In addition, an RNA-DNA chimeric dumbbell-shaped nucleic acid is disclosed (JP11-137260A(1999)). This nucleic acid is not for use in RNA interference. The RNA portion of the dumbbell-shaped nucleic acid is cleaved by an enzyme in cells, and the resulting antisense DNA portion binds to a mRNA in cells to inhibit it. JP11-137260A(1999) discloses that a nucleic acid possesses a high resistance to nucleic acid degrading enzymes in cells and remains stable in cells until ribonuclease H acts, owning to its dumbbell-shaped structure. It further discloses that, because a single-chain oligonucleotide containing the antisense DNA portion can be released into the cytoplasm of cells only after cleaving the complementary RNA portion with the effect of ribonuclease H, the antisense effect per dose is believed to be enhanced.

Moreover, regarding the method of synthesizing a circular nucleic acid having a dumbbell-shaped structure, for example, it is disclosed that linear oligonucleotides are synthesized, a stem region and a hairpin loop region are formed to obtain a nick dumbbell-shaped oligonucleotide, wherein one site of the target circular dumbbell-shaped oligonucleotide (the opposite end of the above hairpin loop region) is unbound, and the 5' end is ligated with a ligase to prepare a circular dumbbell-shaped circular nucleic acid (JP11-137260(1999)).

It is an object of the present invention to provide a single-chain circular RNA and a method of producing the same.

Summary~~Disclosure~~ of the Invention

The present inventors have now found that, surprisingly, a single-chain circular RNA can be obtained with a high yield by separately synthesizing a sense strand and an antisense strand, both comprising a nucleotide sequence with unpaired nucleotides at each end, and allowing ligase to act on the nucleotides at both ends simultaneously, and that the obtained single-chain circular RNA exerts a sustained or slow-releasing RNA interference effect. Based on these scientific findings, the present inventors have accomplished the present invention.

In one aspect, the present invention refers to a single-chain circular RNA having a sustained or slow-releasing RNA interference effect, characterized in that the single-chain circular RNA comprises a sense strand sequence, an antisense strand sequence complementary to the sense strand sequence, identical or different two loop sequences between the sense strand and the antisense strand, connecting both strands, wherein the sense strand and the antisense strand are paired to form a stem, and wherein each of the two loop sequences has 6 to 9 nucleotides in length.

In another aspect, the present invention refers to a method of producing the single-chain circular RNA as defined above, comprising synthesizing a sense strand and an antisense strand, both comprising a nucleotide sequence with unpaired nucleotides at the 5` end and 3` end, and simultaneously ligating the nucleotide at the 5' end of the nucleotide sequence with unpaired nucleotides in the sense strand, with the nucleotide at the 3' end of the nucleotide sequence with unpaired nucleotides in the antisense strand, and vice versa, using a ligase,

wherein the nucleotide sequence with unpaired nucleotides at the 5' end of the sense strand and the nucleotide sequence with unpaired nucleotides at the 3' end of the antisense strand are bound to each other to form a loop, the nucleotide sequence with unpaired nucleotides at the 3` end of the sense strand and the nucleotide sequence with unpaired nucleotides at the 5` end of the antisense strand are bound to each other to form a loop, and the sense strand and the antisense strand are paired to form a stem.

In another aspect, the present invention refers to a method of suppressing expression of a gene encoding a protein *in vitro*, comprising introducing the single-chain circular RNA as defined above to human-derived cells, and impairing a target RNA by the single-chain circular RNA to inhibit translation of the target RNA into the protein in a sustained manner.

In another aspect, the present invention refers to a method of suppressing expression of a gene encoding a protein, comprising introducing *in vitro* the single-chain circular RNA as defined above to cells of non-human animals or plants, and impairing a target RNA by the single-chain circular RNA to inhibit translation of the target RNA into the protein in a sustained manner.

In another aspect, the present invention refers to a pharmaceutical composition comprising the single-chain circular RNA as defined above as an active ingredient.

More specifically, the present ~~invention~~disclosure includes the following examples~~inventions~~.

(8) The method according to any one of the above (4) to (7), wherein the stem length is 19 to 31 nucleotides.

(9) A method of suppressing expression of a gene encoding a protein *in vitro*, comprising introducing the single-chain circular RNA according to any one of the above (1) to (3) to human-derived cells, and impairing a target RNA by the single-chain circular RNA to inhibit translation of the target RNA into the protein in a sustained manner.

