DESCRIPTION

GENE EXPRESSION SYSTEM USING STEALTHY RNA, AND GENE INTRODUCTION/EXPRESSION VECTOR INCLUDING SAID RNA

Technical Field

[0001]

The present invention relates to a vector for introducing and persistently expressing exogenous genes in animal cells.

Background Art

[0002]

The techniques of externally introducing any given gene into animal cells including human cells, and expressing the gene persistently in the cells are essential techniques in various industries utilizing biotechnologies. For example, industrial mass production of human monoclonal antibodies for use as pharmaceuticals requires the technique of persistently expressing genes of H-chain and L-chain of immunoglobulin at the same level. In gene therapy of congenital metabolic diseases, the technique of introducing a therapeutic gene into human tissue cells, and stably expressing the gene in the body for a long term is required.

[0003]

1. Regarding cell-reprogramming technology

Recently, a cell-reprogramming technology for producing useful cells by genetically converting the characteristic of

normal tissue cells attracts attention. The technique of introducing genes into an animal cell and persistently expressing the genes is also a base technology essential for cell-reprogramming. For example, it is possible to prepare human induced pluripotent stem cells (iPS cells) by introducing a combination of four genes, OCT4, SOX2, KLF4, and c-MYC, or OCT4, SOX2, NANOG, and LIN28 into human normal fibroblasts, and expressing the genes persistently for 21 days (Patent Document 1, Patent Document 2, Non-Patent Document 1, and Non-Patent Document 2). Also, it is possible to prepare hepatic cells by introducing three genes, FOXA3, HNF1A, and HNF4A into human fibroblasts and expressing the gene persistently for 14 days (Non-Patent Document 3). It is also reported that a dopaminergic neuron can be prepared by introducing five genes, ASCL1, BRN2, MYT1L, LMX1A, and FOXA2 into human fibroblasts, and expressing the gene persistently for 24 days (Non-Patent Document 4). Thus, in various cell-reprogramming, there is a need for a technique capable of simultaneously introducing and expressing plural genes into a cell, and keeping the expression for a period required for reprogramming.

[0004]

It is known that cell-reprogramming can be induced in vivo. For example, it has been reported that when three genes, GATA4, MEF2C, and TBX5, or four genes, GATA4, HAND2, MEF2C, and TBX5 are administered to an infarcted site in a mouse myocardial

infarction model, infiltrated fibroblasts transdifferentiate into cardiomyocytes (Non-Patent Document 5, and Non-Patent Document 6). Therefore, the cell-reprogramming technology is expected to become the basis of regenerative medicine for myocardial infarction, spinal cord injury and the like in future.

[0005]

2. Improvement in cell-reprogramming efficiency

Assuming that in vitro cell-reprogramming is used for medicine, it is desired that the material cells can be collected from a human body without invasion, and can be collected in the condition that they are not contaminated with microorganisms outside the living body. Cells that satisfy these requirements are almost limited to mononuclear cells in peripheral blood, and a gene introduction vector adapted to these cells is desired.

[0006]

In general, the efficiency with which animal cells are reprogrammed by externally introduced genes is very low, however, the efficiency can be raised by carrying all the genes on one vector, and introducing the genes into cells at once (Patent Document 3, Patent Document 4, Non-Patent Document 7, and Non-Patent Document 8).

Also it is known that the efficiency is raised by increasing the number of genes used in cell-reprogramming. For example, in the technique of converting mouse fibroblasts to induced pluripotent stem cells (iPS cells), it is known that the

efficiency of conversion rises five times by using a total of six genes by adding two genes, BRG1 and BAF155, to four genes, OCT4, SOX2, KLF4, and c-MYC (Non-Patent Document 9). Also, in the technique of reprogramming human fibroblasts into motor nerves, it is known that the efficiency of reprogramming rises 100 times by using a total of seven genes by adding three genes, HB9, ISL1, and NGN2 to four genes, LHX3, ASCL1, BRN2, and MYT1L (Non-Patent Document 10).

