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(54) **Title:** RNA INTERFERENCE INDUCTION ELEMENT AND USE THEREOF

(57) **Abstract:** The present invention provides an RNA interference induction element containing a nucleotide sequence selected from among the nucleotide sequences (a) to (c) below: (a) a nucleotide sequence containing SEQ ID NO:1 or a sequence complementary thereto; (b) a nucleotide sequence containing at least 15 continuous nucleotides present in the nucleotide sequence (a) above, and possessing RNA interference induction potential; (c) a nucleotide sequence having a homology of at least 70% to any one of the nucleotide sequences (a) and (b) above, and possessing RNA interference induction potential. Using the RNA interference induction element of the present invention, it is easily possible to knock down a desired target gene, and to produce a siRNA for a desired target gene.

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DESCRIPTION

RNA INTERFERENCE INDUCTION ELEMENT AND USE THEREOF

TECHNICAL FIELD OF THE INVENTION

The present invention relates to an RNA interference induction element and a use thereof. More specifically, the present invention relates to an RNA interference induction element comprising a nucleotide sequence comprising SEQ ID NO:1 or a sequence complementary thereto or the like, a vector harboring the element, cells containing the vector, a method of producing cells wherein the expression of a target gene is suppressed or a siRNA for the target gene using the element, and the like.

BACKGROUND OF THE INVENTION

RNA interference (RNAi) is a phenomenon in which mRNA is degraded by double-stranded RNA (dsRNA) and the like with specificity for the sequence thereof, resulting in suppression of gene expression. RNA interference has been shown to be conserved across various organisms, including nematodes, yeast and other fungi, insects, plants, and mammals, suggesting that it is a biological system common to all organisms.

Known biological roles of RNA interference include heterochromatin control in fission yeast and the like, control of DNA deletion in *Tetrahymena* and the like, and the like. It has been reported that deletion of Dicer (dcr1), Argonaute (ago1), or RdRp (rdp1) (these are genes playing important roles in the RNAi pathway) in fission yeast (mutants dcr1$^-$, ago1$^-$, and rdp1$^-$, respectively) resulted in the aberrant accumulation of complementary transcripts from outer centromeric heterochromatic repeats, and this was accompanied by transcriptional de-repression of transgenes integrated at the centromere, loss of histone H3 lysine-9 methylation, and impairment of centromere function (Science, Vol. 297, pp. 1833-1837, 2002). Additionally, it was suggested that a short

RNA derived from centromeric repeats is present in fission yeasts (Science, Vol. 297, p. 1831, 2002).

Because RNA interference enables the selective knockdown of a desired gene, it is highly expected to find new applications in biotechnological areas such as breed improvement of crop and medical areas such as gene therapy, as well as in basic sciences such as biochemistry.

There are two major methods of knocking down a gene by RNA interference: direct transfer of siRNA (short interfering RNA) into cells and transfer of siRNA expression vector into cells. Although the former method is quite simple, it is faulty in that the effect of the siRNA introduced does not persist for a long time when it is degraded. The latter siRNA expression vector method, on the other hand, is advantageous in that it enables the preparation of knockdown cell lines or knockdown animals thanks to the long persisting effect thereof. Because RNA interference in cells is triggered by the formation of double-stranded RNA, however, many siRNA expression vectors produce double-stranded RNAs such as hairpin RNAs, which in turn can cause the vector DNA itself to have a stem loop structure and hence become unstable in *Escherichia coli*; it has been difficult to construct a siRNA expression vector.

SUMMARY OF THE INVENTION

In view of the above-described circumstances, the present invention is directed to provide a method of easily inducing RNA interference for a desired gene.

The present inventors diligently investigated to solve the problem described above, mapped centromeric siRNAs of fission yeast in centromeric repeats by Northern blotting, and found that the siRNAs are abundant in the vicinity of a particular shared nucleotide sequence. When a polynucleotide incorporating a desired gene connected with the nucleotide sequence was transferred to cells, RNA interference for the

gene was induced. The inventors thus found that the nucleotide sequence serves as an RNA interference induction element, and developed the present invention. Accordingly, the present invention relates to the following:

[1] An RNA interference induction element comprising a nucleotide sequence selected from among the nucleotide sequences (a) to (c) below:

(a) a nucleotide sequence comprising SEQ ID NO:1 or a sequence complementary thereto;

(b) a nucleotide sequence comprising at least 15 continuous nucleotides present in the nucleotide sequence (a) above, and possessing RNA interference induction potential;

(c) a nucleotide sequence having a homology of at least 70% to any one of the nucleotide sequences (a) and (b) above, and possessing RNA interference induction potential.

[2] A polynucleotide comprising the element described in [1] above, wherein a nucleotide sequence comprising at least 15 continuous nucleotides present in the nucleotide sequence that encodes the transcript of a target gene, or a sequence complementary thereto, is connected so that RNA interference induction potential for the target gene can be exhibited.

[3] The polynucleotide described in [2] above, wherein the nucleotide sequence is connected to the 5′ side of the element.

[4] The polynucleotide described in [2] above, which comprises plural copies of the element as connected in tandem.

[5] A vector harboring the element described in [1] above.

[6] The vector described in [5] above, which comprises plural copies of the element as connected in tandem.

[7] The vector described in [5] or [6] above, which further harbors a promoter joined to the element so that the expression of the element can be controlled.

[8] The vector described in [5] or [6] above, which further harbors at least one cloning site connected to the element so that RNA interference induction potential for a target gene

can be exhibited when a nucleotide sequence comprising at least 15 continuous nucleotides present in the nucleotide sequence that encodes the transcript of the target gene or a sequence complementary thereto is inserted to the cloning site.

[9] The vector described in [8] above, wherein the cloning site is connected to the 5′ side of the element.

[10] The vector described in [8] or [9] above, which further harbors a promoter joined to the element or the cloning site so that the expression of the element and the cloning site can be controlled.

[11] A vector harboring the polynucleotide described in any of [2] to [4] above.

[12] The vector described in [11] above, which further harbors a promoter joined to the polynucleotide so that the expression of the polynucleotide can be controlled.

[13] A cell incorporating the polynucleotide described in any of [2] to [4] above.

[14] A cell incorporating the vector described in any of [5] to [12] above.

[15] A method of producing a cell wherein the expression of a target gene is suppressed, which comprises a step for transferring the polynucleotide described in any of [2] to [4] above, or the vector described in [11] or [12] above, into cells, and a step for selecting a cell incorporating the polynucleotide or the vector.

[16] A method of suppressing the expression of a target gene, which comprises a step for transferring the polynucleotide described in any of [2] to [4] above, or the vector described in [11] or [12] above, into cells.

[17] A method of producing a siRNA for a target gene, which comprises a step for transferring the polynucleotide described in any of [2] to [4] above, or the vector described in [11] or [12] above, into cells, and a step for obtaining the siRNA for the target gene from the cells incorporating the

polynucleotide or the vector.

[18] An RNA interference inducing agent comprising the polynucleotide described in any of [2] to [4] above, or the vector described in [11] or [12] above.

[19] A gene knockdown polynucleotide library comprising a plurality of polynucleotides, each of which comprises a nucleotide sequence comprising at least 15 continuous nucleotides present in the nucleotide sequence that encodes each of the transcripts of a plurality of genes or a sequence complementary thereto, wherein each nucleotide sequence is connected to the element described in [1] above so that RNA interference induction potential for the gene can be exhibited.

[20] The library described in [19] above, wherein the each polynucleotide is harbored in a vector.

[21] A cellular population incorporating the library described in [19] or [20] above.

[22] A method of screening for a functional gene, which comprises the steps (a) to (c) below:

(a) analyzing the phenotype of a cellular population incorporating the library described in [19] or [20] above;

(b) isolating cells with an altered phenotype from the cellular population; and

(c) obtaining a functional gene based on a nucleotide sequence in the polynucleotide or the vector incorporated in the isolated cells.

[23] An RNA-dependent RNA synthesis reaction induction element comprising a nucleotide sequence selected from among the nucleotide sequences (a) to (c) below:

(a) a nucleotide sequence comprising SEQ ID NO:1 or a sequence complementary thereto;

(b) a nucleotide sequence comprising at least 15 continuous nucleotides present in the nucleotide sequence (a) above, and possessing RNA-dependent RNA synthesis reaction induction potential;

(c) a nucleotide sequence having a homology of at least 70% to any one of the nucleotide sequences (a) and (b) above, and possessing RNA-dependent RNA synthesis reaction induction potential.

[24] A template for an RNA-dependent RNA synthesis reaction comprising the element described in [23] above.

[25] A vector capable of expressing the template described in [24] above.

[26] A cell incorporating the vector described in [25] above.

[27] A method of synthesizing an RNA, which comprises the steps shown below:

(a) a step for providing a template for an RNA-dependent RNA synthesis reaction comprising the element of [23] above;

(b) a step for bringing the template of (a) in contact with RNA-dependent RNA polymerase to cause the RNA-dependent RNA synthesis reaction.

[28] A gene expression suppression element comprising a nucleotide sequence selected from among the nucleotide sequences (a) to (c) below:

(a) a nucleotide sequence comprising SEQ ID NO:1 or a sequence complementary thereto;

(b) a nucleotide sequence comprising at least 15 continuous nucleotides present in the nucleotide sequence (a) above, and possessing gene expression suppression potential;

(c) a nucleotide sequence having a homology of at least 70% to any one of the nucleotide sequences (a) and (b) above, and possessing gene expression suppression potential.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the results of Northern blotting using Regions 1 to 8, obtained by dividing the otr repeat (pRS140) in the left arm of the first chromosome centromere of fission yeast into the eight portions, as the probes, to detect small-

molecule RNAs in the fission yeast.

Figure 2 shows the schematic structures of the centromere DNAs of the three chromosomes of fission yeast.

Figure 3 shows the suppression of the expression of the endogenous ura4$^+$ gene by the expression of the SIRE-incorporating ura4 gene.

Figure 4 shows the presence of a ura4-derived siRNA in the strain expressing the SIRE-incorporating ura4 gene.

Figure 5 shows the presence of a ura4-derived siRNA in the strain expressing the SIRE-incorporating ura4 gene.

Figure 6 shows the RNA interference mechanism dependency of the suppression of the expression of ura4$^+$ by SIRE.

Figure 7 schematically shows the constructs used in Example 6.

Figure 8(A-D) shows the results of Northern blotting detection of a c10orf96- or ura4-derived siRNA in the strain expressing the c10orf96-ura4 fusion gene incorporating SIRE or ERIS. SIRE or ERIS induced a c10orf96-derived siRNA (A to D). A siRNA derived from c10orf96 connected to the 5′ side of ERIS was preferentially induced (B). In Δeri1, an siRNA derived from the c10orf96 gene was observed even when the c10orf96-ura4 fusion gene incorporating one unit of SIRE or ERIS was expressed (C and D).

Figure 9 schematically shows the constructs used in Example 7.

Figure 10 shows the growth of the fission yeasts on complete medium (EMM2+aa) or histidine-free medium (EMM2+aa-His). In the figure, lanes a to d corresponds to the constructs of a to d in Figure 9. The growth of the strain incorporating the construct-d is observed on the histidine-free medium.

Figure 11 shows the growth of the fission yeasts on complete medium (EMM2+aa) or histidine-free medium (EMM2+aa-His). In the figure, lanes a to d corresponds to the

constructs of a to d in Figure 9. Even when the construct-d was transferred to Δrdp1, no growth was observed on the histidine-free medium.

Figure 12 schematically shows the RNA-dependent RNA reverse transcription reaction induced by SIRE.

Figure 13 schematically shows the constructs used in Example 8.

Figure 14 shows the results of fluorescent microscopic examination of Hela cells and SVts8 cells that stably express GFP-Cenp-A. Transfer of ΨGFP^{2-238}-SIREx3 or ΨGFP^{2-163}-SIREx3 weakened GFP fluorescence.

Figure 15 shows the results of Western blotting analysis of the GFP-Cenp-A protein amount in Hela cells and SVts8 cells that stably express GFP-Cenp-A. The graph shows the relative signal intensity of GFP as standardized with HEC1. α-GFP: anti-GFP antibody, α-Cenp-A: anti-Cenp-A antibody, α-HEC1: anti-HEC1 antibody.

EFFECT OF THE INVENTION

Using the RNA interference induction element of the present invention, it is easily possible to knock down a desired target gene and produce a siRNA for a desired target gene.

BEST MODE FOR EMBODYING THE INVENTION

The present invention is hereinafter described in detail. Throughout this description, a singular form can include the concept of the plural form thereof unless otherwise stated. Additionally, the terms as used herein are used to have ordinary meanings in the art unless otherwise stated.

Terms that frequently appear herein are defined below.

The term "polynucleotide" as used herein has the same meaning as "oligonucleotide", "nucleic acid", and "nucleic acid molecule", and refers to a nucleotide polymer of an optionally chosen length. Although the polynucleotide may be a

DNA, an RNA, or a DNA/RNA chimera, it is preferably a DNA or an RNA. Additionally, the polynucleotide may be double-stranded or single-stranded. In the case of a double-stranded polynucleotide, it may be a double-stranded DNA, a double-stranded RNA, or a DNA:RNA hybrid. Furthermore, the polynucleotide may be an unmodified polynucleotide (or unmodified oligonucleotide); a polynucleotide with a known modification, for example, one with a label known in the art, one with a cap, one methylated, one with one or more naturally occurring nucleotides substituted by analogues; or a polynucleotide with an intramolecularly modified nucleotide for example, one with an uncharged bond (e.g., methyl phosphonate, phosphotriester, phosphoramidate, carbamate and the like), one with a charged bond or sulfur-containing bond (e.g., phosphorothioate, phosphorodithioate and the like), and one with a modified bond (e.g., α-anomeric nucleic acids and the like). Here, "nucleoside", "nucleotide" and "nucleic acid" may comprise not only the purine and pyrimidine bases, but also other modified heterocyclic bases. Such modified products may comprise methylated purine and pyrimidine, acylated purine and pyrimidine, or another heterocyclic ring. The modified nucleoside and modified nucleotide may have a modification in the sugar moiety thereof; for example, one or more hydroxyl groups may be substituted by halogens, aliphatic groups and the like, or may be converted into functional groups such as ethers and amines.

Nucleotide sequences are herein described as DNA sequences unless otherwise specified; however, when the polynucleotide is an RNA, thymine (T) should read as uracil (U) as appropriate.

The term "gene" as used herein refers to a factor that determines a genetic character. Genes are usually placed in chromosomes in a particular order. A gene that determines the primary structure of a protein is called a structural gene,

and a gene that controls the expression thereof is called a regulator gene (e.g., promoter). Herein genes encompass both structural genes and regulator genes unless otherwise stated. The term "gene" as used herein may also refer to "a polynucleotide", "an oligonucleotide" and "a nucleic acid" and/or "a protein", "a polypeptide", "an oligopeptide" and "a peptide". The term "gene product" as used herein encompasses "a polynucleotide", "an oligonucleotide" and "a nucleic acid" and/or "a protein", "a polypeptide", "an oligopeptide" and "a peptides" expressed by the genes. Those skilled in the art can understand what is the gene product according to the situation.

The term "homology" as used herein with respect to genes (e.g., nucleotide sequences, amino acid sequences and the like) refers to the extent of mutual identity of two or more gene sequences. Accordingly, as the homology of two particular genes increases, the extent of mutual identity or similarity of the sequences thereof increases. Whether or not two kinds of genes possess a homology can be determined by a direct comparison of the sequences, or, in the case of a polynucleotide, by the hybridization method under stringent conditions. Referring to a direct comparison of two gene sequences, these genes are judged to possess a homology when their nucleotide sequences are typically at least 50% identical, preferably at least 70% identical, and more preferably at least 80%, 90%, 95%, 96%, 97%, 98% or 99% identical, to each other. The term "similarity" of genes (e.g., nucleotide sequences, amino acid sequences and the like) as used herein refers to the extent of mutual identity of two or more gene sequences, provided that conservative substitutions are deemed positive (identical) in the above-described homology. Accordingly, if there is a conservative substitution, identity and similarity differ from each other depending on the presence of the conservative substitution. Additionally, if there is no conservative substitution, identity and

similarity show the same numerical value.

Algorithms to determine gene homology include, for example, but are not limited to, the algorithm described in Karlin et al., Proc. Natl. Acad. Sci. USA, 90: 5873-5877 (1993) [the algorithm is incorporated in the NBLAST and XBLAST programs (version 2.0) (Altschul et al., Nucleic Acids Res., 25: 3389-3402 (1997))], the algorithm described in Needleman et al., J. Mol. Biol., 48: 444-453 (1970) [the algorithm is incorporated in the GAP program in the GCG software package], the algorithm described in Myers and Miller, CABIOS, 4: 11-17 (1988) [the algorithm is incorporated in the ALIGN program (version 2.0), which is part of the CGC sequence alignment software package], the algorithm described in Pearson et al., Proc. Natl. Acad. Sci. USA, 85: 2444-2448 (1988) [the algorithm is incorporated in the FASTA program in the GCG software package] and the like. Gene homology can be calculated as appropriate with the above-described program using default parameters thereof. For example, nucleotide sequence homology can be calculated using the homology calculation algorithm NCBI BLAST (National Center for Biotechnology Information Basic Local Alignment Search Tool) under the following conditions (expectancy=10; gap allowed; filtering=ON; match score=1; mismatch score=-3).