(10) A method of suppressing expression of a gene encoding a protein, comprising introducing the single-chain circular RNA according to any one of the above (1) to (3) to non-human animals, plants or cells thereof, and impairing a target RNA by the single-chain circular RNA to inhibit translation of the target RNA into the protein in a sustained manner.

(11) A pharmaceutical composition comprising the single-chain circular RNA according to any one of the above (1) to (3) as an active ingredient.

As used herein, the term "single-chain circular RNA" may be referred to as dumbbell-shaped RNA. A dumbbell-shaped RNA refers to a single-chain circular RNA, wherein a sense strand and an antisense strand are complementarily paired to form a stem, loops are formed at both sides of the stem by a nucleotide sequence with unpaired nucleotides, and the entire shape of the single-chain circular RNA is in a dumbbell.

According to the present ~~invention~~disclosure, the dumbbell-shaped synthetic RNA, which is excellent in stability, sustainability and slow-releasing property, can be efficiently produced.

In particular, the dumbbell-shaped RNA for use in the RNA interference method can be produced by cyclizing two RNA strands. Because both ends are closed in a loop shape, it has no RNA end and is therefore not likely to serve as a substrate for enzymes, exonuclease (RNase) and the like, except for specific enzymes such as Dicer in cells. Thus, it is less susceptible to enzymatic degradation and has significantly increased stability in the cell. As a result, there is no necessity to use non-natural nucleic acids to enhance stability.

Moreover, the dumbbell-shaped RNA is specifically recognized by *in vivo* enzymes such as Dicer in cells, and the loop regions at both sides are cleaved to form a naturally occurring type double-stranded RNA (Figure 1). As a result, it can have activity equivalent

to that of a double-stranded RNA, and exerts a more sustainable or slow-releasing effect than RNA interference effect by conventional double-stranded RNAs.

The dumbbell-shaped RNA of the present ~~invention~~disclosure is also more stable than conventional double-stranded RNAs in human serum.

Brief Description of the Drawings

Figure 1 is a schematic view of the dumbbell-shaped RNA of the present invention.

Figure 2 shows the structure of RNAs and an electrophoresis image of each RNA.

Figure 3 shows the structure of siRNA and dumbbell-shaped RNAs.

Figure 4 shows electrophoresis images of dumbbell-shaped RNAs and liner double-stranded RNAs cleaved by Dicer.

Figure 5 shows an interference effect of each dumbbell-shaped RNA 24 hours after transfection.

Figure 6 shows a sustainable RNA interference effect of the dumbbell-shaped RNA.

Figure 7 shows stability of the dumbbell-shaped RNA in human serum.

Best Mode for Carrying Out the Invention

The single-chain circular RNA of the present invention includes a sense strand sequence homologuous to the nucleotide sequence of a target RNA or part thereof, an antisense strand sequence which is complementary to the sense strand sequence and is capable of pairing with it, and loop sequences which cannot form pairing between the strands.

The sense strand and the antisense strand are paired to form a stem. The length of the stem, not specifically limited, can be determined depending on the type, structure or the like of a target RNA. For example, the stem is composed of 19 to 31 base pairs, preferably 21 to 25 base pairs, more preferably 22 to 24 base pairs, and even more preferably 23 base pairs.

The nucleotide sequences with unpaired nucleotides are present at the 5' end and 3' end of both the sense strand and the antisense strand. The nucleotide at the 5' end of the nucleotide sequence with unpaired nucleotides in the sense strand and the nucleotide at the 3' end of the nucleotide sequence with unpaired nucleotides in the antisense strand are ligated

1. A single-chain circular RNA having a sustained or slow-releasing RNA interference effect, characterized in that the single-chain circular RNA comprises a sense strand sequence, an antisense strand sequence complementary to the sense strand sequence, identical or different two loop sequences between the sense strand and the antisense strand, connecting both strands, wherein the sense strand and the antisense strand are paired to form a stem.

2. The single-chain circular RNA according to claim 1, wherein the expression of a target RNA is 0.4 or less 24 hours after introduction of the single-chain circular RNA into eukaryotic cells, compared to control cells whose expression level of the target RNA is set to 1.

3. The single-chain circular RNA according to claim 1 or 2, wherein 70% or more thereof is retained after 8 hours in human serum.