When the number of genes used in cell-reprogramming is increased, the size of genes that should be carried also increases. Illustrating preparation of iPS cells as an example, the total size of four genes, KLF4, OCT4, SOX2, and c-MYC is 4,774 base pairs, whereas the total size of genes after adding the two genes, BRG1 (5,040 base pairs) and BAF155 (3,318 base pairs) is 13,132 base pairs (Non-Patent Document 9). By adding CHD1 gene (5,133 base pairs) encoding a chromatin remodeling factor that is specifically expressed in embryonic stem cells and is expected to accelerate reprogramming of cells to iPS cells to four genes, KLF4, OCT4, SOX2, and c-MYC, the total size amounts to 9,907 base pairs, and by adding TET1 gene (6,429 base pairs) encoding a DNA demethylase to four genes, KLF4, OCT4, SOX2, and c-MYC, the total size amounts to 11,203 base pairs. The total size of the seven genes, LHX3, ASCL1, BRN2, MYT1L, HB9, ISL1, and NGN2 that are used in the technique of reprogramming human fibroblasts into motor nerves is 9,887 base pairs (Non-Patent

Document 10).

Thus, in order to raise the efficiency of the cell-reprogramming, it is desired to use at least six or more genes, and a vector capable of carrying all of these genes at once is desired. Also, desired is a vector capable of expressing introduced exogenous genes even when the total size of the genes is 5,000 or more nucleotides, desirably 8,000 or more nucleotides.

The term vector used herein refers to a recombinant viral or non-viral nucleic acid-macromolecular substance complex that is composed of nucleic acid including exogenous genes, and is capable of introducing the nucleic acid into animal cells and expressing the genes.

[0007]

It is known that in reprogramming of animal cells by expression of exogenous genes, the expression levels of the genes seriously affect the characteristics of the reprogrammed cells. For example, when four genes, OCT4, SOX2, KLF4, and c-MYC are expressed in mouse fibroblasts, it is known that iPS cells are generated when expression of the genes is weak, whereas cells having a totally different characteristic from iPS cells are generated when the expression of the genes is strong (Non-Patent Document 11). Thus, for the technique of reprogramming animal cells including human cells by expressing externally introduced genes, there is a need for a vector capable of setting the

expression of the genes at an optimum level depending on the purpose.

[0008]

3. Removal of genes for reprogramming

Further, in order to make the reprogrammed cells prepared by externally introducing genes completely exert their function, it is necessary to completely remove the reprogramming genes from the cells. Also, when the prepared human cells are used as a material for regenerative medicine, it is necessary to completely remove the genes from the cells for ensuring the safety. For example, in induced pluripotent stem cells (iPS cells) prepared by using four genes, OCT4, SOX2, KLF4, and c-MYC, the pluripotency cannot be functional in the condition that these four genes are expressed, and hence, it is necessary to at least completely suppress the expression of these genes, or preferably completely remove these genes from the cells (Patent Document 1, Patent Document 2, Patent Document 3, Non-Patent Document 1, Non-Patent Document 2, and Non-Patent Document 7). It is also known that if the c-MYC gene used in preparing the iPS cells is left in the iPS cells, the tissue cells that are prepared by differentiation of the iPS cells become tumorigenic with high frequency (Non-Patent Document 12). Therefore, it is necessary to completely remove the c-MYC gene from the iPS cells for ensuring the safety.

Thus, the gene expression technique required for

cell-reprogramming needs to have the mutually contradictory characteristics: persistent expression of genes at an optimum levels is desired for achieving the reprogramming, while it can be removed easily and completely once the reprogramming has completed.

[0009]

4. Importance of avoiding activation of innate immune system

Most of the gene introduction/expression vectors that are currently used in animal cells are constructed using an animal viruses or plasmid DNAs prepared from microorganisms such as Escherichia coli as materials. However, an animal cell has an innate immune system that eliminates invading pathogens from outside (Non-Patent Document 13), and nucleic acids derived from viruses or microorganisms introduced from outside the cell are recognized as foreign substances, and the innate immune system is activated. When the degree of activation of the innate immune system exceeds a certain level, cell death by the apoptosis is induced, and thus the efficiency of the reprogramming is deteriorated. When expression of interferon or inflammatory cytokines is induced by the activation of the innate immune system, inflammation is caused in the living body. In order to prevent such an undesired reaction, gene introduction/expression technique for cell-reprogramming is required to be capable of avoiding the activation of the innate immune system. This characteristic is important particularly in application to the

regenerative medicine including in vivo cell-reprogramming as described in the above section 1..