Although the length of a polynucleotide can herein be shown by the number of nucleotide units, the number is not unconditional; the number as the upper or lower limit is intended to include several units (or, for example, 10% above and below) straddling the number, as long as the same function is retained. To express this intent, the number may herein be preceded by the adjective "about". It should be understood, however, that the presence or absence of "about" herein does not influence the interpretation of the numerical value.

The term "transcript" as used herein refers to an RNA produced by gene transcription (mRNA and the like).

Transcripts include initial transcripts (immature mRNA), mature transcripts resulting from post-transcriptional processing (splicing) (mature mRNA), and splicing variants thereof.

The term "expression" of a gene or a gene product such as a polynucleotide or a polypeptide as used herein refers to a phenomenon in which the gene and the like undergoes a particular action in vivo (intracellularly) to turn into another form. Preferably, "expression" refers to a phenomenon in which a gene, a polynucleotide, and the like undergoes transcription and translation to turn into the form of a polypeptide; transcription to produce a transcript (mRNA and the like) can also be a form of expression.

Accordingly, the term "suppression" of the "expression" of a gene, a polynucleotide, a polypeptide, and the like as used herein refers to a significant reduction in the amount expressed when a particular factor is allowed to act compared to the amount expressed without the action. Preferably, suppression of the expression includes a reduction in the amount of polypeptide expressed. The term "induction" of the "expression" of a gene as used herein refers to increasing the amount of the gene expressed by allowing a particular factor to act on a cell. Therefore, induction of the expression encompasses allowing the gene to be expressed in cases where no expression of the gene has been observed, and increasing the expression of the gene in cases where the expression of the gene has been observed.

The term "detection" or "quantitation" of gene expression (e.g., mRNA expression, polypeptide expression) can, for example, be accomplished using an appropriate method, including mRNA assay and immunological assay methods. Examples of molecular biological assay methods include Northern blotting, dot blotting, PCR and the like. Examples of immunological assay methods include ELISA using microtiter

plates, RIA, fluorescent antibody method, Western blotting, immunohistological staining and the like. Additionally, examples of methods of quantitation include ELISA, RIA and the like. The detection or quantitation can also be performed by genetic analyses using arrays (e.g., DNA arrays, protein arrays). An extensive overview of DNA arrays is given in "DNA Microarrays and Current PCR Techniques", extra issue, Saibo Kogaku (Cell Engineering), published by Shujunsha. Protein arrays are described in detail in Nat Genet. 2002 Dec.; 32 Suppl: 526-32. In addition to these methods, methods of gene expression analysis include, but are not limited to, RT-PCR, RACE, SSCP, immunoprecipitation, two-hybrid system, in vitro translation and the like. Such analytical methods are described in, for example, Genomu Kaiseki Jikkenhou - Yusuke Nakamura's Lab Manual, edited by Yusuke Nakamura, Yodosha (2002) and elsewhere.

The term "RNA interference (also referred to as RNAi)" as used herein refers to a phenomenon in which homologous mRNA is specifically degraded and the expression (synthesis) of a gene product is suppressed by transferring a factor that causes RNA interference, such as double-stranded RNA (also called dsRNA) or siRNA, to cells, and a technology used therefor.

The term "siRNA" as used herein is an abbreviation for short interfering RNA, referring to a short, double-stranded RNA of 10 base pairs or more, that has been synthesized artificially, chemically, biochemically or intracellularly, or that has resulted from intracellular degradation of a double-stranded RNA of about 40 bases or more; a siRNA normally has the 5'-phosphoric acid and 3'-OH structure, with about two bases protruding at the 3' end. The length of siRNA is normally about 20 bases (e.g., typically about 21 to 23 bases) or less, and is not subject to limitation, as long as RNA interference can be induced.

While not being restrained theoretically, a likely mechanism for RNA interference is such that when a molecule that induces RNA interference, like dsRNA, is transferred into a cell, an RNase III-like nuclease with a helicase domain, known as a dicer, cleaves the molecule by about every 20 base pairs from the 3' end thereof in the presence of ATP, to produce a short dsRNA (siRNA), in the case of a relatively long (e.g., 40 base pairs or more) RNA. A specific protein binds to this siRNA to form an RNA-induced-silencing-complex (RISC). This complex recognizes and binds to an mRNA having the same sequence as siRNA, and cleaves the mRNA at the center of the siRNA by RNase III-like enzyme activity. Regarding the relationship between the sequence of the siRNA and the sequence of the mRNA cleaved as the target, a 100% identity is preferred. However, regarding mutations in bases at positions off the center of the siRNA (the mutations can be in the range of homology of at least 70%, preferably 80%, more preferably 90%, and most preferably 95% or more), the cleavage activity by RNAi is not completely lost, but the activity can remain partially. On the other hand, mutations in bases at the center of the siRNA have a major influence; the mRNA cleavage activity by RNAi may decline extremely.

Additionally, while not being restrained theoretically, another pathway for siRNA has been proposed. The antisense strand of siRNA binds to mRNA and acts as a primer for RNA-dependent RNA polymerase (RdRP) to synthesize a dsRNA. This dsRNA again serves as a dicer substrate to produce a new siRNA and enhance the action.

The term "cell" as used herein is defined as of the broadest sense used in the art, referring to an organism wrapped by a membranous structure isolating it from the outer world, capable of self-regeneration therein, and having genetic information and a mechanism for its expression, as the individual unit of a single-cell organism or the structural

unit of a tissue of a multicellular organism. The cell used in the present invention may be a naturally occurring cell, or an artificially altered cell (e.g., fusion cell, genetically altered cell). The source of the cell can, for example, be a single cell culture, or includes, but is not limited to, embryos, blood, or somatic tissue of a normally grown wild type or transgenic animal, or a cell mixture like cells derived from a normally grown cell line.

The term "isolated" as used herein refers to a condition wherein substances that naturally accompany the object product in ordinary environments have been at least reduced, preferably substantially no such substances are contained in the object product. Accordingly, an isolated cell refers to a cell substantially free from other substances that naturally accompany the cells of interest in ordinary environment (e.g., other cells, proteins, nucleic acids and the like). The term "isolated" as used with respect to a polynucleotide or polypeptide refers to a polynucleotide or polypeptide substantially free from cellular substances and culture media when prepared by recombinant DNA technology, or a polynucleotide or polypeptide substantially free from precursor chemical substances or other chemical substances when chemically synthesized. The isolated polynucleotide is preferably free from sequences naturally flanking to the polynucleotide (i.e., sequences located at the 5′ and 3′ ends of the nucleic acid) in the organism from which the nucleotide is derived.

The term "purified" biological factor (e.g., a polynucleotide or polypeptide and the like) as used herein refers to a biological factor deprived of at least a portion of the factors naturally accompanying the biological factor. Therefore, the purity of a biological factor in a purified biological factor is normally higher than that of the biological factor in ordinary state (i.e., the biological

factor has been concentrated).

The terms "purified" and "isolated" as used herein mean that preferably at least 75% by weight, more preferably at least 85% by weight, still more preferably at least 95% by weight, and most preferably at least 98% by weight, of the same type of biological factor is present.

Preferred modes of embodiment of the present invention are hereinafter described. The following modes of embodiment are understood to be given for the purpose of better understanding of the present invention, and not to be construed as limiting the scope of the invention. Accordingly, it is evident that those skilled in the art can alter these modes as appropriate within the scope of the present invention, in view of the description herein.

1. RNA interference induction element

In one aspect, the present invention provides an RNA interference induction element comprising a nucleotide sequence selected from among the nucleotide sequences (a) to (c) below:

(a) a nucleotide sequence comprising SEQ ID NO:1 or a sequence complementary thereto;

(b) a nucleotide sequence comprising at least 15 continuous nucleotides present in the nucleotide sequence (a) above, and possessing RNA interference induction potential;

(c) a nucleotide sequence having a homology of at least 70% to any one of the nucleotide sequences (a) and (b) above, and possessing RNA interference induction potential.

The term "element" as used herein refers to a nucleotide sequence (or polynucleotide) having a particular function, or a region thereof.

The term "RNA interference induction potential" as used herein refers to the potential for inducing RNA interference for a functionally connected target gene. More specifically,

"RNA interference induction potential" refers to the potential of a nucleotide sequence (or polynucleotide) for inducing RNA interference for an optionally chosen target gene, inducing siRNA for the target gene, or suppressing the expression of the target gene when transferred to cells, while being connected to a nucleotide sequence (target nucleotide sequence) comprising at least 15 continuous nucleotides present in the nucleotide sequence that encodes the transcript (mRNA) of the target gene, or a sequence complementary thereto. Accordingly, the terms "siRNA induction potential" and "gene expression suppression potential" can be used herein interchangeably with "RNA interference induction potential".

Accordingly, the term "RNA interference induction element" as used herein refers to a nucleotide sequence (or polynucleotide) having the above-described RNA interference induction potential, or a region thereof. The terms "siRNA induction element" and "gene expression suppression element" can be used herein interchangeably with "RNA interference induction element " as described above.

Additionally, when a single-stranded RNA comprising the RNA interference induction element of the present invention is transferred to cells, transcription of an RNA complementary to the RNA transferred is induced in the vicinity of the element (5′ or 3′ side); as a result, a double-stranded RNA comprising the RNA transferred and the RNA complementary thereto can be produced. In an embodiment, the RNA-dependent RNA synthesis (extension) reaction can proceed in the direction from the element of the present invention as the initiation site to the 3′ side (this direction is the direction in the strand complementary to the RNA transferred). Accordingly, the RNA interference induction element of the present invention can be an "initiation site (element)" for the RNA-dependent RNA synthesis (extension) reaction, a "site (element) with priming function", or an "RNA-dependent RNA synthesis (extension)

reaction induction element".

In a preferred mode of embodiment, the nucleotide sequence (b) above is preferably a nucleotide sequence comprising at least 15, for example, 50 or more, 100 or more, 150 or more, 200 or more, 250 or more, 300 or more, 310 or more, 320 or more, 330 or more, 340 or more, 350 or more, 360 or more, or 370 or more continuous nucleotides present in SEQ ID NO:1 or a sequence complementary thereto, and possessing RNA interference induction potential. Although a longer nucleotide sequence is preferred, the nucleotide sequence may be short, as long as it possesses RNA interference induction potential.

In another preferred mode of embodiment, the nucleotide sequence (c) above is preferably a nucleotide sequence having a homology of at least 70%, for example, 80% or more, 85% or more, 90% or more, 95% or more, 96% or more, 97% or more, 98% or more, or 99% or more, to any one of the nucleotide sequences (a) and (b) above, and possessing RNA interference induction potential. Although a higher homology is preferred, the nucleotide sequence may be of low homology, as long as it possesses RNA interference induction potential.

In (b) and (c) above, the potency of RNA interference induction potential is preferably equivalent (e.g., about 0.01 to 100 times, preferably about 0.1 to 10 times, more preferably about 0.5 to 2 times) to that of an RNA interference induction element comprising SEQ ID NO:1 or a sequence complementary thereto.

Because the nucleotide sequence of SEQ ID NO:1 is a sequence derived from the centromeric region of the chromosome DNA of fission yeast, a polynucleotide comprising the RNA interference induction element of the present invention can be obtained by a commonly known PCR method with the fission yeast chromosome DNA as the template using a synthetic DNA primer comprising a portion of the nucleotide sequence of SEQ ID NO:1.

Alternatively, the same can also be obtained from a fission yeast chromosome DNA library by a hybridization method. This hybridization can be performed according to, for example, the method described in Molecular Cloning 2nd (J. Sambrook et al., Cold Spring Harbor Lab. Press, 1989) and the like. Alternatively, the same can also be obtained by chemical synthesis using a commercially available nucleic acid synthesizer. Additionally, these methods may be used in combination with a site-directed mutagenesis method known per se (ODA-LA PCR method, gapped duplex method, Kunkel method and the like) or a method based thereon.

The presence/absence or potency of RNA interference induction potential in the polynucleotide obtained can be confirmed by connecting the polynucleotide to a nucleotide sequence (target nucleotide sequence) comprising at least 15 continuous nucleotides present in the nucleotide sequence that encodes the transcript (mRNA) of an optionally chosen target gene or a sequence complementary thereto to obtain the polynucleotide of the present invention described below, transferring this polynucleotide to a cell, and detecting or quantifying the presence/absence or potency of the induction of RNA interference for the target gene, induction of siRNA for the target gene, or suppression of the expression of the target gene.

Using the RNA interference induction element of the present invention, it is possible to induce RNA interference for a desired gene, to induce siRNA, and to suppress the expression.

2. A polynucleotide comprising an RNA interference induction element

In one aspect, the present invention provides a polynucleotide comprising the above-described RNA interference induction element of the present invention, wherein a

nucleotide sequence (also referred to as the target nucleotide sequence) comprising at least 15 continuous nucleotides present in the nucleotide sequence that encodes the transcript of a target gene or a sequence complementary thereto is connected so that RNA interference induction potential for the target gene can be exhibited.

The term "target gene" as used herein refers to a gene intended to have the expression thereof suppressed by RNA interference, and the target gene can be selected optionally. As such, the target gene selected is preferably a gene of known sequence whose function is to be clarified, a gene whose expression is considered to be a cause of disease, or the like. The target gene selected may be a gene whose full-length genome sequence or full-length mRNA sequence remains unknown, provided that a portion, at least 15 bases or more, of the nucleotide sequence of the transcript (mRNA and the like) thereof is known. Therefore, a gene whose mRNA has been partially known but whose full-length remains unknown, such as expressed sequence tag (EST), can also be selected as a target gene in the present invention.

Although the transcript used may be any of an initial transcript, a mature transcript, and a splicing variant thereof, a mature transcript is preferably used.

Although the length of the target nucleotide sequence is not subject to limitation, as long as the polynucleotide of the present invention is capable of inducing RNA interference for the target gene when transferred to cells, the target nucleotide sequence is preferably a nucleotide sequence comprising at least 15, for example, 20 or more, 21 or more, 23 or more, 40 or more, 60 or more, or 100 or more continuous nucleotides present in the nucleotide sequence that encodes the transcript of a target gene or a sequence complementary thereto, considering that the length of siRNA is about 20 bases (e.g., typically about 21 to 23 bases). From the

viewpoint of more potently inducing RNA interference for a target gene, the length of the target nucleotide sequence is preferably longer; examples of preferable target nucleotide sequences include, but are not limited to, the full-length of the nucleotide sequence that encodes the transcript of a target gene or a sequence complementary thereto, the full-length of the ORF region of the nucleotide sequence of the transcript of a target gene or a sequence complementary thereto and the like.

Additionally, the target nucleotide sequence may be connected adjacently to the RNA interference induction element of the present invention (not via a spacer region), or may be connected via a spacer region, as long as RNA interference induction potential for the target gene can be exhibited. Although the length of the spacer region is not subject to limitation, as long as the individual constituents, from the target nucleotide sequence to the RNA interference induction element of the present invention, can be stably present without interruption in one polynucleotide chain, and RNA interference induction potential for the target gene can be exhibited, it is preferably at most 10 Kbp, for example, 5 Kbp or less, 3 Kbp or less, 1 Kbp or less, 500 bp or less, 200 bp or less, 100 bp or less, 50 bp or less, or 25 bp or less. The nucleotide sequence that constitutes the spacer region is not subject to limitation, and may be an optionally chosen sequence.

Although the target nucleotide sequence may be connected to any of the 5′ and 3′ sides of the RNA interference induction element of the present invention, as long as RNA interference induction potential for the target gene can be exhibited, it is preferably connected to the 5′ side.

Here, for the sake of convenience for the designation of the RNA interference induction element of the present invention,

an element comprising:

(a') a nucleotide sequence comprising SEQ ID NO:1,

(b') a nucleotide sequence comprising at least 15 continuous nucleotides present in the nucleotide sequence (a') above, and possessing RNA interference induction potential, or

(c') a nucleotide sequence having a homology of at least 70% to any one of the nucleotide sequences (a') and (b') above, and possessing RNA interference induction potential,

is referred to as "a sense element", and

an element comprising

(a'') a nucleotide sequence comprising a sequence complementary to SEQ ID NO:1,

(b'') a nucleotide sequence comprising at least 15 continuous nucleotides present in the nucleotide sequence (a'') above, and possessing RNA interference induction potential, or

(c'') a nucleotide sequence having a homology of at least 70% to any one of the nucleotide sequences (a'') and (b'') above, and possessing RNA interference induction potential,

is referred to as "an antisense element".

Additionally, with respect to the polynucleotide of the present invention, "a nucleotide sequence comprising at least 15 continuous nucleotides present in the nucleotide sequence that encodes the transcript of a target gene" is referred to as "a sense target nucleotide sequence", and "a nucleotide sequence comprising at least 15 continuous nucleotides present in a sequence complementary to the nucleotide sequence that encodes the transcript of a target gene" is referred to as "an antisense target nucleotide sequence".

