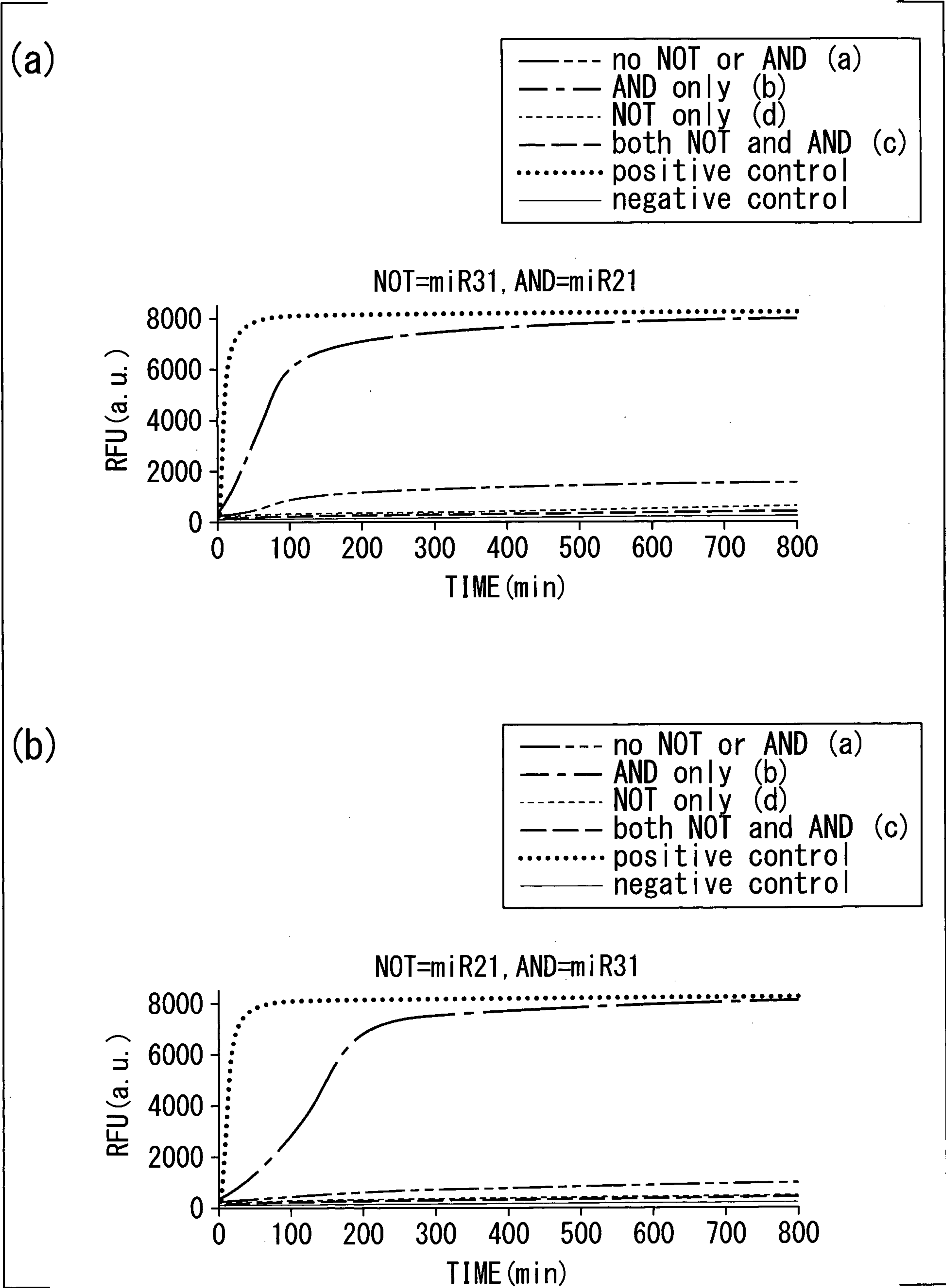


FIG. 11B



INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2019/048835

A. CLASSIFICATION OF SUBJECT MATTER

A61K 31/7105(2006.01)i; A61K 48/00(2006.01)i; C12N 15/09(2006.01)i; C12N 15/11(2006.01)i; C12N 15/63(2006.01)i; C12Q 1/68(2018.01)i; A61K 38/46(2006.01)i
FI: C12N15/09 110; C12N15/11 Z ZNA; C12N15/63 Z; A61K48/00; A61K31/7105; A61K38/46; C12Q1/68