[0010]

5. Gene introduction/expression system for ideal cell-reprogramming

From the foregoing investigation, there is a need for a gene introduction/expression technique satisfying the following at least five requirements as discussed in the above sections 1. to 4. so as to further ameliorate the cell-reprogramming technique for animal cells including human cells by using genes for industrial application.

(1) Capability of efficiently introducing exogenous genes into animal cells including human peripheral blood cells.

(2) Capability of persistently expressing the genes for any required period.

(3) Capability of avoiding the innate immune system possessed by cells in expression of the genes.

(4) Capability of expressing the genes even if the total length of the introduced exogenous genes is 5,000 or more nucleotides, desirably 8,000 or more nucleotides.

(5) Capability of simultaneously expressing at least six, desirably eight or more genes.

Also, it is greatly desired to further achieve the following points.

(6) Capability of regulating the expression levels of the

genes. In particular, it is preferred that the expression level of each gene can be regulated individually when plural genes are introduced.

In applying gene-introduced cells, in particular, to transplantation techniques, the following point is also very important.

(7) Capability of removing the gene by a simple technique when the genes become unnecessary.

[0011]

6. Technique of introducing plural genes into animal cells

As a technique for introducing plural genes into animal cells including human cells from outside, and expressing the genes persistently in the cells, that has been reported to be applicable to cell-reprogramming, the following three techniques are known.

(1) Method of integrating the genes into nuclear genomic DNA.

(2) Method of carrying the genes on DNA capable of existing stably and independently from genomic DNA in a nucleus.

(3) Method of carrying the genes on RNA capable of existing in cytoplasm.

[0012]

6-1. Method for integrating plural genes into nuclear genomic DNA

In the method of integrating an exogenous gene into genomic DNA existing in a nucleus of cell by using a lentivirus vector

(Non-Patent Document 8, and Non-Patent Document 14), transposon (Non-Patent Document 15, and Non-Patent Document 16), non-homologous recombination, homologous recombination or the like, the gene can exist stably as with the genomic DNA. However, once the gene is integrated into the genomic DNA, complicated operations such as introducing a sequence specific recombinase into cells are required for selectively removing the gene from the genomic DNA, and the gene cannot be removed securely from every cell (Non-Patent Document 15). Further, since integration of exogenous genes into genomic DNA requires DNA replication of host cells, the efficiency of gene introduction into cells having poor proliferation potency such as blood cells is very low. Further, the phenomenon of "insertional mutagenesis" that random integration of exogenous gene into genomic DNA causes disruption or abnormal activation of genes of the host is known, and hence, there exists a concern about the safety for medical application (Non-Patent Document 17).

[0013]

6-2. Method for carrying plural genes on a DNA that is independent from genomic DNA in nucleus

As a method for carrying a exogenous gene on a DNA capable of existing stably in a nucleus of cell independently from genomic DNA, a method of using a circular DNA carrying a replication origin of genome of Epstein-Barr virus (Non-Patent Document 18), and a method of using an artificial chromosome containing a

straight-chain giant DNA (Non-Patent Document 19) are known. These DNA molecules continue replication and are kept stably in nuclei of human cells, and the mechanism of this relies on the mechanism with which genomic DNA of host cells is replicated. Therefore, it is impossible to specifically inhibit only replication of the DNA carrying exogenous genes, and a technique for actively removing the DNA from cells has not been reported. Additionally, since division of a host cell is required for introducing the DNA molecule into a cell nucleus, the efficiency of gene introduction into cells having poor proliferation potency such as blood cells is very low. Further, since it is known that circular DNA in a cell nucleus is frequently incorporated into genomic DNA of the cell, the risk of insertional mutagenesis cannot be eliminated (Non-Patent Document 20).

[0014]

6-3. Technique of expressing plural genes from single vector DNA

Further, as described in the above sections 6-1. and 6-2., when DNA is used as a platform for gene expression, a technique of expressing plural genes from the single vector DNA is required. As such a technique, the following three methods are known: 1) a method of simply linking plural independent genes, and expressing the genes, 2) a method of expressing plural proteins from one messenger RNA (mRNA) by using an RNA structure called Internal Ribosome Entry Site (IRES), and 3) a method of expressing

a fusion protein in which plural proteins are linked by 2A peptide.