Provided that the target nucleotide sequence is connected to the 5' side of the RNA interference induction element of the present invention, the following four forms (A) to (D) are available:

(A) 5'-sense target nucleotide sequence-sense element-3'

(B) 5'-antisense target nucleotide sequence-sense element-3'

(C) 5′-sense target nucleotide sequence-antisense element-3′

(D) 5′-antisense target nucleotide sequence-antisense element-3′

Provided that the target nucleotide sequence is connected to the 3′ side of the RNA interference induction element of the present invention, the following four forms (A′) to (D′) are available:

(A′) 5′-sense element-sense target nucleotide sequence-3′

(B′) 5′-sense element-antisense target nucleotide sequence-3′

(C′) 5′-antisense element-sense target nucleotide sequence-3′

(D′) 5′-antisense element-antisense target nucleotide sequence-3′

The polynucleotide of the present invention may be in a form in which the RNA interference induction element of the present invention is inserted in the midst of the target nucleotide sequence. In this case, it is preferable that at least one of the nucleotide sequence on the 5′ side of the RNA interference induction element insertion site in the target nucleotide sequence, and the nucleotide sequence on the 3′ side of the insertion site, have the same length as the above-described "length of the target nucleotide sequence".

Additionally, as long as RNA interference induction potential for the target gene can be exhibited, the RNA interference induction element inserted may be connected adjacently to the target nucleotide sequence (not via a spacer region), or may be joined via a spacer region, on the 5′ and/or 3′ side thereof. The length/sequence of the spacer region are the same as those described above.

When the polynucleotide of the present invention, which is a single-stranded RNA, is transferred to a cell, transcription of an RNA complementary to the target nucleotide sequence is induced in the vicinity (5′ or 3′ side) of the element; as a result, a double-stranded RNA having the target nucleotide sequence can be synthesized. In an embodiment, this

RNA-dependent RNA synthesis (extension) reaction can proceed in the direction from the element of the present invention as the initiation site to the 3′ side (this direction is the direction in the strand complementary to the RNA transferred). The double-stranded RNA undergoes various modifications, including cleavage, via intracellular siRNA synthesis mechanisms (Dicer (dcr1) and the like), so that siRNA for the target gene can be produced, and RNA interference for the target gene can be induced.

Accordingly, the term RNA-dependent RNA synthesis (extension) reaction initiation function, RNA-dependent RNA synthesis (extension) reaction priming function or RNA-dependent RNA synthesis (extension) reaction induction potential can be used herein interchangeably with "RNA interference induction potential".

Additionally, with respect to the polynucleotide of the present invention, the number of copies of the RNA interference induction element of the present invention present in one polynucleotide chain is not subject to limitation; only one copy of the RNA interference induction element may be present in one polynucleotide chain, or plural copies of the RNA interference induction element may be present in one polynucleotide chain as connected in tandem. Using plural copies of the RNA interference induction element as connected in tandem, more potent RNA interference induction potential can be obtained. When plural copies of the RNA interference induction element are used as connected in tandem, the number of copies of the RNA interference induction element connected is not subject to limitation, as long as RNA interference induction potential for the target gene can be obtained; the number of copies is, for example, 2 to 50 copies, preferably 2 to 20 copies, and more preferably 2 to 10 copies. In view of the ease of polynucleotide connecting procedures and other factors, the number of copies is preferably about 2

to 5 copies.

When plural copies of RNA interference induction element are used as connected in tandem, the nucleotide sequences of the individual units of the RNA interference induction element may be identical or not. The units of the RNA interference induction element may be connected adjacently (not via a spacer region), or may be connected via a spacer region. Although the length of the spacer region is not subject to limitation, as long as the individual constituents, from the target nucleotide sequence to the plural copies of the RNA interference induction element connected, can be stably present without interruption in one polynucleotide chain, and RNA interference induction potential for the target gene can be exhibited, it is preferably at most 10 Kbp, for example, 5 Kbp or less, 3 Kbp or less, 1 Kbp or less, 500 bp or less, 200 bp or less, 100 bp or less, 50 bp or less, or 25 bp or less. The nucleotide sequence that constitutes the spacer region is not subject to limitation, and may be an optionally chosen sequence.

3. Vector harboring RNA interference induction element (I)

In one aspect, the present invention provides a vector harboring the above-described RNA interference induction element of the present invention (the vector of the present invention (I)). Using the vector, it is easily possible to induce RNA interference and produce a siRNA for a desired target gene.

The term "vector" as used herein refers to a nucleic acid construct capable of transferring a target polynucleotide sequence to a target cell. Examples of such vectors include those capable of self-replication in host cells such as prokaryotic cells, yeast, animal cells, plant cells, insect cells, animal individuals, and plant individuals, or those capable of being incorporated in chromosome.

The kind of vector is not subject to limitation, and an appropriate vector can be optionally selected according to the intended use, the kind of target cells and the like. Useful vectors include, but are not limited to, plasmid vectors (*Escherichia coli*-derived plasmids (e.g., pBR322, pBR325, pUC12, pUC13), *Bacillus subtilis*-derived plasmids (e.g., pUB110, pTP5, pC194), yeast-derived plasmids (e.g., pSH19, pSH15, pAU001) and the like), bacteriophages such as lambda phage, viral vectors (animal viruses such as retrovirus, vaccinia virus, and baculovirus, and the like) and the like.

With respect to the vector of the present invention (I), the number of copies of the RNA interference induction element of the present invention present in one vector, like the above-described polynucleotide of the present invention, is not subject to limitation; only one copy of the RNA interference induction element may be present in one vector, or plural copies of the RNA interference induction element may be present in one vector as connected in tandem. When plural copies of the RNA interference induction element are used as connected in tandem, the range of the copy number of the RNA interference induction element connected is the same as the above-described polynucleotide of the present invention. When plural copies of the RNA interference induction element are used as connected in tandem, the nucleotide sequences of the individual units of the RNA interference induction element may be identical or not. The units of the RNA interference induction element may be connected adjacently (not via a spacer region), or may be connected via a spacer region. The range of the length of the spacer region, and the nucleotide sequence that constitutes the spacer region are the same as the above-described polynucleotide of the present invention.

In a preferred mode, the vector of the present invention (I) further comprises a promoter, which promoter is preferably connected to the RNA interference induction element of the

present invention so that the expression of the element can be controlled. Hence, the promoter can be connected to the element and placed in the vector so that the RNA interference induction element of the present invention can be contained in the transcript (RNA) that can be produced by the function of the promoter.

The term "promoter" as used herein refers to a region in DNA that determines the initiation site for gene transcription and directly regulates the frequency thereof, and is usually a nucleotide sequence to which RNA polymerase binds to initiate the transcription.

Although the promoter may be placed at any position in the vector, as long as the expression of the RNA interference induction element of the present invention can be controlled, the promoter is preferably bound to the 5′ side of the RNA interference induction element of the present invention because the promoter is usually located about 20 to 30 bp upstream (5′ side) of the transcription initiation point. Additionally, the RNA interference induction element of the present invention is preferably located downstream (3′ side) of the transcription initiation point defined by the promoter.

The kind of promoter is not subject to limitation, and an appropriate promoter can be optionally selected according to the intended use, the kind of target cells and the like. Useful promoters include pol I promoters, pol II promoters, pol III promoters and the like. When used in animal cells, the SRα promoter, the SV40 promoter, the LTR promoter, the CMV promoter, the HSV-TK promoter and the like can be used. When used in *Escherichia coli*, the trp promoter, the lac promoter, the recA promoter, the λPL promoter, the lpp promoter, the T7 promoter and the like can be used. When used in yeast, the PHO5 promoter, the PGK promoter, the GAP promoter, the ADH promoter, the NMT1 promoter and the like can be used. When used in insect cells, the polyhedrin promoter, the P10

promoter and the like can be used. Additionally, when in vitro transcription is performed, the SP6, T3, and T7 promoters and the like can be used.

In another preferred mode, the vector of the present invention (I) can further comprise at least one cloning site, which cloning site can be connected to the element so that RNA interference induction potential for a target gene can be exhibited when a nucleotide sequence (target nucleotide sequence) comprising at least 15 continuous nucleotides present in the nucleotide sequence that encodes the transcript of the target gene or a sequence complementary thereto is inserted to the site. By inserting a desired target gene and the like to the cloning site, it is easily possible to induce RNA interference and produce a siRNA for the desired target gene.

A cloning site generally means a continuous nucleotide sequence comprising one kind or more of restriction enzyme recognition sequence for incorporating an exogenous gene. The cloning site preferably comprises one kind or more of restriction enzyme recognition sequence that forms a cohesive end upon cleavage with restriction enzyme. The aforementioned restriction enzyme recognition sequence present in the cloning site is preferably a unique restriction enzyme sequence that presents in the vector only at one position. The cloning site is preferably a multiple-cloning site comprising plural restriction enzyme recognition sequences.

Additionally, the cloning site may be connected adjacently to the RNA interference induction element of the present invention (not via a spacer region), or may be connected via a spacer region, as long as RNA interference induction potential for the target gene can be exhibited when the target nucleotide sequence is inserted to the site. Although the length of the spacer region is not subject to limitation, as long as the individual constituents, from the

target nucleotide sequence to the RNA interference induction element, can be stably present without interruption in one vector (or transcript), and RNA interference induction potential for the target gene can be exhibited, when the target nucleotide sequence is inserted to the cloning site, it is preferably at most 10 Kbp, for example, 5 Kbp or less, 3 Kbp or less, 1 Kbp or less, 500 bp or less, 200 bp or less, 100 bp or less, 50 bp or less, or 25 bp or less. The nucleotide sequence that constitutes the spacer region is not subject to limitation, and may be an optionally chosen sequence.

Although the cloning site may be connected to any of the 5′ and 3′ sides of the RNA interference induction element of the present invention, as long as RNA interference induction potential for the target gene can be exhibited when the target nucleotide sequence is inserted in the site, it is preferably connected to the 5′ side.

Additionally, the vector in this mode can further harbor a promoter, in addition to the cloning site, which promoter can be joined to the element or the cloning site so that the expression of the RNA interference induction element of the present invention and cloning site can be controlled. Accordingly, the promoter that can be harbored in the vector can be connected to the element or the cloning site and placed in the vector so that the RNA interference induction element of the present invention and the cloning site can be present in the transcript (RNA) that can be produced by the function of the promoter. Hence, in the transcript that can be produced by the function of the promoter, the cloning site is joined to the RNA interference induction element of the present invention so that RNA interference induction potential for the target gene can be exhibited when the target nucleotide sequence is inserted to the site.

Although the promoter may be placed at any position in

the vector, as long as the expression of the RNA interference induction element of the present invention and the cloning site can be controlled, the promoter is preferably bound to the 5′ side of the RNA interference induction element of the present invention and the cloning site because the promoter is usually located about 20 to 30 bp upstream (5′ side) of the transcription initiation point. Additionally, the RNA interference induction element of the present invention and the cloning site are preferably located downstream (3′ side) of the transcription initiation point defined by the promoter. Because the cloning site is preferably connected to the 5′ side of the RNA interference induction element of the present invention, it is more preferable that the promoter, the cloning site, and the RNA interference induction element be placed in the order of 5′-promoter-cloning site-RNA interference induction element-3′.

The promoter used may be the same as that described above.

The vector of the present invention (I) may further harbor a terminator, an enhancer, a selection marker (genes that confer resistance to drugs such as tetracycline, ampicillin, kanamycin, hygromycin, and phosphinothricin, genes that compensate for auxotrophic mutations, genes that encode fluorescent proteins, and the like) and the like.

The term "terminator" as used herein refers to a sequence located downstream of the region that encodes a transcribable gene (nucleotide sequence), and involved in transcription termination and polyA sequence addition in DNA transcription to mRNA. Terminators are known to participate in the stability of mRNA and influence the amount of gene expressed. The term "enhancer" as used herein refers to a nucleotide sequence used to increase the expression efficiency for an objective gene. Such enhancers are well known to those skilled in the art. Although plural enhancers can be used, a

single enhancer may be used, or no enhancers may be used.

4. Vector harboring RNA interference induction element (II)

In another aspect, the present invention provides a vector harboring the above-described polynucleotide of the present invention (the vector of the present invention (II)). Using the vector, it is easily possible to induce RNA interference and produce a siRNA for a target gene.

Applicable modes of vector are the same as the vector of the present invention (I) described above.

In a preferred mode, the vector of the present invention further harbors a promoter, which promoter is preferably joined to the polynucleotide of the present invention so that the expression of the polynucleotide can be controlled. Accordingly, the above-described polynucleotide of the present invention, which is a single-stranded RNA, can be produced as the transcript (RNA) by the action of the promoter that can be present in the vector.

Although the promoter may be placed at any position in the vector, as long as the expression of the polynucleotide of the present invention can be controlled, the above-described promoter is preferably bound to the 5′ side of the polynucleotide of the present invention because promoters are usually located about 20 to 30 bp upstream (5′ side) of the transcription initiation point. Additionally, the polynucleotide of the present invention is preferably located downstream (3′ side) of the transcription initiation point defined by the promoter. Because the target nucleotide sequence is preferably connected to the 5′ side of the RNA interference induction element in the polynucleotide of the present invention, it is more preferable that the promoter, the target nucleotide sequence, and the RNA interference induction element be placed in the order of 5′-promoter-target nucleotide sequence-RNA interference

induction element-3'.

The kind of promoter used may be the same as that of the vector of the present invention (I) described above. Additionally, the vector in this mode can also further harbor a terminator, an enhancer, a selection marker and the like as described above.

5. Cell incorporating the polynucleotide or the vector of the present invention

In one aspect, the present invention provides a cell incorporating the above-described polynucleotide or the vector of the present invention (the cell of the present invention (I)).

The cell used in the present invention may be derived from any organism (prokaryotic organisms, eukaryotic organisms and the like). Prokaryotic organisms include bacteria such as *Escherichia coli* and *Salmonella* and the like. Eukaryotic organisms include fungi (molds, mushrooms, yeasts (fission yeast, budding yeast and the like) and the like), plants (monocotyledons, dicotyledons and the like), animals (invertebrates, vertebrates and the like) and the like. Invertebrates include nematodes, crustaceans (insects and the like) and the like. Vertebrates include hagfishes, lampreys, chondrichthians, osteichthians, amphibians, reptiles, birds, mammals and the like. Examples of mammals include monotremes, marsupials, edentates, dermapterans, chiropters, carnivores, insectivores, proboscideans, perissodactyles, artiodactyles, tubulidentata, squamatas, sirenians, cetaceans, primates, rodents, lagomorphs and the like. Rodents include mice, rats and the like. Examples of primates include chimpanzees, Japanese macaques, humans and the like.

Polynucleotide transfer into the cell may be achieved by any technique; examples include transformation, transduction, transfection and the like. These techniques for transferring

nucleic acid molecules are well known and in common use in the art, and are described in, for example, Ausubel F.A. et al. eds. (1988), Current Protocols in Molecular Biology, Wiley, New York, NY; Sambrook J. et al. (1987), Molecular Cloning: A Laboratory Manual, 2nd Ed. and 3rd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, Bessatsu Jikken Igaku, Experimental Methods in Gene Transfer & Expression Analysis, Yodosha Co., Ltd., 1997 and elsewhere. Polypeptide transfer can be confirmed using the methods described herein, such as Northern blotting and Western blot analysis, or other known techniques in common use.

Additionally, any method of vector transfer can be used, as long as it comprises transferring a polynucleotide to a cell as described above; examples include transfection, transduction, transformation and the like (e.g., calcium phosphate method, liposome method, DEAE-dextran method, electroporation method, method using particle gun (gene gun) and the like).

To obtain a stable transformant cell incorporating a polynucleotide or a vector, for example, a polynucleotide or a vector incorporating a selection marker may be used, and the cell may be cultured by a method suitable for the selection marker. For example, when the selection marker is a gene that confers drug resistance to a selection drug lethal to the host cell, the cell incorporating the polynucleotide or the vector may be cultured using a medium supplemented with the drug. Examples of useful combinations of a drug resistance gene and a selection drug include a combination of an ampicillin resistance gene and ampicillin, a combination of a neomycin resistance gene and neomycin, a combination of a hygromycin resistance gene and hygromycin, a combination of a blasticidin S resistance gene and blasticidin S, and the like. When the selection marker is a gene that encodes a fluorescent protein (GFP, YFP and the like), a cell of high fluorescence intensity

may be selected from among the cells incorporating the polynucleotide or the vector using a cell sorter and the like.

6. Method of producing cell wherein the expression of target gene is suppressed

In one aspect, the present invention provides a method of producing a cell wherein the expression of a target gene is suppressed, which comprises a step for transferring the above-described polynucleotide of the present invention, or a vector harboring the polynucleotide, into cells, and a step for selecting a cell incorporating the polynucleotide or the vector. The present invention also provides a method of suppressing the expression of a target gene, which comprises a step for transferring the above-described polynucleotide of the present invention, or a vector harboring the polynucleotide, into a cell. By transferring the above-described polynucleotide of the present invention or a vector harboring the polynucleotide into a cell, a siRNA for the target gene is produced, RNA interference for the target gene is induced, and the expression of the target gene is suppressed.

The cell used here may be the above-described cell with a described target gene expressed therein. The polynucleotide or the vector transfer to the cell can be achieved using the same methods as those described above.

In a preferred mode, the polynucleotide of the present invention, which is a single-stranded RNA wherein the target nucleotide sequence is connected to the 5′ side of the RNA interference induction element, is transferred into a cell. As a result, a siRNA for the target gene is produced, RNA interference for the target gene is induced, and the expression of the target gene is suppressed.