4. A method of producing the single-chain circular RNA according to any one of claims 1 to 3, comprising synthesizing a sense strand and an antisense strand, both comprising a nucleotide sequence with unpaired nucleotides at the 5' end and 3' end, and simultaneously ligating the nucleotide at the 5' end of the nucleotide sequence with unpaired nucleotides in the sense strand, with the nucleotide at the 3' end of the nucleotide sequence with unpaired nucleotides in the antisense strand, and vice versa, using a ligase,

wherein the nucleotide sequence with unpaired nucleotides at the 5' end of the sense strand and the nucleotide sequence with unpaired nucleotides at the 3' end of the antisense strand are bound to each other to form a loop, the nucleotide sequence with unpaired nucleotides at the 3' end of the sense strand and the nucleotide sequence with unpaired nucleotides at the 5' end of the antisense strand are bound to each other to form a loop, and the sense strand and the antisense strand are paired to form a stem.

5. The method according to claim 4, further comprising phosphorylating each 5' end of the nucleotide sequence with unpaired nucleotides in the sense strand and the nucleotide sequence with unpaired nucleotides in the antisense strand.

6. The method according to claim 4 or 5, wherein the loop sequences are identical to or different from each other.

7. The method according to any one of claims 4 to 6, wherein the loop length is 2 to 20 nucleotides.

8. The method according to any one of claims 4 to 7, wherein the stem length is 19 to 31 nucleotides.

9. A method of suppressing expression of a gene encoding a protein *in vitro*, comprising introducing the single-chain circular RNA according to any one of claims 1 to 3 to human-derived cells, and impairing a target RNA by the single-chain circular RNA to inhibit translation of the target RNA into the protein in a sustained manner.

10. A method of suppressing expression of a gene encoding a protein, comprising introducing the single-chain circular RNA according to any one of claims 1 to 3 to non-human animals, plants or cells thereof, and impairing a target RNA by the single-chain circular RNA to inhibit translation of the target RNA into the protein in a sustained manner.

11. A pharmaceutical composition comprising the single-chain circular RNA according to any one of claims 1 to 3 as an active ingredient.

CLAIMS

1. A single-chain circular RNA having a sustained or slow-releasing RNA interference effect, characterized in that the single-chain circular RNA comprises a sense strand sequence, an antisense strand sequence complementary to the sense strand sequence, identical or different two loop sequences between the sense strand and the antisense strand, connecting both strands, wherein the sense strand and the antisense strand are paired to form a stem, and wherein each of the two loop sequences has 6 to 9 nucleotides in length.

2. The single-chain circular RNA according to claim 1, wherein the expression of a target RNA is 0.4 or less 24 hours after introduction of the single-chain circular RNA into eukaryotic cells, compared to control cells whose expression level of the target RNA is set to 1.

3. The single-chain circular RNA according to claim 1 or 2, wherein 70% or more thereof is retained after 8 hours in human serum.

4. A method of producing the single-chain circular RNA according to any one of claims 1 to 3, comprising synthesizing a sense strand and an antisense strand, both comprising a nucleotide sequence with unpaired nucleotides at the 5' end and 3' end, and simultaneously ligating the nucleotide at the 5' end of the nucleotide sequence with unpaired nucleotides in the sense strand, with the nucleotide at the 3' end of the nucleotide sequence with unpaired nucleotides in the antisense strand, and vice versa, using a ligase,

wherein the nucleotide sequence with unpaired nucleotides at the 5' end of the sense strand and the nucleotide sequence with unpaired nucleotides at the 3' end of the antisense strand are bound to each other to form a loop, the nucleotide sequence with unpaired nucleotides at the 3' end of the sense strand and the nucleotide sequence with unpaired nucleotides at the 5' end of the antisense strand are bound to each other to form a loop, and the sense strand and the antisense strand are paired to form a stem.

5. The method according to claim 4, further comprising phosphorylating each 5' end of the nucleotide sequence with unpaired nucleotides in the sense strand and the nucleotide sequence with unpaired nucleotides in the antisense strand.

6. The method according to claim 4 or 5, wherein the loop sequences are identical to or different from each other.

7. The method according to any one of claims 4 to 6, wherein the stem length is 19 to 31 nucleotides.

8. A method of suppressing expression of a gene encoding a protein *in vitro*, comprising introducing the single-chain circular RNA according to any one of claims 1 to 3 to human-derived cells, and impairing a target RNA by the single-chain circular RNA to inhibit translation of the target RNA into the protein in a sustained manner.

9. A method of suppressing expression of a gene encoding a protein, comprising introducing *in vitro* the single-chain circular RNA according to any one of claims 1 to 3 to cells of non-human animals or plants, and impairing a target RNA by the single-chain circular RNA to inhibit translation of the target RNA into the protein in a sustained manner.

10. A pharmaceutical composition comprising the single-chain circular RNA according to any one of claims 1 to 3 as an active ingredient.