[0015]

It is known that in the method of linking plural independent genes, expression of genes is strongly suppressed due to mutual interference between genes (Non-Patent Document 21). In order to prevent this, it is necessary to insert a structure called an insulator between genes, and the insertion increases the size of the vector DNA, and complicates the structure of the vector DNA. While the case of expressing four genes installed on one DNA molecule has been reported in this method (Non-Patent Document 22), the case of simultaneously expressing five or more genes has not been reported.

[0016]

In the method of expressing plural proteins from one messenger RNA (mRNA) by using IRES sequence, the translation efficiency of the protein positioned downstream IRES sequence is lower than, or sometimes 10% or less compared with the translation efficiency of the protein positioned upstream IRES sequence (Non-Patent Document 23). Additionally, since IRES sequence has a relatively large size and has a complicated structure, the method of using IRES sequence is mainly used for simultaneously expressing two proteins.

[0017]

2A peptide has a structure consisting of 18 to 22 amino acid residues found in a positive-sense single-stranded RNA virus,

and a fusion protein in which plural proteins are connected by 2A peptide are automatically cleaved at the time of synthesis and dissociated into the original plural proteins. In this technique, one proline residue is left at the N-terminus of each protein arising after cleavage, and 17 to 21 amino acid residues are left at the C-terminus, and these excess amino acid residues can influence on the function of the protein (Non-Patent Document 24). In addition, since the efficiency of cleavage at a 2A peptide site is largely influenced by the structure of the fusion protein, it is necessary to make trial and error requiring labors for preparing plural proteins efficiently (Non-Patent Document 25). In the method of connecting plural proteins by 2A peptide, the case of simultaneously expressing four proteins (Non-Patent Document 8) and the case of simultaneously expressing five proteins (Non-Patent Document 16) have been reported. Also the case of expressing four proteins by combining IRES sequence and 2A peptide has been reported (Non-Patent Document 14).

[0018]

6-4. Method for carrying plural genes on one RNA existing in cytoplasm

As described in the above sections 6-1. to 6-3., in the existing gene introduction/expression technique that uses DNA as a platform for gene expression, cell-reprogramming using four to five genes has been reported. However, as long as DNA is used as a platform for gene expression, it is not easy to

simultaneously carry six or more genes and to achieve removal of the genes in a convenient way, and a technique satisfying at least all the five requirements required for ideal reprogramming shown in the above section 5. has not been reported.

[0019]

Meanwhile, as a technique of cell-reprogramming by expressing plural genes that are externally introduced into animal cells including human cells using RNA as a platform, techniques of using a positive-sense RNA (Non-Patent Document 26, and Non-Patent Document 27), and techniques of using a negative-sense RNA (Patent Document 3, Patent Document 4, Patent Document 5, Patent Document 6, Non-Patent Document 7, Non-Patent Document 28, Non-Patent Document 29, and Non-Patent Document 30) have been reported.

[0020]

6-4-1. Method of using positive-sense RNA

As a technique of cell-reprogramming by using a positive-sense RNA capable of existing stably in cytoplasm, a technique of using a positive-sense single-stranded genomic RNA derived from Venezuelan equine encephalomyelitis virus (VEEV) (Non-Patent Document 26) has been reported. In this technique, expression of four proteins is realized by replacing a structural gene on 3′ side of genomic RNA of VEEV with genes encoding proteins that are linked by 2A peptide. This system induces extremely strong expression of interferon, and then combination with an

anti-interferon substance (B18R protein derived from vaccinia virus) is necessarily required (Non-Patent Document 26). The efficiency of gene introduction depends on the gene introducing reagent to be applied, and cells capable of being reprogrammed is limited to adhesive cells such as fibroblasts. An RNA carrying exogenous genes is unstable, and disappears by removing B18R protein from the culture medium.

[0021]

As a technique of cell-reprogramming by using a positive-sense RNA, a technique using a chemically synthesized messenger RNA (mRNA) (Non-Patent Document 27) has been reported. In this prior art, after mixing plural mRNAs separately carrying up to five exogenous genes, the plural mRNAs are introduced into cells by using a gene introducing reagent. Since the expressions of the genes are transient, it is necessary to newly introduce the genes into the cells every day. Also, the gene introduction is limited to adhesive cells such as fibroblasts. Also in this technique, since the innate immune system is activated strongly, it is necessary to combine an anti-interferon substance (B18R protein derived from vaccinia virus) (Non-Patent Document 27).