In another preferred mode, the above-described vector of the present invention harboring a promoter, by which promoter

the polynucleotide of the present invention, which is a single-stranded RNA wherein the target nucleotide sequence is connected to the 5′ side of the RNA interference induction element, can be produced as the transcript, is transferred into a cell. A promoter that can act in the objective cell is selected as appropriate. When the vector is transferred into a cell, the polynucleotide of the present invention, which is a single-stranded RNA wherein the target nucleotide sequence is connected to the 5′ side of the RNA interference induction element, is produced as the transcript. As a result, a siRNA for the target gene is produced, RNA interference for the target gene is induced, and the expression of the target gene is suppressed.

Selection of cells incorporating the polynucleotide of the present invention or a vector harboring the polynucleotide can be achieved by a commonly known method, such as hybridization or PCR with a nucleotide sequence specific for the polynucleotide of the present invention or a vector harboring the polynucleotide as the probe or primer; when a polynucleotide or vector provided with a selection marker is used, selection can be performed using the phenotype by the selection marker as the index.

Additionally, it may be confirmed whether or not the expression of a target gene is suppressed in the cell incorporating the polynucleotide of the present invention or a vector harboring the polynucleotide. This confirmation can be achieved by, for example, comparing the expression of the target gene in the cells incorporating the polynucleotide of the present invention or a vector harboring the polynucleotide with the expression of the target gene in a control cell not incorporating the polynucleotide of the present invention or a vector harboring the polynucleotide. Although any method can be used for this confirmation, a commonly known method such as hybridization or PCR is available. Alternatively, a phenotype

difference in cells between the presence and absence of the expression of the target gene may also be examined. The presence or absence of siRNA for the target gene may also be examined by hybridization and the like.

A cell incorporating the polynucleotide of the present invention or a vector harboring the polynucleotide as described above is a cell having the expression of the target gene suppressed (knockdown cells). Such "knockdown cell" include both a cell wherein the expression of the target gene has been completely suppressed and a cell wherein the expression of the target gene has been reduced, though not completely been suppressed. Conventionally, such cell has been generated by deleting or modifying the target gene or the control region thereof; it is possible to produce a cell wherein the expression of the target gene is suppressed, by a simple method comprising transferring the polynucleotide of the present invention or a vector harboring the polynucleotide into cells, and selecting the cell incorporating the same, without modifying the target gene in a chromosome, using the present invention. The knockdown cell thus generated can be used as a research material for functional analysis of the target gene; additionally, a cell wherein the expression of a causal gene for a disease as the target gene is suppressed can be used as a disease model cell and the like. Additionally, by transferring the polynucleotide of the present invention or a vector harboring the polynucleotide to a germ cell or multipotent stem cell, and allowing the target gene knockdown germ cell or the target gene knockdown multipotent stem cell to develop into an individual organism or tissue, it is also possible to generate a target gene knockdown animal, a disease model animal, a target gene knockdown tissue and the like. The present invention also includes the above-described knockdown cell produced by the present invention; furthermore, individual organisms retaining the above-described

polynucleotide of the present invention or the vector (e.g., a target gene knockdown non-human animal and the like), a tissue (a target gene knockdown tissue) and the like are also included in the present invention. As examples of the above-described organism in the present invention, mice, rats, rabbits, cattle, horses, swine, sheep, monkeys, or chimpanzees and the like can be mentioned.

Additionally, by obtaining (isolating, purifying, or the like) a siRNA from the cell incorporating the polynucleotide of the present invention or a vector harboring the polynucleotide, a siRNA for a target gene can be produced. Accordingly, the present invention provides a method of producing a siRNA for a target gene, which comprises a step for transferring the polynucleotide of the present invention or a vector harboring the polynucleotide into a cell, and a step for obtaining a siRNA for the target gene from the cell incorporating the aforementioned polynucleotide or the vector. siRNA isolation and purification from a cell can be performed by a method known per se such as RNA purification or gel filtration column chromatography.

7. RNA interference inducing agent

Using the polynucleotide of the present invention or a vector harboring the polynucleotide as described above, it is possible to induce RNA interference for a desired target gene and suppress the expression of the gene. Accordingly, the present invention provides an RNA interference inducing agent comprising the above-described polynucleotide of the present invention or a vector harboring the polynucleotide.

The agent of the present invention can comprise an optionally chosen carrier, for example, a physiologically acceptable carrier, in addition to an effective amount of the above-described polynucleotide of the present invention or a vector harboring the polynucleotide.

Examples of the physiologically acceptable carrier include, but are not limited to, excipients such as sucrose, starch, mannitol, sorbitol, lactose, glucose, cellulose, talc, calcium phosphate and calcium carbonate; binders such as cellulose, methylcellulose, hydroxypropylcellulose, polypropylpyrrolidone, gelatin, acacia, polyethylene glycol, sucrose and starch; disintegrants such as starch, carboxymethylcellulose, hydroxypropyl starch, sodium-glycol-starch, sodium hydrogen carbonate, calcium phosphate and calcium citrate; lubricants such as magnesium stearate, aerosil, talc and sodium lauryl sulfate; flavoring agents such as citric acid, menthol, glycyrrhizin ammonium salt, glycine and orange flour; preservatives such as sodium benzoate, sodium hydrogen sulfite, methyl paraben and propyl paraben; stabilizers such as citric acid, sodium citrate and acetic acid; suspending agents such as methylcellulose, polyvinylpyrrolidone and aluminum stearate; dispersing agents such as surfactants; diluents such as water, physiological saline and orange juice; base waxes such as cacao butter, polyethylene glycol and refined kerosene; and the like.

To promote the transfer of the polynucleotide and the vector into cells, the agent of the present invention can further comprise a nucleic acid transfer reagent. When the active ingredient is a viral vector, particularly a retroviral vector, retronectin, fibronectin, polybrene or the like can be used as the transfection reagent. When the active ingredient is a polynucleotide, a plasmid vector or the like, a cationic lipid such as lipofectin, lipfectamine, DOGS (transfectam; dioctadecylamidoglycylspermine), DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine), DOTAP (1,2-dioleoyl-3-trimethylammoniumpropane), DDAB (dimethyldioctadecylammonium bromide), DHDEAB (N,N-di-n-hexadecyl-N,N-dihydroxyethylammonium bromide), HDEAB (N-n-hexadecyl-N,N-dihydroxyethylammonium bromide), polybrene, or

poly(ethyleneimine) (PEI) can be used.

Because the expression of a desired target gene can be suppressed using the agent of the present invention, it is possible to treat or prevent a disease by, for example, administering the agent of the present invention to a patient to suppress the gene expression that causes the disease. Furthermore, the agent of the present invention is also useful as a reagent for investigating the function of a target gene.

8. Gene knockdown polynucleotide library

In one aspect, the present invention provides a gene knockdown polynucleotide library comprising a plurality of polynucleotides each of which comprises a nucleotide sequence comprising at least 15 continuous nucleotides present in the nucleotide sequence that encodes each of the transcripts of a plurality of gene or a sequence complementary thereto, wherein each nucleotide sequence is connected to the RNA interference induction element of the present invention so that RNA interference induction potential for the gene can be exhibited. Each polynucleotide present in the library may be harbored in a vector. Each polynucleotide or vector present in the library of the present invention can be in the same mode as the above-described polynucleotide of the present invention or a vector harboring the polynucleotide. By transferring the library of the present invention to a cellular population, it is possible to search for a functional gene.

"A plurality of genes" can be selected as appropriate according to the intended purpose and the like; for example, an assembly of genes expressed in desired cells, tissues and the like (gene library) and the like can be used.

The library of the present invention can, for example, be prepared by synthesizing a cDNA library by an ordinary method, and functionally connecting the cDNA library to the RNA interference induction element of the present invention. A

commonly used method of synthesizing a cDNA library comprises synthesizing a cDNA by a reverse transcriptase reaction using oligodT (oligodeoxythymidine) or a random hexamer as the primer with an mRNA purified from total RNA extracted from tissue and the like as the template, enzymatically treating the reaction product cDNA to render it a double-stranded DNA, and binding the DNA to a cloning vector (Molecular Cloning: A Laboratory Manual-Second Edition, 1989).

In this case, it is easily possible to prepare the library of the present invention by, for example, using the above-described vector of the present invention, wherein the individual constituents are placed in the order of 5′-promoter-cloning site-RNA interference induction element-3′, and inserting a cDNA library to the cloning site. Additionally, when the library is transferred to a cellular population, and the insert is expressed by the action of the promoter in the vector, a single-stranded RNA wherein the RNA interference induction element is bound to the 3′ side of the cDNA sequence of any one gene in the cDNA library or a sequence complementary thereto is expressed in each cell, and a siRNA for the gene is produced by the action of the element. Subsequently, this siRNA inhibits the expression of the gene to alter the cell function or phenotype.

The present invention provides a method of screening for a functional gene, which comprises a step for analyzing the phenotype of a cellular population incorporating the above-described gene knockdown nucleotide library, a step for isolating a cell with a change in the phenotype from the cellular population, and a step for obtaining a functional gene on the basis of the target nucleotide sequence in the polynucleotide or the vector transferred to the isolated cell.

In the above-described method, a cellular population incorporating a gene knockdown nucleotide library can be produced in the same manner as described above.

Subsequently, the phenotype of the cellular population is analyzed. This phenotype analysis can be performed by, for example, comparing the phenotype with that of a population of control cells not incorporating the gene knockdown nucleotide library. This phenotype includes not only those occurring on the cell surface, but also, for example, intracellular changes and the like. Subsequently, cells with a desired change in phenotype are isolated from the cellular population. Isolation of the cells can be performed using a means known per se such as a cell sorter.

It is highly likely that a polynucleotide (or vector) that produces a siRNA capable of suppressing the expression of a functional gene has been transferred to a cell with a change in phenotype. Hence, to screen for the functional gene, for example, a probe and primer are constructed on the basis of the target nucleotide sequence in the polynucleotide or the vector transferred in this isolated cell. Then, hybridization or PCR is performed using this probe or primer; cloning of the functional gene can thereby be performed. Additionally, on the basis of the target nucleotide sequence, a functional gene can also be searched for in a database.

Using the above-described method, the "forward genetic" approach with a step of isolating a cell with a mutated phenotype of interest from a cellular population having random knockdown mutated phenotypes, and cloning the causal gene, can be applied to the cells of higher organisms, so that the methodology of genetic analysis improves dramatically.

9. Template for the RNA-dependent RNA synthesis reaction

As described above, when a single-stranded RNA comprising the RNA interference induction element of the present invention is transferred to cells, the RNA acts favorably as a template for an RNA-dependent RNA synthesis reaction, and transcription of an RNA complementary to the RNA

transferred is induced in the vicinity of the element (5′ or 3′ side). Hence, the RNA interference induction element of the present invention is capable of functioning as an "RNA-dependent RNA synthesis reaction induction element."

Accordingly, in still another aspect, the present invention provides a template for an RNA-dependent RNA synthesis reaction comprising the RNA-dependent RNA synthesis reaction induction element of the present invention. The template is an RNA.

The template of the present invention further comprises "a target template sequence," in addition to the above-described RNA-dependent RNA synthesis reaction induction element. "A target template sequence" refers to a nucleotide sequence intended to cause a synthesis reaction of an RNA complementary thereto, and can be chosen optionally. The target template sequence is connected to the RNA-dependent RNA synthesis reaction induction element so that a synthesis reaction of an RNA complementary thereto is caused when an RNA-dependent RNA synthesis reaction is performed using the template of the present invention.

The length of the target template sequence is not subject to limitation, as long as a synthesis reaction of an RNA complementary to the sequence can be caused when an RNA-dependent RNA synthesis reaction is performed using the template of the present invention.

Additionally, the target template sequence may be connected adjacently to the RNA-dependent RNA synthesis reaction induction element of the present invention (not via a spacer region), or may be connected via a spacer region, as long as a synthesis reaction of an RNA complementary to the sequence can be caused when an RNA-dependent RNA synthesis reaction is performed using the template of the present invention. Although the length of the spacer region is not subject to limitation, as long as the individual constituents,

from the target template sequence to the element, can be stably present without interruption in one polynucleotide chain, and as long as a synthesis reaction of an RNA complementary to the target template sequence can be caused, it is preferably at most 10 Kbp, for example, 5 Kbp or less, 3 Kbp or less, 1 Kbp or less, 500 bp or less, 200 bp or less, 100 bp or less, 50 bp or less, or 25 bp or less. The nucleotide sequence that constitutes the spacer region is not subject to limitation, and may be an optionally chosen sequence.

The target template sequence may be connected to any of the 5′ and 3′ sides of the RNA-dependent RNA synthesis reaction induction element of the present invention, as long as a synthesis reaction of an RNA complementary to the target template sequence can be caused. However, the target template sequence is preferably connected to the 5′ side of the RNA-dependent RNA synthesis reaction induction element of the present invention, because an RNA-dependent RNA synthesis reaction is caused with dependence on RNA-dependent RNA polymerase from the vicinity of the element toward the 3′ side (this is the direction in the strand complementary to the template) when the template of the present invention is used.

Additionally, with respect to the template of the present invention, the number of copies of the RNA-dependent RNA synthesis reaction induction element present in one template chain is not subject to limitation; only one copy of the element may be present in one template chain, or plural copies of the element may be present in one template chain as connected in tandem. Using plural copies of the element as connected in tandem, more potent RNA-dependent RNA synthesis reaction induction potential can be obtained. When plural copies of the element are used as connected in tandem, the number of copies of the element connected is not subject to limitation, as long as a synthesis reaction of an RNA

complementary to the target template sequence can be caused; the number of copies is, for example, 2 to 50 copies, preferably 2 to 20 copies, and more preferably 2 to 10 copies. In view of the ease of polynucleotide connecting procedures and other factors, the number of copies is preferably about 2 to 5 copies.

When plural copies of RNA-dependent RNA synthesis reaction induction element are used as connected in tandem, the nucleotide sequences of the individual units of the element may be identical or not. The units of the element may be connected adjacently (not via a spacer region), or may be connected via a spacer region. Although the length of the spacer region is not subject to limitation, as long as the individual constituents, from the target template sequence to the plural copies of the RNA-dependent RNA synthesis reaction induction element connected, can be stably present without interruption in one polynucleotide chain, and as long as a synthesis reaction of an RNA complementary to the target template sequence can be caused, it is preferably at most 10 Kbp, for example, 5 Kbp or less, 3 Kbp or less, 1 Kbp or less, 500 bp or less, 200 bp or less, 100 bp or less, 50 bp or less, or 25 bp or less. The nucleotide sequence that constitutes the spacer region is not subject to limitation, and may be an optionally chosen sequence.

10. A vector capable of expressing a template for the RNA-dependent RNA synthesis reaction

In still another aspect, the present invention provides a vector capable of expressing the above-described template of the present invention (the vector of the present invention (III)). Using the vector, it is easily possible to produce a template of the present invention and induce an RNA-dependent RNA synthesis reaction in a cell.

Applicable modes of vector are the same as the vector of

the present invention (I) or (II) described above.

In a preferred mode, the vector of the present invention (III) further harbors a promoter, which promoter is preferably joined to a region encoding the template of the present invention so that the expression of the template can be controlled. Accordingly, the template of the present invention can be produced as the transcript (RNA) by the action of the promoter that can be present in the vector.

Although the promoter may be placed at any position in the vector, as long as the expression of the template of the present invention can be controlled, the above-described promoter is preferably bound to the 5′ side of the region encoding the template of the present invention because promoters are usually located about 20 to 30 bp upstream (5′ side) of the transcription initiation point. Additionally, the region encoding the template of the present invention is preferably located downstream (3′ side) of the transcription initiation point defined by the promoter. Because the target template sequence is preferably connected to the 5′ side of the RNA-dependent RNA synthesis reaction induction element of the present invention in the template of the present invention, it is more preferable that the promoter, the target template sequence, and the RNA-dependent RNA synthesis reaction induction element be placed in the order of 5′-promoter-target template sequence-RNA-dependent RNA synthesis reaction induction element-3′.

The kind of promoter used may be the same as that of the vector of the present invention (I) or (II) described above. Additionally, the vector in this mode can also further harbor a terminator, an enhancer, a selection marker and the like as the vector of the present invention (I) or (II) described above.

11. Cell incorporating the vector capable of expressing the

template for the RNA-dependent RNA synthesis reaction

In still another aspect, the present invention provides a cell incorporating the above-described vector of the present invention (III) (the cell of the present invention (II)).

The kinds of cells useful in the present invention are the same as those mentioned with respect to the above-described cell of the present invention (I). Because the template of the present invention is expressed from the vector transferred in the cell, making it possible to cause an RNA-dependent RNA synthesis reaction based on the template, it is preferable to use a cell having RNA-dependent RNA polymerase.

The cell of the present invention (II) can be produced using the above-described method of vector transfer.

12. Method of synthesizing an RNA

In still another aspect, the present invention provides a method of synthesizing an RNA comprising the steps shown below:

(a) a step for providing a template for an RNA-dependent RNA synthesis reaction comprising the RNA-dependent RNA synthesis reaction induction element of the present invention;

(b) a step for bringing the template of (a) in contact with RNA-dependent RNA polymerase to cause the RNA-dependent RNA synthesis reaction.