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)
C12N15/00-15/90; C12Q1/68-1/6897

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Published examined utility model applications of Japan 1922-1996
Published unexamined utility model applications of Japan 1971-2020
Registered utility model specifications of Japan 1996-2020
Published registered utility model applications of Japan 1994-2020

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
JSTPlus/JMEDPlus/JST7580 (JDreamIII); CAlus/MEDLINE/BIOSIS/WPIDS (STN)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JAKIMO N et al., ssRNA/DNA-Sensors via Embedded Strand-Displacement Programs in CRISPR/Cas9 Guides, bioRxiv, 2018.02.12, https://www.biorxiv.org/content/10.1101/264424v1.full.pdf , [retrieved on 2020-03-03] Abstract, Fig. 3C	1-6, 10-17, 19-20
Y		1-20
Y	JIN M et al., Programmable CRISPR-Cas Repression, Activation, and Computation with Sequence-Independent Targets and Triggers, ACS Synth. Biol., 2019, 8(7), pp.1583-1589, Epub 2019 Jul 10 Abstract, Fig. 3	1-20
Y	WO 2017/223449 A1 (PRESIDENT AND FELLOWS OF HARVARD COLLEGE) 28 December 2017 (2017-12-28) pp. 30-31	1-20
Y	WO 2015/168404 A1 (MASSACHUSETTS INSTITUTE OF TECHNOLOGY) 05 November 2015 (2015-11-05) [0056], [0065]	1-20

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:
“A” document defining the general state of the art which is not considered to be of particular relevance
“E” earlier application or patent but published on or after the international filing date
“L” document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
“O” document referring to an oral disclosure, use, exhibition or other means
“P” document published prior to the international filing date but later than the priority date claimed

“T” later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
“X” document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
“Y” document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
“&” document member of the same patent family

Date of the actual completion of the international search
03 March 2020

Date of mailing of the international search report
17 March 2020

Name and mailing address of the ISA/JP
**Japan Patent Office
3-4-3, Kasumigaseki, Chiyoda-ku, Tokyo
100-8915, Japan**

Authorized officer
TAKEUCHI Yuki 4B 5082
Telephone No. **+81-3-3581-1101 Ext. 3448**

INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2019/048835

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LI Y et al., RNA Strand Displacement Responsive CRISPR/Cas9 System for mRNA Sensing, Anal. Chem., 2019, 91(6), pp.3989-3996, Epub 2019 Feb 27 Abstract, Figs. 1-2	1-20
A	SIU KH et al., Riboregulated toehold-gated gRNA for programmable CRISPR-Cas9 function, Nat. Chem. Biol., 2019, 15(3), pp.217-220, Epub 2018 Dec 10 Abstract, Fig. 1	1-20
A	WO 2018/231730 A2 (CALIFORNIA INSTITUTE OF TECHNOLOGY) 20 December 2018 (2018-12-20) Examples	1-20
A	GREEN AA et al., Complex cellular logic computation using ribocomputing devices, Nature, 2017, 548(7665), pp.117-121 Abstract	1-20

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. ☐ forming part of the international application as filed:
- ☐ in the form of an Annex C/ST.25 text file.
- ☐ on paper or in the form of an image file.
- b. ☒ furnished together with the international application under PCT Rule 13^{ter}.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
- c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
- ☐ in the form of an Annex C/ST.25 text file (Rule 13^{ter}.1(a)).
- ☐ on paper or in the form of an image file (Rule 13^{ter}.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/JP2019/048835

Patent document cited in search report			Publication date (day/month/year)	Patent family member(s)			Publication date (day/month/year)
WO	2017/223449	A1	28 December 2017	US	2019/0233806	A1	
WO	2015/168404	A1	05 November 2015	(Family: none)			
WO	2018/231730	A2	20 December 2018	US	2018/0362944	A1	