[0022]

6-4-2. Method of using negative-sense RNA

As a technique of cell-reprogramming using negative-sense RNAs, a method of using mixed vectors separately carrying an exogenous gene on a wild-type strain of Sendai virus which is

one species of paramyxoviruses(Patent Document 5, Non-Patent Document 28, and Non-Patent Document 29), and a method of using a vector carrying three genes simultaneously (Patent Document 6, and Non-Patent Document 30) have been reported as priorarts. In these gene expression systems using negative-sense RNA(s), autonomous replication ability of the wild-type virus is attenuated by deleting F gene, and exogenous genes are installed respectively as single gene expression cassettes. Although activation of the innate immune system was not mentioned, the vectors are expected to have ability to activate the innate immune system correspondingly because it has been known that Sendai virus which is a material has strong interferon inducibility (Non-Patent Document 31). Also it has been reported that the vector can be removed by introducing a temperature sensitive mutation into genome of the wild-type virus, and thus increasing the cultivation temperature (Patent Document 6, Non-Patent Document 29, and Non-Patent Document 30). The size of gene that can be expressed by a vector based on wild-type Sendai virus has been reported to be from 3078 base pairs (beta galactosidase from Escherichia coli) (Non-Patent Document 32) to 3450 base pairs (sum of three genes, KLF4, OCT4, and SOX2) (Patent Document 6, Non-Patent Document 30).

[0023]

As a technique of reprogramming cells by using a negative-sense RNA, a technique based on a mutant Sendai virus

capable of persistent infection has been reported (Patent Document 3, Patent Document 4, and Non-Patent Document 7). In this technique, plural point mutations responsible for long-term persistence are identified in genome of the virus which is a material of the vector, and it is indicated that these mutations are involved in avoidance of activation of the innate immune system (deterioration in interferon expression). Also by deleting three genes from virus genome, and carrying new genes, it is possible to express four exogenous genes simultaneously. Further, it has been reported that vectors are actively removed from cells by suppressing expression of L gene that encodes an RNA-dependent RNA polymerase by short interfering RNA (siRNA). It has been reported that the size of gene that can be expressed with the use of a vector based on a mutant Sendai virus capable of persistent infection is 4774 base pairs (sum of four genes, KLF4, OCT4, SOX2, and c-MYC) (Patent Document 3, Patent Document 4,and Non-Patent Document 7).

[0024]

7. Future challenge in plural gene introducing techniques

In existent gene introduction/expression techniques using RNA as a platform for gene expression as described in the above section 6-4., cell-reprogramming using four to five genes has been reported. Among these techniques, a defective and persistent expression Sendai virus vector described in the above section 6-4-2. has the most excellent characteristic, however,

the number of genes that can be installed on the vector has been reported to be at most four. In the technique using an RNA virus as a material, it is difficult to alter the level of gene expression.

[0025]

As shown in the above section 6., the technique of externally introducing plural genes into animal cells including human cells and persistently expressing the genes in the cells has been variously modified toward optimization for cell-reprogramming that converts the characteristics of normal tissue cells using genes, and produces useful cells. However, a technique satisfying all the five requirements required for ideal reprogramming shown in the above section 5. has not been reported heretofore.

Citation List

Patent Document

[0026]

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Patent Document 2: WO 2008/118820

Patent Document 3: WO 2010/134526

Patent Document 4: WO 2012/063817

Patent Document 5: WO 2010/008054

Patent Document 6: WO 2012/029770

Patent Document 7: US 8,326,547

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Patent Document 9: US 7,561,973

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[0027]

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Summary of the Invention

Problems to be Solved by the Invention

[0028]

As described above, a problem to be solved by the present invention is developing a gene introduction/expression technique desired for reprogramming animal cells including human cells by the use of genes, and a vector for the technique. It

is also an object of the present invention to provide a vector capable of carrying a total length of 5,000 or more nucleotides or at least six or more exogenous genes besides reprogramming genes, and capable of persistently expressing the genes without activating an innate immune system in animal cells. Also provided is an efficient technique for carrying six or more exogenous genes on a vector.