For example, when the RNA synthesis reaction is performed in vitro, the template of the present invention for an RNA-dependent RNA synthesis reaction is prepared using a nucleic acid synthesizer, in vitro transcription and the like (step (a)). The template obtained is brought into contact with RNA-dependent RNA polymerase under conditions allowing the RNA-dependent RNA synthesis reaction, to cause the RNA-dependent RNA synthesis reaction and an RNA complementary to the target template sequence is synthesized (step (b)). The RNA-dependent RNA synthesis reaction is preferably performed

in an appropriate buffer solution supplemented with a substrate essential to the RNA-dependent RNA synthesis reaction (e.g., NTPs and the like).

A method of synthesizing an RNA in cells is also encompassed in the scope of the present invention. For example, the template of the present invention for an RNA-dependent RNA synthesis reaction is expressed by transferring the above-described vector of the present invention (III) into a cell having RNA-dependent RNA polymerase (step (a)). The resulting template comes into contact with the RNA-dependent RNA polymerase in the cell to cause the RNA-dependent RNA synthesis reaction and an RNA complementary to the target template sequence is synthesized (step (b)).

Using the method of the present invention, it is possible to produce an RNA complementary to a desired target template sequence from an RNA having the sequence. For example, when using the nucleotide sequence of the reverse transcript of a structural gene as the target template sequence in the template used in the step (a), an RNA-dependent RNA synthesis reaction occurs, resulting in the forward transcript (RNA) of the structural gene. Therefore, provided that the reaction has been performed in a cell, the translation product (protein) of the structural gene will be produced from the forward transcript of the structural gene.

The present invention is hereinafter described in more detail by means of the following Examples, which, however, are not to be construed as limiting the scope of the present invention.

EXAMPLES

Example 1: Fission yeast centromeric siRNA is derived from the vicinity of SIRE

The fission yeast wild type strains (h^- and h^{90}) used were common laboratory strains 972 and 968. Mutants of the RNAi apparatus (Δago1, Δrdp1, and Δdcr1) were prepared by

replacing the SPCC736.11, SPAC6F12.09, and SPCC584.10c genes with the G418 resistance gene, respectively.

The otr repeats in the left arm of the first chromosome centromere of fission yeast were divided into eight portions (regions 1 to 8), and small-molecule RNAs in the fission yeast were detected by Northern blotting using regions 1 to 8 as the probes (Figure 1).

The probe of region 1 corresponds to the base number 19814-21497 region of the cosmid SPAP7G5 (GenBank accession number: AL353014),
the probe of region 2 corresponds to the EcoRI-HindIII fragment region of the centromeric plasmid pRS140,
the probe of region 3 corresponds to the HindIII-AatII fragment region of the centromeric plasmid pRS140,
the probe of region 4 corresponds to the AatII-BamHI fragment region of the centromeric plasmid pRS140,
the probe of region 5 corresponds to the BamHI-SpeI fragment region of the centromeric plasmid pRS140,
the probe of region 6 corresponds to the SpeI-KpnI fragment region of the centromeric plasmid pRS140,
the probe of region 7 corresponds to the KpnI-HindIII fragment region of the centromeric plasmid pRS140, and
the probe of region 8 corresponds to the HindIII-EcoRI fragment region of the centromeric plasmid pRS140.

As a result, when probes 2, 6 and 7 were used, siRNA accumulation was detected specifically in RNAs extracted from the wild type strains (h^- and h^{90}). No such accumulation was observed in any RNA extracted from the mutants (Δago1, Δrdp1, and Δdcr1) of the RNAi apparatus.

Although it is not present in pRS140, a sequence comprising the RNA interference induction element of the present invention (hereinafter referred to as SIRE) comprising SEQ ID NO:1 or a sequence homologous thereto is inserted in the probe 2 region of the ordinary dh repeat unit of the

fission yeast (see Figure 2). With this in mind, two probes surrounding the insertion site were prepared (probes 2.1 and 2.2), and Northern blotting was performed in the same way; a larger amount of siRNA was found with probe 2.2 (Figure 1). This agrees with the difference in the amount detected between probes 6 and 7 relative to SIRE.

Figure 2 shows the schematic structures of the centromere DNAs of the three chromosomes of fission yeast. The upper panel against the gray background illustrates the entire centromere, the lower panel shows the features of the otr repeat, which is a common unit shared by the three centromeres, and the homologous portions to sequences other than the centromeres. SIRE is present in dh units other than the dh unit contained in pRS140. In the third chromosome centromere otr, in particular, dg and dh often occur in mixture to form a single repeat unit with SIRE as the transition point. Additionally, a otr-like region containing SIRE is also found in the cenH and SPAC212.11 of mat2-3.

Example 2: Expression of SIRE-incorporating ura4 gene suppresses the expression of endogenous ura4$^+$ gene

One to three SIRE units comprising the sequence of SEQ ID NO:1 were inserted to the ura4$^+$ gene under the control of the nmt1 promoter in the pAU001 vector in two directions (differentially designated as SIRE for the insertion in the forward direction, and as ERIS for the insertion in the reverse direction), and expressed in a fission yeast wherein the endogenous ura4$^+$ gene is functioning normally, to examine the effect (Figure 3). The ura4$^+$ gene used was the genome sequence (a region covering the initiation codon, the ORF full-length and the terminator sequence) of the ura4$^+$ gene (SEQ ID NO:2). In SEQ ID NO:2, the region of base numbers 1 to 795 corresponds to ORF. SIRE or ERIS was inserted to the EcoRV restriction site at the 679 position in SEQ ID NO:2.

Nucleotide sequences functionally connected to the nmt1 promoter are shown by SEQ ID NO:2 to 7, respectively.

A liquid culture of each yeast strain expressing the insert was serially diluted 10 fold and spotted onto each medium at six dilution rates, and this was followed by incubation at 33°C and examination for viability. Referring to Figure 3, panel YES shows control results obtained by spotting to a complete medium; panel YES+FOA shows results obtained with a complete medium supplemented with 5-FOA, a drug that makes the strains expressing the ura4$^+$ gene to be incapable of growing; panel EMM2+AA+FOA shows results obtained with a synthetic medium supplemented with 5-FOA; in the latter two panels, the ratio of cells with suppressed expression of the ura4$^+$ gene is shown. EMM2+AA-Ura is a synthetic medium lacking uracil, in which only cells expressing the ura4$^+$ gene can grow.

In the control cases without expression (none) or with the expression (ura4$^+$) of the ura4$^+$ gene, the endogenous ura4$^+$ gene remained normally expressed, and no growth on the FOA medium was observed. When the SIRE-incorporating ura4 gene was expressed, cells that also grow on the FOA medium were identified (ura4SIRE(RV)). This demonstrates that the expression of endogenous ura4$^+$ gene was suppressed by the expression of the SIRE-incorporating ura4 gene. Additionally, the number of cells that grow on the FOA medium increased (ura4SIREx2(RV)) as the number of SIRE units inserted increased. This demonstrates that the expression of endogenous ura4$^+$ gene is more potently suppressed as the number of SIRE units inserted increases. Furthermore, even when the ura4 gene, incorporating ERIS, a sequence complementary to SIRE, was expressed, cells that also grow on the FOA medium were identified, demonstrating the suppression of the expression of the endogenous ura4$^+$ gene (ura4ERIS(RV)). Additionally, it was demonstrated that, as with SIRE, the number of cells that grow on the FOA medium increased, and the expression of the

endogenous ura4$^+$ gene was more potently suppressed, as the number of ERIS units inserted increased (ura4ERISx2(RV) and ura4ERISx3(RV)). Such an effect was not observed when an irrelevant stuffer was inserted (ura4stuffer(RV)).

Example 3: A ura4-derived siRNA is detected in the strain expressing ura4ERISx2

RNA was extracted from a wild type strain wherein the endogenous ura4$^+$ gene was normally functioning, and a strain expressing ura4ERISx2 (a strain wherein the ura4$^+$ gene incorporating two ERIS units was expressed under the control of the nmt1 promoter), and analyzed for ura4-derived siRNA by Northern blotting to detect small-molecule RNA. The probe used was the ORF region of the ura4$^+$ gene. For the strain expressing ura4ERISx2, two cultures were used for RNA extraction: liquid culture (1) obtained with an ordinary complete medium, and liquid culture (2) under selection conditions of a complete medium supplemented with 5-FOA. The results are shown in Figure 4.

A band not found in the wild type strain was detected in the two RNAs extracted from the strain expressing ura4ERISx2. This demonstrates the presence of an ura4-derived siRNA in the strain expressing ura4ERISx2. For control, the results with centromere-derived siRNA (probe 6 used) are shown in the lower panel. Although the amount detected was variable, centromere-derived siRNA was identified in all samples. From this finding, it can be understood that the detection of ura4-derived siRNA only in the strain expressing ura4ERISx2 and its non-detection in the wild strain is not due to the differences in the amount of total RNA used in the experiment.

Example 4: A ura4-derived siRNA is detected in the strain expressing ura4ERISx3

RNA was extracted from a wild type strain wherein the

endogenous ura4$^+$ gene was normally functioning, and a strain expressing ura4ERISx3 (a strain wherein the ura4$^+$ gene incorporating three ERIS units was expressed under the control of the nmt1 promoter), and analyzed for ura4-derived siRNA by Northern blotting to detect small-molecule RNA. The probe used was the ORF region of the ura4$^+$ gene. For the strain expressing ura4ERISx3, two cultures were used for RNA extraction: liquid culture obtained with an ordinary complete medium, and liquid culture (+FOA) under selection conditions of a complete medium supplemented with 5-FOA. The results are shown in Figure 5.

A band not found in the wild strain was detected in the two RNAs extracted from the strain expressing ura4ERISx3. Such a band was not found in the RNA extracted from the ura4stuffer-expressing strain incorporating an irrelevant stuffer. This demonstrates the presence of an ura4-derived siRNA specifically in the strain expressing ura4ERISx3. For control, the results with centromere-derived siRNA (probe 6 used) are shown in the lower panel. Although the amount detected was variable, centromere-derived siRNA was identified in all samples. From this finding, it can be understood that the detection of ura4-derived siRNA only in the strain expressing ura4ERISx3 and its non-detection in the wild strain is not due to the differences in the amount of total RNA used in the experiment.

Example 5: Suppression of endogenous ura4$^+$ gene by SIRE-incorporating ura4 depends on RNA interference mechanism

As in Example 2, in a fission yeast wild type strain (wt) wherein the endogenous ura4$^+$ gene is normally functioning, and strains wherein incorporating mutations of the RNAi apparatus (Δdcr1, Δago1, and Δrdp1), the ura4$^+$ gene incorporating two ERIS units under the control of the nmt1 promoter was expressed, and their effects were examined. The

results are shown in Figure 6.

In the wild type strain (wt), because of the normal function of the ura4$^+$ gene, no growth was observed on the 5-FOA-containing medium. In the strain expressing ura4 incorporating two units of ERIS (wt ura4ERISx2), this endogenous ura4$^+$ gene underwent expression suppression; growth on the 5-FOA medium was observed. It was revealed, however, that the growth on the 5-FOA medium was inhibited in the strains incorporating the mutations of the RNAi apparatus (Δdcr1 ura4ERISx2, Δago1 ura4ERISx2, and Δrdp1 ura4ERISx2).

These findings demonstrate that the suppression of the expression of ura4$^+$ by SIRE (or ERIS) depends on RNA interference mechanism.

Example 6: siRNA induction for human gene by SIRE and siRNA induction potential with one copy of SIRE detectable in a strain lacking siRNA-decomposing enzyme

One to three units of SIRE or ERIS were inserted to the fusion site of the human-cDNA(c10orf96)-ura4$^+$ fusion gene under the control of the nmt1 promoter in the pREP1 vector (Figure 7), and the gene was expressed in a wild type or eri1-deleted mutant (Δeri1) strain of fission yeast. The nucleotide sequences of the inserts in the individual constructs are shown by SEQ ID NO:8 to 14, respectively. Eri1 is a ribonuclease that decomposes siRNA, and Δeri1 was prepared by replacing and hence destroying the SPBC30B4.08 gene with the hygromycin resistance gene. RNA was extracted from the fission yeast incorporating each vector, and examined for c10orf96- or ura4-derived siRNA by Northern blotting to detect small-molecule RNA. The probe used was the ORF region in the cDNA of the c10orf96 gene or full length of ura4 gene (SEQ ID NO:2). The results are shown in Figure 8.

When the c10orf96-ura4$^+$ fusion gene incorporating two or three units of SIRE or ERIS was expressed in the wild type

strain, siRNA derived from the c10orf96 gene was detected (A). This result indicates that siRNA induction by SIRE is not limited to the genes of fission yeast. Northern blotting on the same cell-extracted RNA with the ura4 as the probe revealed that siRNA of the ura4 gene portion, which is placed on the 3′ side from SIRE, was not detected (B). For control, the results of Northern blotting on the centromere-derived siRNA of each sample with the probe 6 are also shown. These results suggest that SIRE may preferentially induce siRNA from a sequence on the 5′ side of SIRE in the template transcript.

Even when the c10orf96-ura4$^+$ fusion gene incorporating one unit of SIRE or ERIS was expressed in the wild strain, siRNA derived from the c10orf96 gene was little detected (A, C, and D). In Δeri1, in contrast, siRNA derived from the c10orf96 gene was detected not only when a plurality of units of SIRE were inserted, but also when one unit of SIRE or ERIS was inserted (C and D). This is attributable to an increase in siRNA recovery efficiency as a result of deletion of the siRNA-decomposing enzyme from the host cell. These results suggest that even a single copy of SIRE and ERIS exhibits siRNA induction potential.

Example 7: SIRE induces RNA reverse transcription with RNA template

The his5$^+$ gene was connected to the pAU001 vector in the reverse orientation under the control of the nmt1 promoter, and three units of SIRE were connected in tandem to the 3′ side thereof (Figure 9, d). The construct obtained (construct-d) was transferred to a wild type fission yeast, and its effect was examined. The his5$^+$ gene is a histidine synthesis gene. The nucleotide sequences of the inserts of the constructs-a to -d are shown by SEQ ID NO:15 to 18, respectively.

A liquid culture of the fission yeast was serially

diluted 10 fold, spotted onto each medium at six dilution rates and incubated at 33°C to examine their viability. EMM2+aa is a complete medium and represents control values of the amount spotted. EMM2+aa-His is a histidine-free medium, in which only the strain expressing the $his5^+$ gene is capable of growing.

As a result, the strain incorporating the construct-d exhibited infrequent but observable growth on the EMM2+aa-His medium (Figure 10, d). In contrast, the strains incorporating a control construct without the nmt1 promoter (Figure 9, a and b) or a control construct without SIRE (Figure 9, a and c) were incapable of growing on the EMM2+aa-His medium like the non-incorporating strain (Figure 10, a-c).

These results show that SIRE promoted the synthesis of the forward transcript of the $his5^+$ gene from the reverse transcript of the $his5^+$ gene, that $his5^+$ functional protein was produced from the forward transcript, and that the strain incorporating the construct-d acquired the ability to grow in histidine-free medium.

Furthermore, the construct-d was transferred to mutants with a mutation in the RNAi apparatus (Δdcr1, Δago1 and Δrdp1), and its effects were examined. As a result, when the construct-d was transferred to Δdcr1 or Δago1, growth on the EMM2+aa-His medium was observed as with the wild type strain. In contrast, when the construct-d was transferred to Δrdp1, i.e., a strain lacking RNA-dependent RNA polymerase (RdRP), remarkably decreased growth on the EMM2+aa-His medium was observed (Figure 11, Δrdp1).

These results suggest that the SIRE-induced synthesis of the forward transcript from the reverse transcript of the $his5^+$ gene is dependent on RNA-dependent RNA polymerase (RdRP).

Hence, it was demonstrated that the RNA interference induction element of the present invention possesses an activity to induce an RNA reverse transcription reaction with

the RNA template, and that this activity is dependent on RdRP (Figure 12).

Example 8: Gene-suppressive effect of SIRE in human cells

Hela cells and SVts8 cells that stably express the GFP-Cenp-A fusion protein were prepared by transferring pBabe-Hygro-EGFP-CENPA, an expression vector encoding the GFP-Cenp-A fusion protein. Cenp-A is a kind of centromere-localized protein.

The full-length cDNA of the GFP gene mutated at the initiation codon (ΨGFP^{2-238}) or a 3'-end-deleted DNA thereof (ΨGFP^{2-163}) was connected to the pcDNA3 vector in the forward orientation under the control of the CMV promoter, and three units of SIRE were connected in tandem to the 3' side thereof (Figure 13, ΨGFP^{2-238}-SIREx3 and ΨGFP^{2-163}-SIREx3). ΨGFP^{2-238} corresponds to the coding region for the 2-238 position amino acids of the GFP gene, and ΨGFP^{2-163} corresponds to the coding region for the 2-163 position amino acids of the GFP gene. Because all constructs undergo transcription but do not undergo translation into protein, the gene products never, by themselves, generate a fluorescent signal in the cells. Each construct obtained was transferred to the above-described GFP expressing transfectant using the calcium phosphate method and cultured for 72 hours, after which GFP fluorescence was examined under a fluorescence microscope. The nucleotide sequences of the inserts of the individual constructs are shown by SEQ ID NO:19 to 22, respectively.

As a result, when ΨGFP^{2-238}-SIREx3 or ΨGFP^{2-163}-SIREx3 was transferred to Hela cells or SVts8 cells expressing the GFP-Cenp-A protein, the GFP fluorescence weakened (Figure 14). When SIRE-free control constructs (Figure 13, ΨGFP^{2-238} or ΨGFP^{2-163}) were used, no such effect was observed (Figure 14). The bar measures 10 μm.

Lysates were prepared from these cells and the GFP-Cenp-

A protein in the cells was quantified by Western blotting. The amount of GFP protein decreased with the transfer of ΨGFP$^{2-238}$-SIREx3 or ΨGFP$^{2-163}$-SIREx3, in agreement with the findings of the fluorescent microscopic examination (Figure 15).

Hence, it was demonstrated that the RNA interference induction element of the present invention exhibits suppressive potential of gene expression also in mammalian cells such as human cells.

INDUSTRIAL APPLICABILITY

Using the RNA interference induction element of the present invention, it is easily possible to knock down a desired target gene, and to produce a siRNA for a desired target gene.

This application is based on a patent application No. 2005-145876 filed in Japan (filing date: May 18, 2005), the contents of which are incorporated in full herein by this reference.

CLAIMS

1. An RNA interference induction element comprising a nucleotide sequence selected from among the nucleotide sequences (a) to (c) below:

(a) a nucleotide sequence comprising SEQ ID NO:1 or a sequence complementary thereto;

(b) a nucleotide sequence comprising at least 15 continuous nucleotides present in the nucleotide sequence (a) above, and possessing RNA interference induction potential;

(c) a nucleotide sequence having a homology of at least 70% to any one of the nucleotide sequences (a) and (b) above, and possessing RNA interference induction potential.

2. A polynucleotide comprising the element of claim 1, wherein a nucleotide sequence comprising at least 15 continuous nucleotides present in the nucleotide sequence that encodes the transcript of a target gene, or a sequence complementary thereto, is connected so that RNA interference induction potential for the target gene can be exhibited.

3. The polynucleotide of claim 2, wherein the nucleotide sequence is connected to the 5′ side of the element.

4. The polynucleotide of claim 2, which comprises plural copies of the element as connected in tandem.

5. A vector harboring the element of claim 1.

6. The vector of claim 5, which comprises plural copies of the element as connected in tandem.

7. The vector of claim 5 or 6, which further harbors a promoter joined to the element so that the expression of the

element can be controlled.

8. The vector of claim 5 or 6, which further harbors at least one cloning site connected to the element so that RNA interference induction potential for a target gene can be exhibited when a nucleotide sequence comprising at least 15 continuous nucleotides present in the nucleotide sequence that encodes the transcript of the target gene or a sequence complementary thereto is inserted to the cloning site.

9. The vector of claim 8, wherein the cloning site is connected to the 5′ side of the element.

10. The vector of claim 8 or 9, which further harbors a promoter joined to the element or the cloning site so that the expression of the element and the cloning site can be controlled.

11. A vector harboring the polynucleotide of any of claims 2 to 4.

12. The vector of claim 11, which further harbors a promoter joined to the polynucleotide so that the expression of the polynucleotide can be controlled.

13. A cell incorporating the polynucleotide of any of claims 2 to 4.

14. A cell incorporating the vector of any of claims 5 to 12.

15. A method of producing a cell wherein the expression of a target gene is suppressed, which comprises a step for transferring the polynucleotide of any of claims 2 to 4, or the vector of claim 11 or 12, into cells, and a step for

selecting a cell incorporating the polynucleotide or the vector.

16. A method of suppressing the expression of a target gene, which comprises a step for transferring the polynucleotide of any of claims 2 to 4, or the vector of claim 11 or 12, into cells.

17. A method of producing a siRNA for a target gene, which comprises a step for transferring the polynucleotide of any of claims 2 to 4, or the vector of claim 11 or 12, into cells, and a step for obtaining the siRNA for the target gene from the cells incorporating the polynucleotide or the vector.

18. An RNA interference inducing agent comprising the polynucleotide of any of claims 2 to 4, or the vector of claim 11 or 12.

19. A gene knockdown polynucleotide library comprising a plurality of polynucleotides, each of which comprises a nucleotide sequence comprising at least 15 continuous nucleotides present in the nucleotide sequence that encodes each of the transcripts of a plurality of genes or a sequence complementary thereto, wherein each nucleotide sequence is connected to the element of claim 1 so that RNA interference induction potential for the gene can be exhibited.

20. The library of claim 19, wherein the each polynucleotide is harbored in a vector.

21. A cellular population incorporating the library of claim 19 or 20.

22. A method of screening for a functional gene, which

comprises the steps (a) to (c) below:

(a) analyzing the phenotype of a cellular population incorporating the library of claim 19 or 20;

(b) isolating cells with an altered phenotype from the cellular population; and

(c) obtaining a functional gene based on a nucleotide sequence in the polynucleotide or the vector incorporated in the isolated cells.

23. An RNA-dependent RNA synthesis reaction induction element comprising a nucleotide sequence selected from among the nucleotide sequences (a) to (c) below:

(a) a nucleotide sequence comprising SEQ ID NO:1 or a sequence complementary thereto;

(b) a nucleotide sequence comprising at least 15 continuous nucleotides present in the nucleotide sequence (a) above, and possessing RNA-dependent RNA synthesis reaction induction potential;

(c) a nucleotide sequence having a homology of at least 70% to any one of the nucleotide sequences (a) and (b) above, and possessing RNA-dependent RNA synthesis reaction induction potential.

24. A template for an RNA-dependent RNA synthesis reaction, which comprises the element of claim 23.

25. A vector capable of expressing the template of claim 24.

26. A cell incorporating the vector of claim 25.

27. A method of synthesizing an RNA, which comprises the steps shown below:

(a) a step for providing a template for an RNA-dependent RNA synthesis reaction comprising the element of claim 23;

(b) a step for bringing the template of (a) into contact with RNA-dependent RNA polymerase to cause the RNA-dependent RNA synthesis reaction.

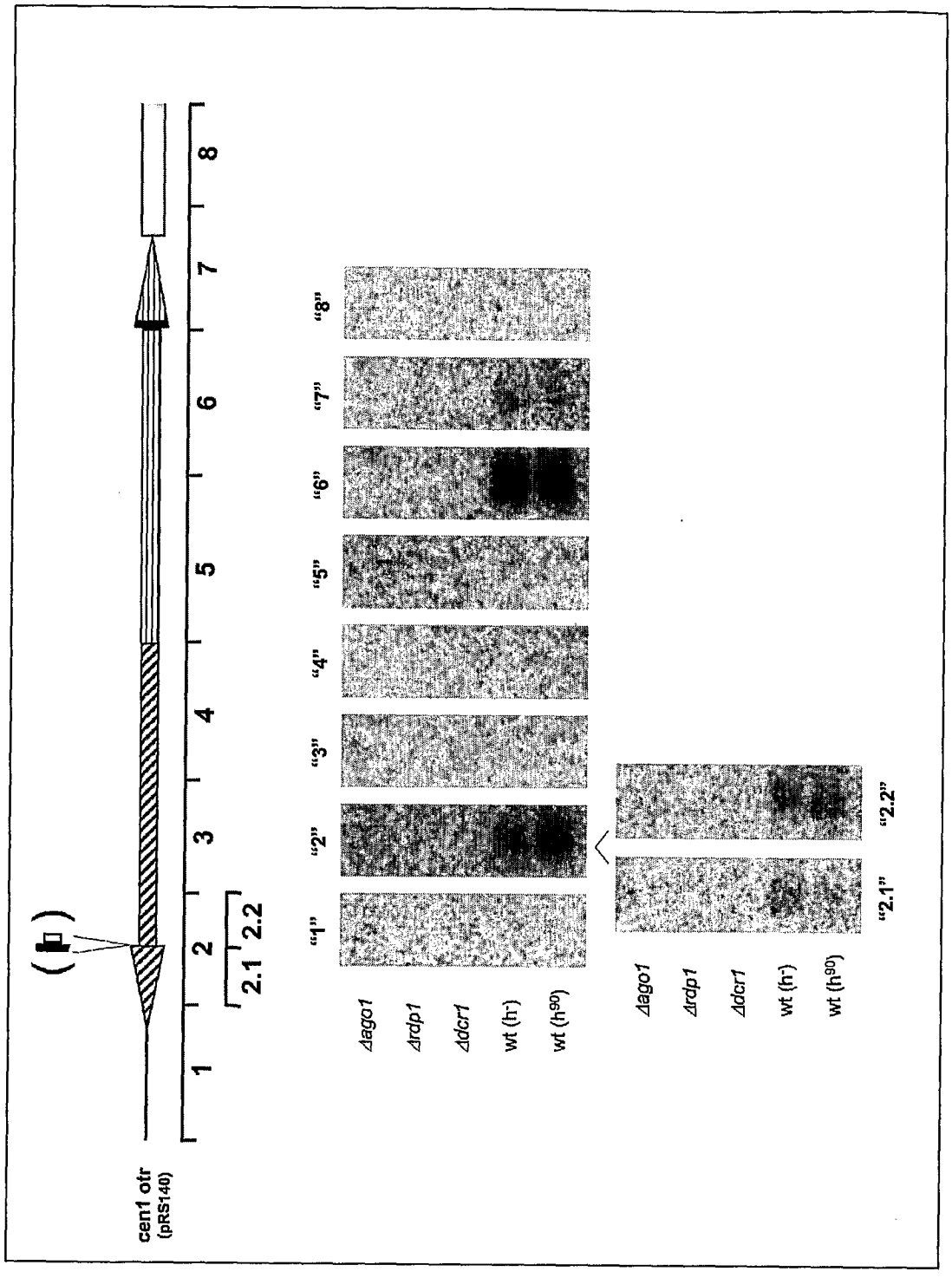
28. A gene expression suppression element comprising a nucleotide sequence selected from among the nucleotide sequences (a) to (c) below:

(a) a nucleotide sequence comprising SEQ ID NO:1 or a sequence complementary thereto;

(b) a nucleotide sequence comprising at least 15 continuous nucleotides present in the nucleotide sequence (a) above, and possessing gene expression suppression potential;

(c) a nucleotide sequence having a homology of at least 70% to any one of the nucleotide sequences (a) and (b) above, and possessing gene expression suppression potential.

Fig. 1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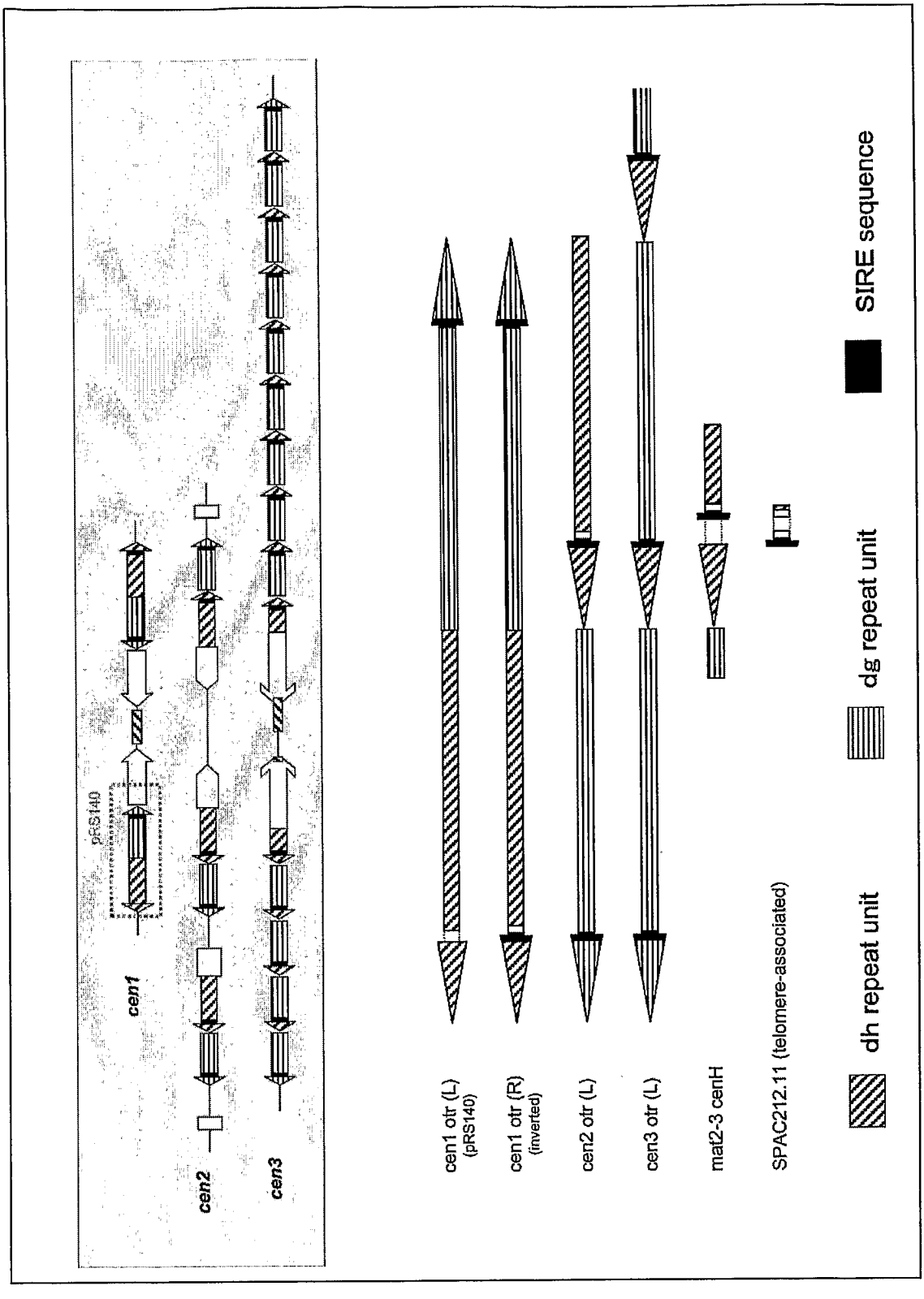
(pRS140)
1
2
3
4
5
6
7
8
2.1
2.2
"1"
"2"
"3"
"4"
"5"
"6"
"7"
"8"
Δago1
Δrdp1
Δdcr1
wt (h-)
wt (h90)
"2.1"
"2.2"

Fig. 2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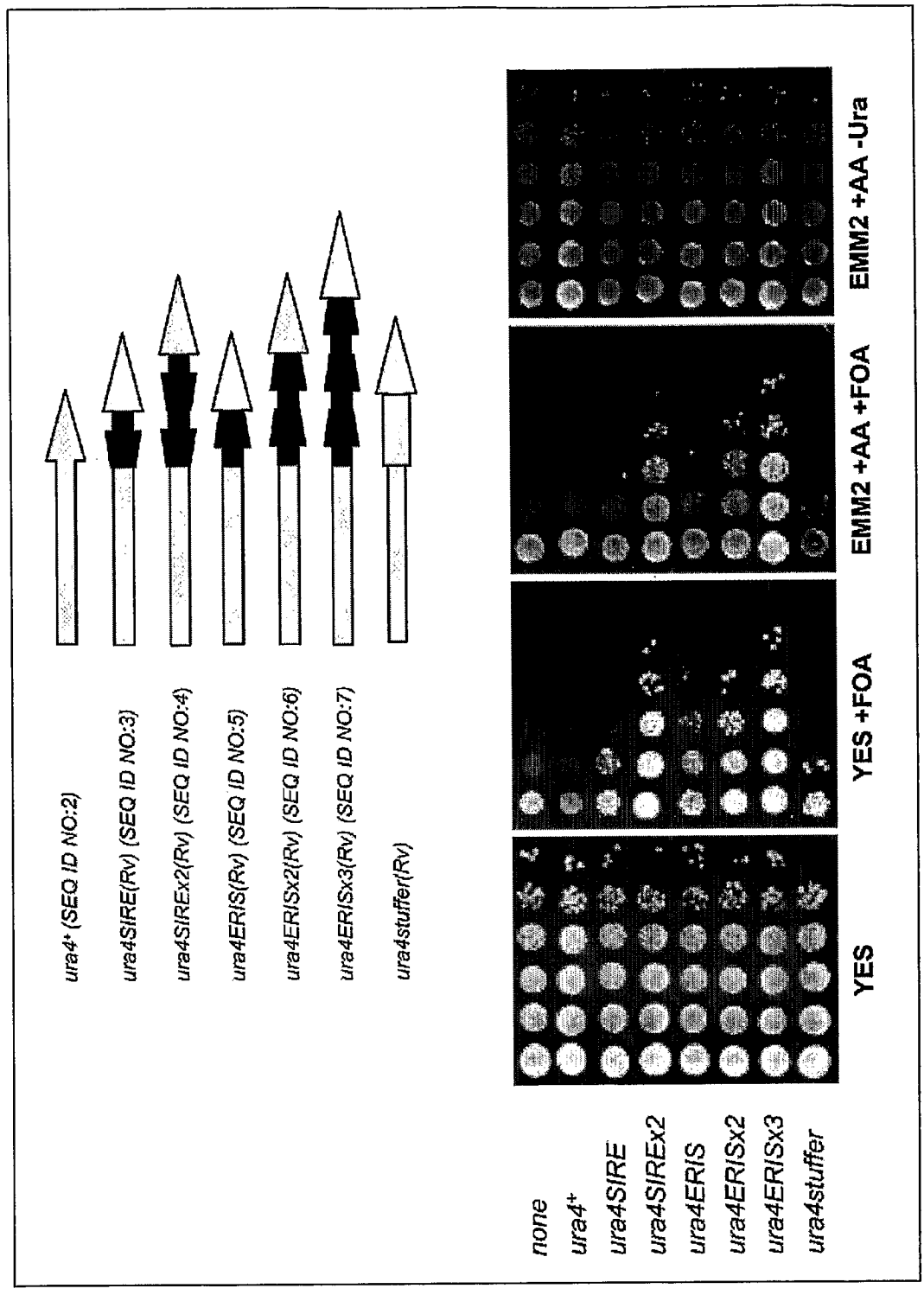
cen1
cen2
cen3
cen1 otr (L) (pRS140)
cen1 otr (R) (inverted)
cen2 otr (L)
cen3 otr (L)
mat2-3 cenH
SPAC212.11 (telomere-associated)
dh repeat unit
dg repeat unit
SIRE sequence