Also provided is a gene introduction/expression technique satisfying the requirements (1) to (5) that are desired especially for reprogramming technology, preferably satisfying the requirements further including the requirements (6) and (7).

(1) Capability of efficiently introducing exogenous genes into animal cells including human peripheral blood cells.

(2) Capability of persistently expressing the genes for any required period.

(3) Capability of avoiding the innate immune system possessed by cells in expression of the genes.

(4) Capability of expressing the genes even if the total length of the introduced exogenous genes is 5,000 or more nucleotides, desirably 8,000 or more nucleotides.

(5) Capability of simultaneously expressing at least six, desirably eight or more genes.

(6) Capability of regulating the levels of the expression of the genes. In particular, when plural genes are introduced, the expression level can be regulated individually.

In applying, in particular, to transplantation techniques, the following point is also important.

(7) Capability of removing the gene expression system by a simple technique when the genes are no longer necessary.

Means for Solving the Problems

[0029]

As described in the sections 6-1. to 6-3. of the background art, when DNA is used as a platform for gene expression, it is theoretically very difficult to satisfy all the five requirements, more preferably all the seven requirements required for ideal reprogramming shown as the "Problems to be Solved by the Invention". On the other hand, as described in the section 6-4., when RNA is used as a platform for gene expression, it becomes the primary issue how to avoid the problem of activation of the intracellular innate immune system caused by a virus-derived RNA while increasing the number of genes that can be installed on the single vector to six or more, and the total length of genes to 5,000 or more nucleotides.

[0030]

Thus, in the present invention, first, using mRNA fragments derived from animal cells that does not activate an innate immune system as materials, a negative-sense single-stranded RNA in which the RNA fragments are combined with transcription start signals, transcription termination signals, and a replication

origin that are recognized by an RNA-dependent RNA polymerase was designed. Then in the negative-sense single-stranded RNA, genes encoding four proteins required for transcription and replication such as an RNA-dependent RNA polymerase were installed after the structures thereof were optimized so as not to be recognized as foreign substances by the innate immune system. Further, the present inventors developed a novel method of binding ten genes as designed by using five restriction endonucleases, and ten cRNAs complementary to these ten genes were bound and then installed on the negative-sense single-stranded RNA.

The present inventors succeeded in carrying at least ten exogenous genes (a total size of at least 13.5 kilo nucleotides) and expressing them persistently for a long term without activating the innate immune system by using the negative-sense single-stranded RNA completed by the above method as a platform for gene expression. Further, the present inventors made the levels of expression of the installed genes regulatable within the range of up to 80 times by modifying the expression efficiency of N protein or C protein required for gene expression. Thus, by eliminating the RNA elements having a structure derived from virus as much as possible, the present inventors succeeded in preparing a novel gene expression system greatly beyond the limit of the capability of the conventional gene expression system using genome of RNA virus.

[0031]

Further, by expressing an envelope protein and a matrix protein of paramyxovirus in cells transfected with the negative-sense single-stranded RNA carrying exogenous genes, prepared in the present invention, according to the method described in Patent Document 3, Non-Patent Document 33, and Non-Patent Document 7, a particle that encapsulates the RNA molecule, and has activity of introducing the RNA molecule into another cell was prepared. This particle could persistently express ten genes installed on the RNA molecule while keeping activation of the innate immune system low in various animal cells including human blood cells. Further, by introducing siRNA that is complementary to the gene of the RNA-dependent RNA polymerase installed on the RNA molecule and of which structure has been optimized, into the cells, the RNA molecule carrying exogenous genes could be eliminated. In the manner as described above, the present inventors confirmed that all the seven requirements including the five requirements (1) to (5) shown in the "Problems to be Solved by the Invention" and the requirements (6) and (7) in the aforementioned preferable case could be satisfied, and accomplished the present invention.

[0032]

Since the RNA molecule used in the present invention lacks a specific structure required for the innate immune system to recognize as "pathogen-associated molecular pattern, PAMP", the

RNA molecule is difficult to be captured by the innate immune system, namely it is "stealthy". Therefore, hereinafter, the RNA molecule is referred to as "stealthy RNA", the gene expression system using the RNA as a material is referred to as "stealth RNA gene expression system", the construct including the gene expression system and having the activity of introducing the gene expression system into animal cells is referred to as "stealth RNA vector".