Fig. 3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ura4SIRE(Rv) (SEQ ID NO:3)
ura4SIREx2(Rv) (SEQ ID NO:4)
ura4ERIS(Rv) (SEQ ID NO:5)
ura4ERISx2(Rv) (SEQ ID NO:6)
ura4ERISx3(Rv) (SEQ ID NO:7)
ura4stuffer(Rv)
none
ura4+
ura4SIRE
ura4SIREx2
ura4ERIS
ura4ERISx2
ura4ERISx3
ura4stuffer
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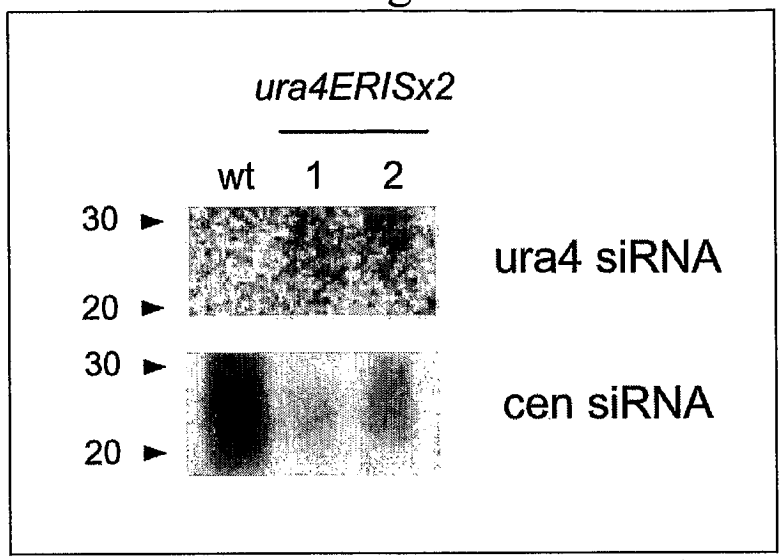
YES +FOA
EMM2 +AA +FOA
EMM2 +AA -Ura

Fig. 4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2
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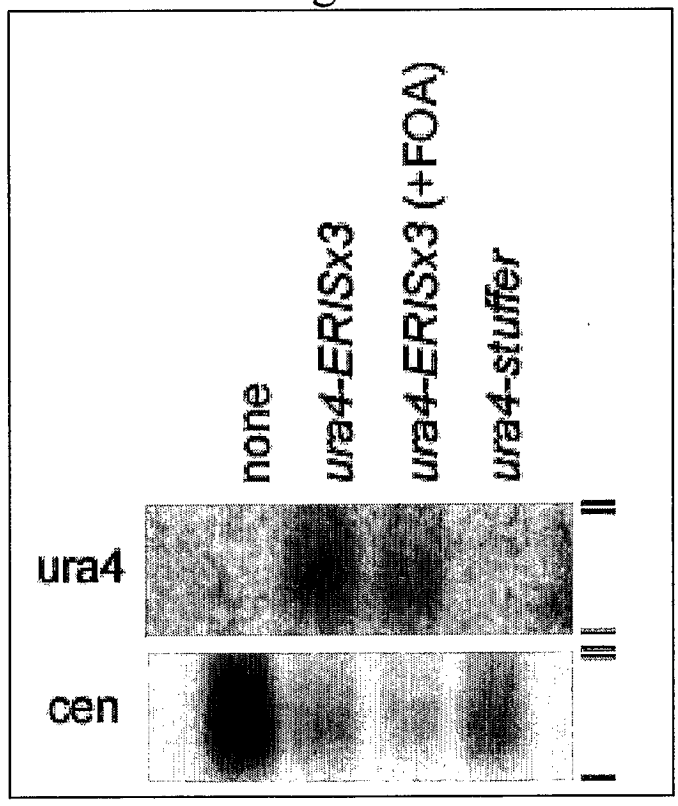
20
ura4 siRNA
30
20
cen siRNA

Fig. 5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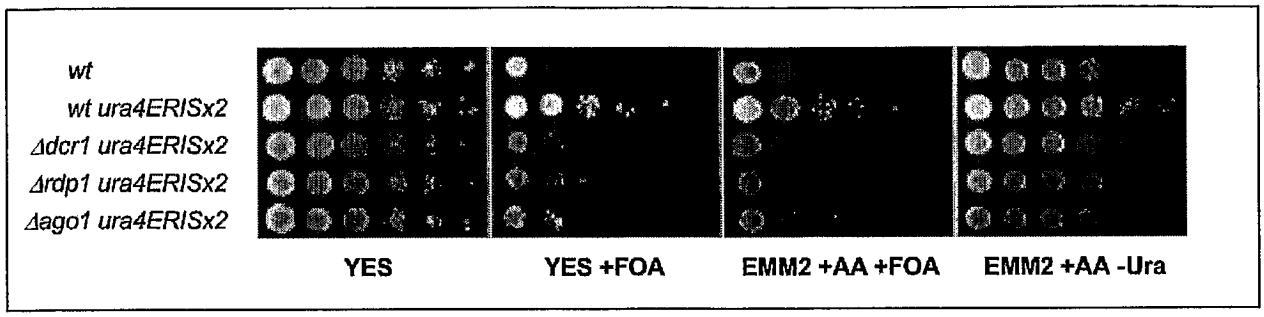
ura4-ERISx3 (+FOA)
ura4-stuffer
ura4
cen
II
III
I

Fig. 6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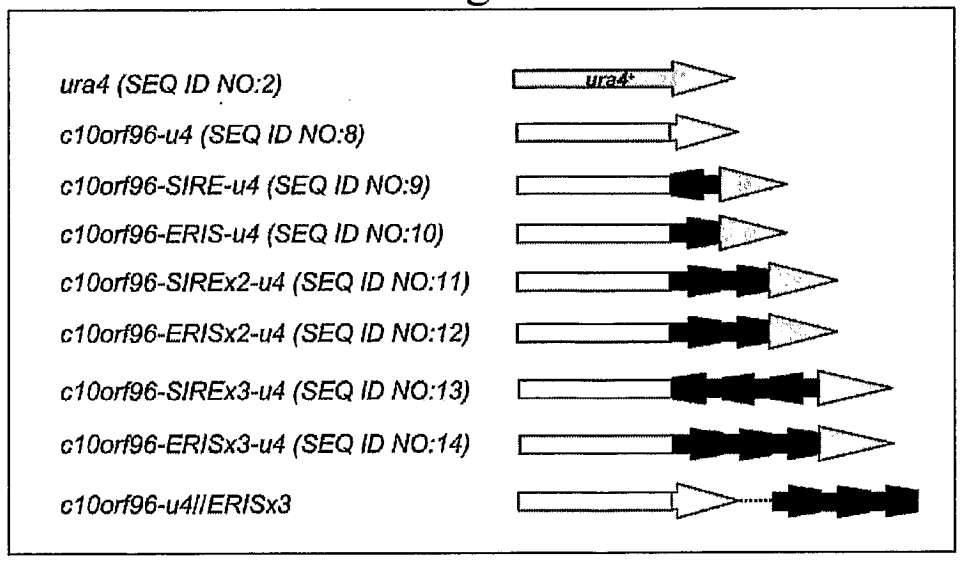
EMM2 +AA -Ura

Fig. 7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c10orf96-SIRE-u4 (SEQ ID NO:9)
c10orf96-ERIS-u4 (SEQ ID NO:10)
c10orf96-SIREx2-u4 (SEQ ID NO:11)
c10orf96-ERISx2-u4 (SEQ ID NO:12)
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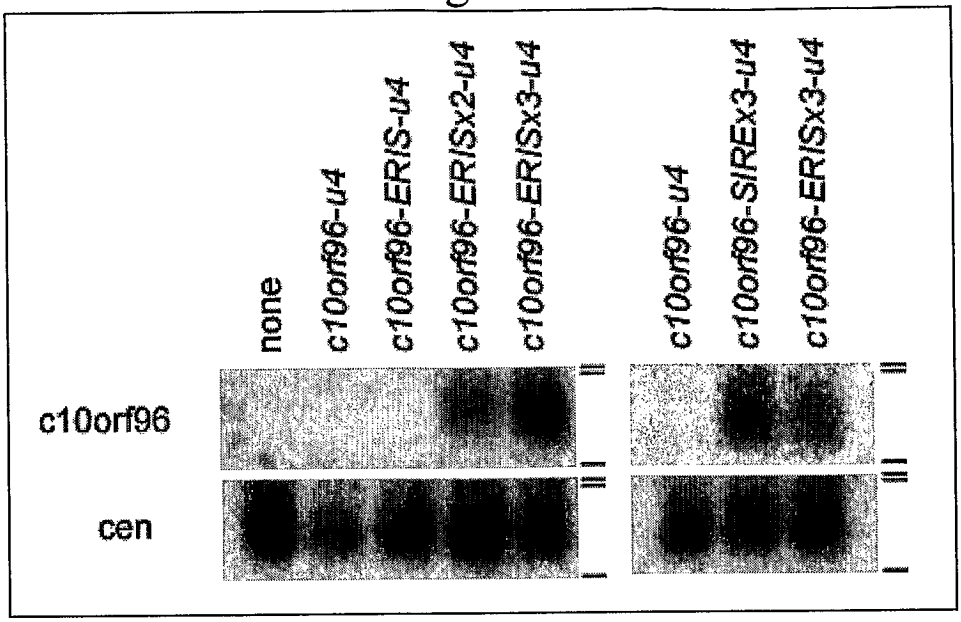
Fig. 8A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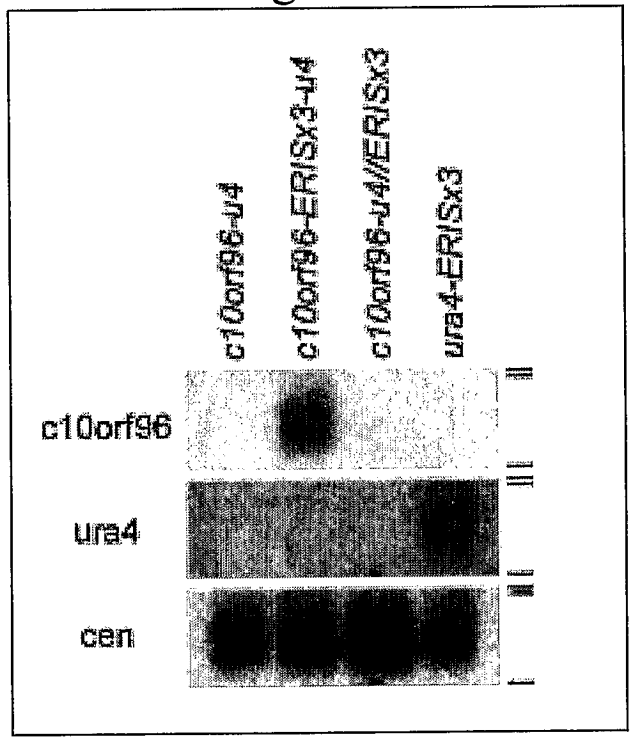
c10orf96
cen

Fig. 8B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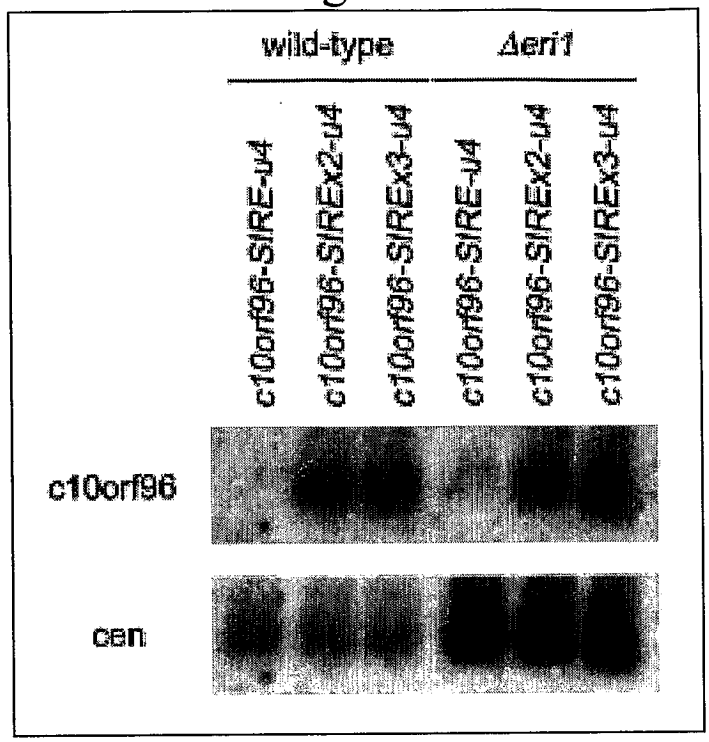
cen

Fig. 8C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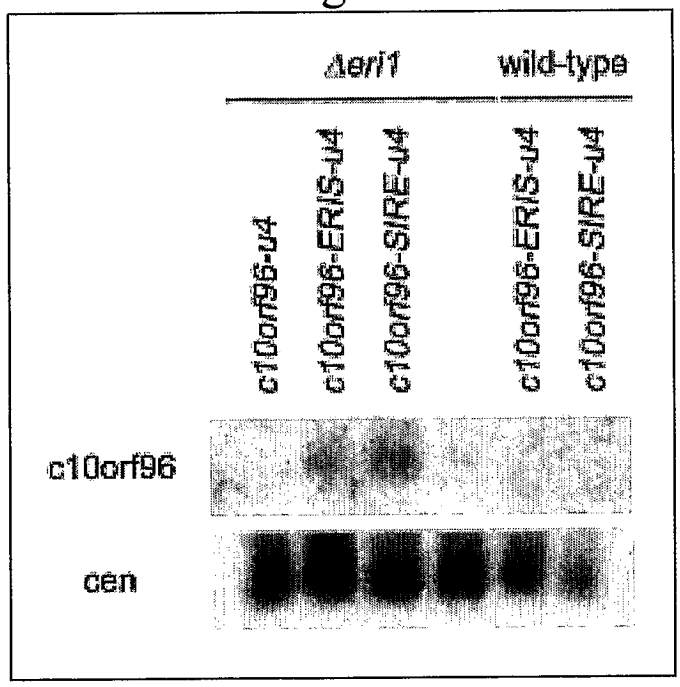
c10orf96-SIREx3-u4
c10orf96
cen

Fig. 8D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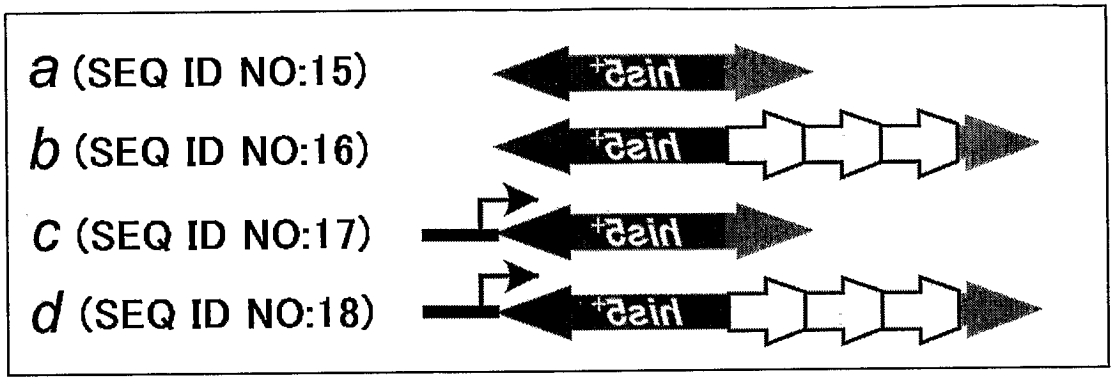
cen

Fig. 9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d (SEQ ID NO:18)
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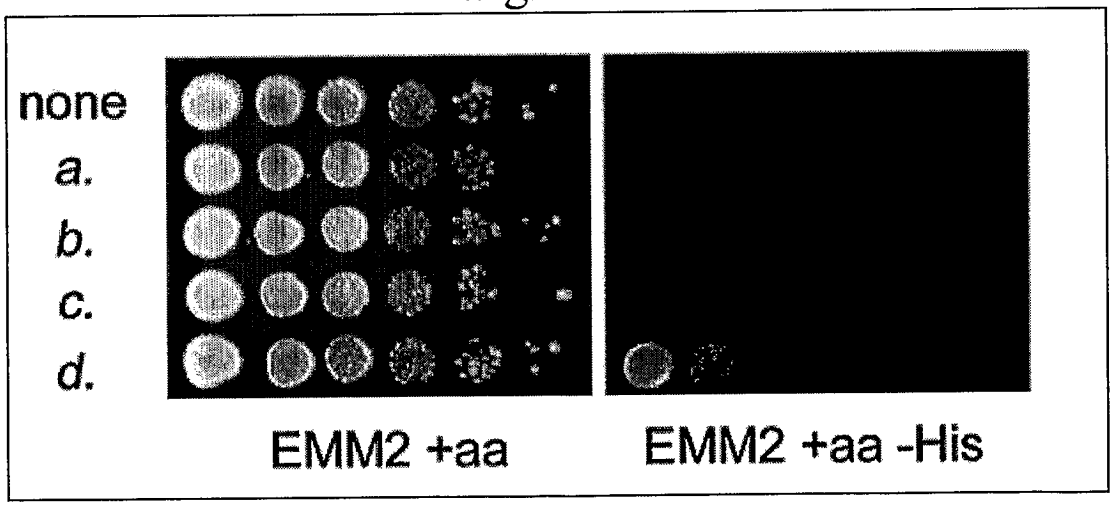
Fig. 10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d.
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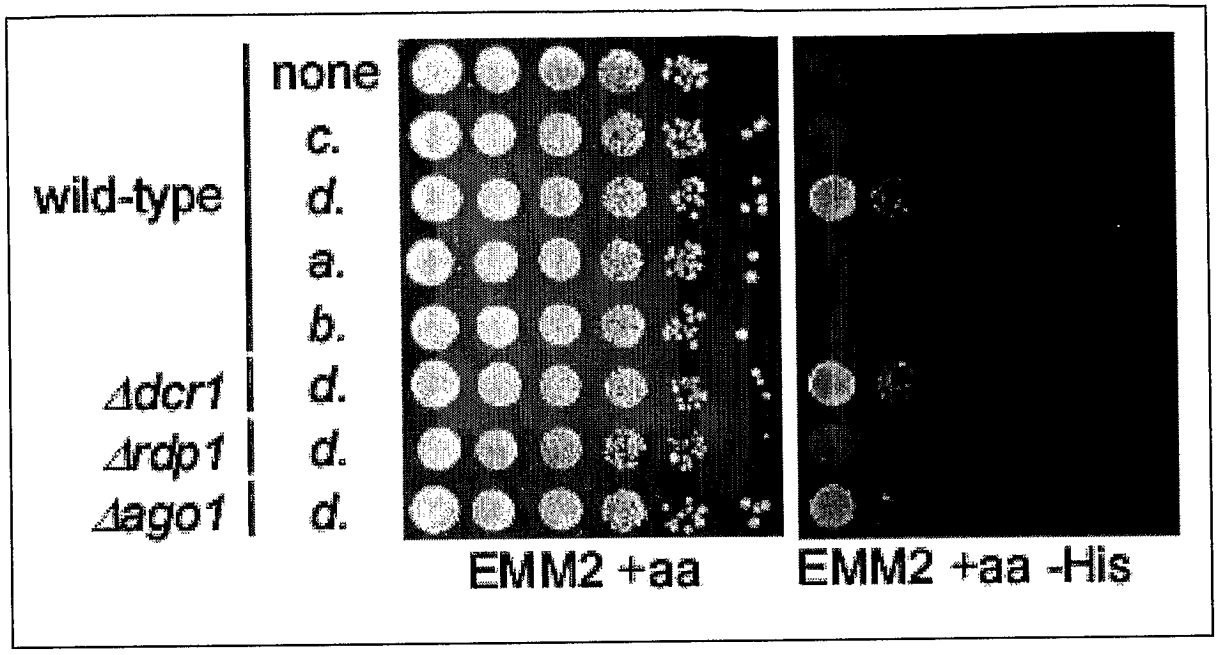
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c.
d.
a.
b.
Δdcr1
d.
Δrdp1
d.
Δago1
d.
EMM2 +aa
EMM2 +aa -His

Fig. 12

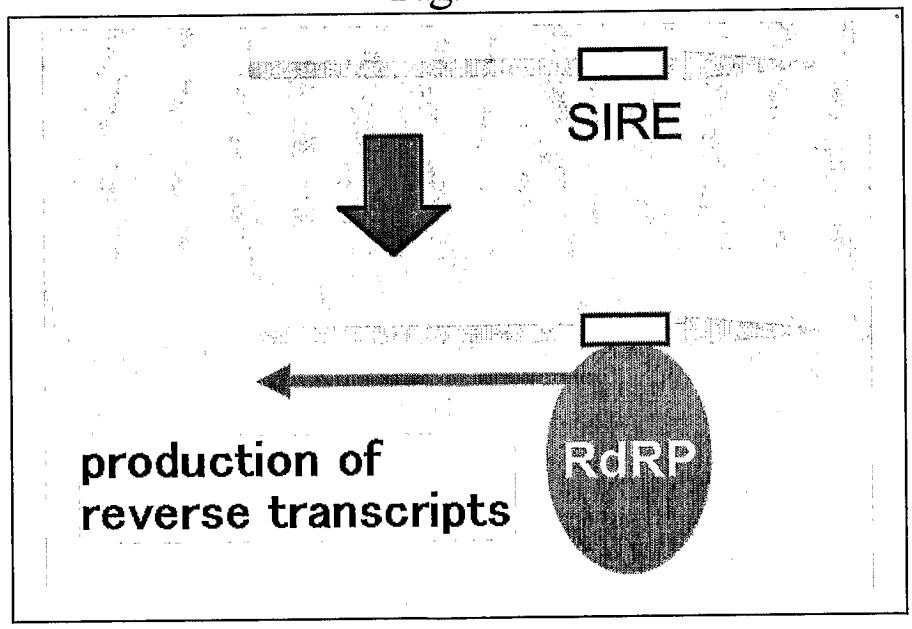
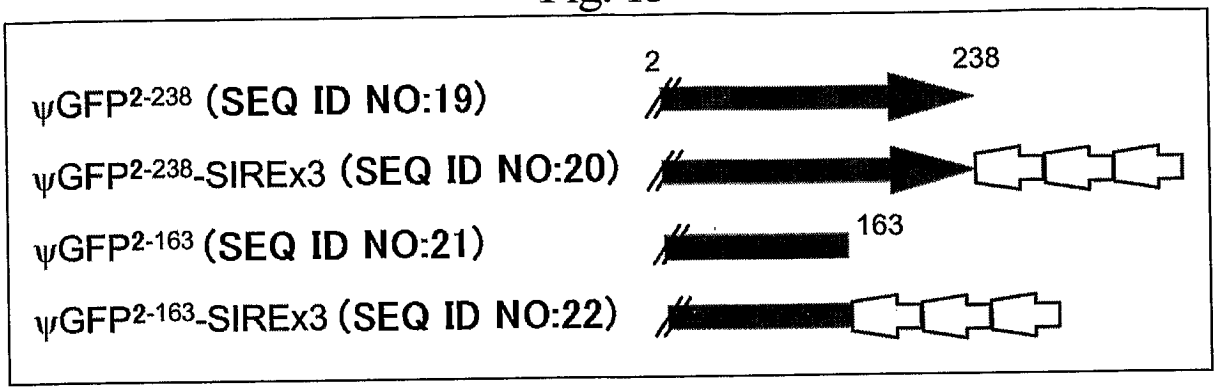

SIRE
RdRP
production of
reverse transcripts

Fig. 13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163

Fig. 14

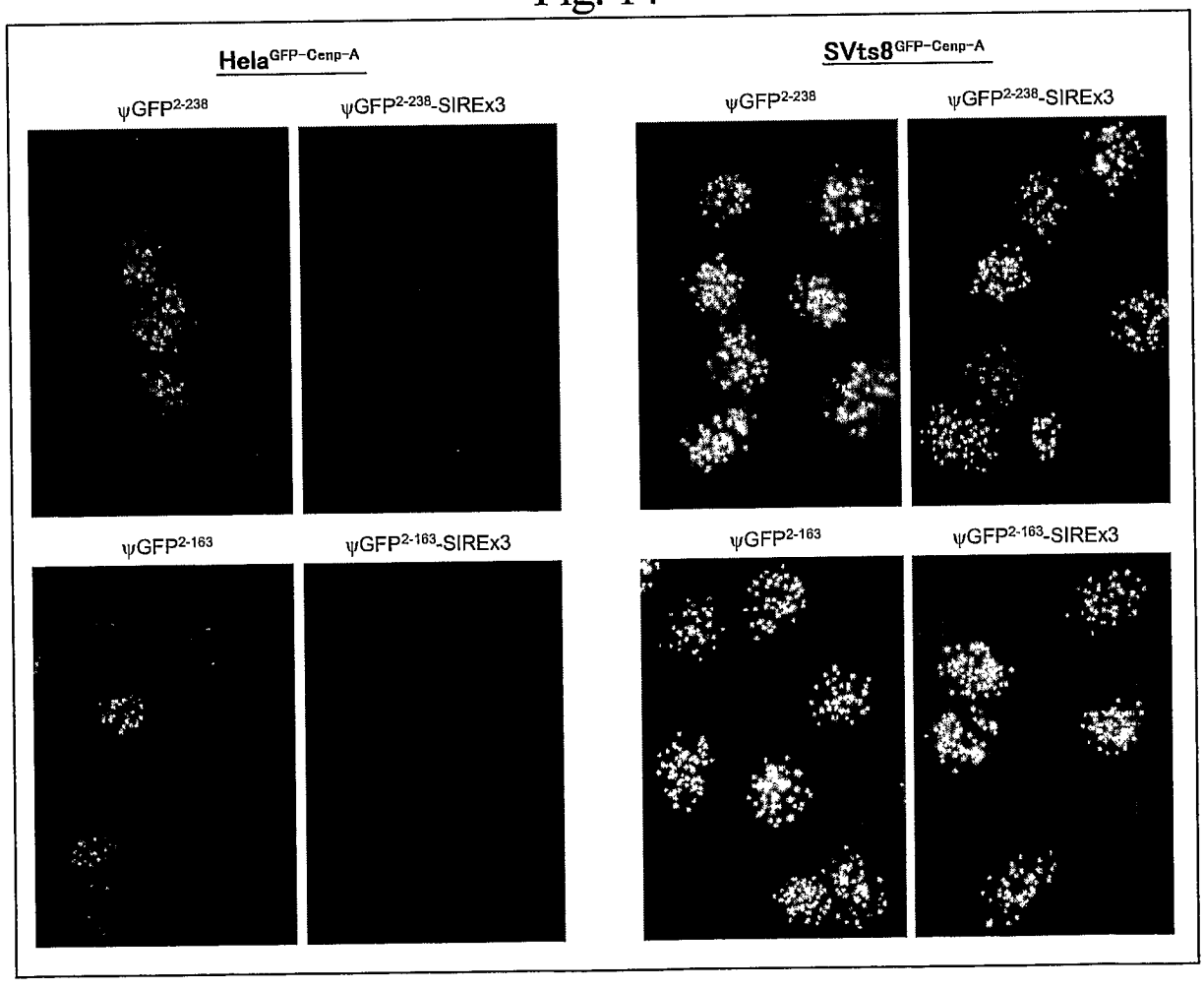
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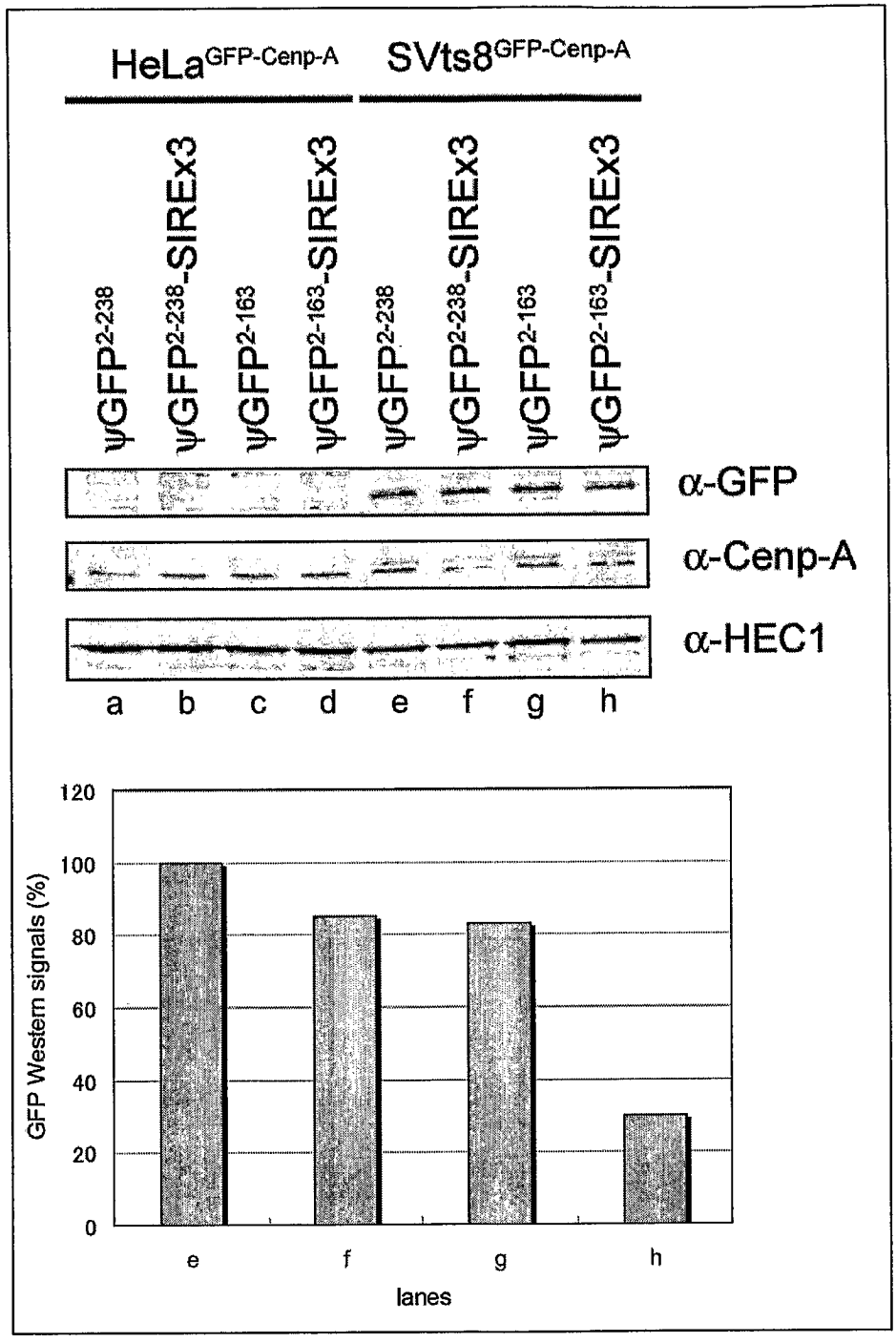
Fig. 15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ψGFP2-238
ψGFP2-238-SIREx3
ψGFP2-163
ψGFP2-163-SIREx3
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α-Cenp-A
α-HEC1
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GFP Western signals (%)
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100
80
60
40
20
0
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lanes

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Japan Science and Technology Agency

<120> RNA interference induction element and use thereof

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tgtacaaagc caatgaaaga tgtatgtaga tgaatgtaaa ata 1963

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<223> ura4ERISx3

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<211> 1487
<212> DNA
<213> Artificial

<220>
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<212> DNA
<213> Artificial

<220>
<223> c10orf96-ERIS-u4

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<212> DNA
<213> Artificial

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tcagagtctg cagttaatcc cgcggtctag tgattaactg cagctcaagt gactgcatta 2400

aagcataata ccgaagcact gacataacag agaggttcag aaaggaattt aaagattgac 2460

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<213> Artificial

<220>
<223> psi-GFP2-238

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ccatgcccga aggttatgta caggaaagaa ctatattttt caaagatgac gggaactaca 360

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caattggcga tggccctgtc cttttaccag acaaccatta cctgtccaca caatctgccc 660

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agacacgtgc tgaagtcaag tttgaaggtg ataccttgt taatagaatc gagttaaaag 420

gtattgattt taaagaagat ggaaacattc ttggacacaa attggaatac aactataact 480

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caattggcga tggccctgtc cttttaccag acaaccatta cctgtccaca caatctgccc 660

tttcgaaaga tcccaacgaa aagagagacc acatggtcct tcttgagttt gtaacagctg 720

ctgggattac acatggcatg gatgaactat acaaataggg cgcgctagac cgcgggatta 780

actgcagact ctgaagacaa cgatgtgttt tctcaagatg ataacggatc tagcttcgcc 840

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atttttgcag gacaaccagc ctgagcacaa gagacatggt gtactagact ttgtgcggaa 960

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agacacgtgc tgaagtcaag tttgaaggtg ataccttgt taatagaatc gagttaaaag 420

gtattgattt taaagaagat ggaaacattc ttggacacaa attggaatac aactataact 480

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cacacaatgt atacatcatg gcagacaaac aaaagaatgg aatcaaagtt ctagaccgcg 540

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