[0033]

In other words, the present invention can be described as follows:

[1] A stealth RNA gene expression system comprising:

a negative-sense single-stranded RNA (A) having RNA sequences (1) to (8) below,

a single-stranded RNA binding protein (B), and

an RNA-dependent RNA polymerase (C),

wherein the stealth RNA gene expression system is a complex that does not activate an innate immune system:

(1) target RNA sequences encoding any given protein or functional RNA,

(2) RNA sequences constituting noncoding region(s) and derived from mRNA(s) expressed in animal cells,

(3) transcription start signal sequences recognized by the RNA-dependent RNA polymerase,

(4) transcription termination signal sequences recognized

by the polymerase enzyme,

(5) RNA sequences containing replication origins recognized by the polymerase enzyme,

(6) RNA sequences encoding the polymerase enzyme with codons optimized for a biological species from which cells for transfection are derived,

(7) an RNA sequence encoding a protein that regulates activity of the polymerase enzyme with codons optimized for a biological species from which cells for transfection are derived, and

(8) an RNA sequence encoding the single-stranded RNA binding protein with codons optimized for a biological species from which cells for transfection are derived.

Here, since typical cells for transfection are human cells, the preferred cases can be described as follows.

[1'] A stealth RNA gene expression system comprising:

a negative-sense single-stranded RNA (A) having RNA sequences (1) to (8) below,

a single-stranded RNA binding protein (B), and

an RNA-dependent RNA polymerase (C),

wherein the stealth RNA gene expression system is a complex that does not activate an innate immune system:

(1) target RNA sequences encoding any given protein or functional RNA,

(2) human mRNA-derived RNA sequences constituting noncoding

region(s),

(3) transcription start signal sequences recognized by the RNA-dependent RNA polymerase,

(4) transcription termination signal sequences recognized by the polymerase enzyme,

(5) RNA sequences containing replication origins recognized by the polymerase enzyme,

(6) RNA sequences encoding the polymerase enzyme with codons optimized for human cells,

(7) an RNA sequence encoding a protein that regulates activity of the polymerase enzyme with codons optimized for human cells, and

(8) an RNA sequence encoding the single-stranded RNA binding protein with codons optimized for human cells.

[2] The stealth RNA gene expression system according to the [1], wherein the target RNA sequences of the (1) contain at least six genes, or are RNA sequences having a total length of 5000 or more nucleotides.

Here, the target RNA sequences can contain seven to ten genes, or are RNA sequences having a total length of 5,000 to 15,000 nucleotides.

[3] The stealth RNA gene expression system according to the [1] or [2], wherein the RNA sequences of the (2) are derived from mRNA of human gene(s) and each of the RNA sequences is having a length of 5 to 49 nucleotides.

Here, as the mRNA sequence of a human gene, preferably a mRNA sequence of a human House-keeping gene, more preferably a noncoding region sequence in a mRNA sequence of a human House-keeping gene, for example, an RNA sequence described in (Table 1), or a partial sequence having a length of consecutive 5 to 49 nucleotides thereof, or a plurality of these sequences linked to each other can be used.

[4] The stealth RNA gene expression system according to any one of the [1] to [3], wherein each of the RNA sequences of the (2) having sequences identical to or different from one another is placed adjacent to 3′ terminal site and/or 5′ terminal site of each of gene sequences contained in the target RNA sequences of the (1).

[5] The stealth RNA gene expression system according to any one of the [1] to [4], wherein

the RNA-dependent RNA polymerase encoded by the RNA sequences of the (6) consists of L protein and P protein derived from an RNA virus belonging to a paramyxovirus family,

the protein that regulates activity of the polymerase enzyme encoded by the RNA sequence of the (7) is C protein derived from the same virus as the RNA virus,

the single-stranded RNA binding protein encoded by the RNA sequence of the (8) is NP protein derived from the same virus as the RNA virus, and

all of the RNA sequences of the (3) to (5) are RNA sequences

containing a transcription start signal, a transcription termination signal, or a replication origin sequence derived from a genome of the same virus as the RNA virus.

[6] The stealth RNA gene expression system according to the [5], wherein the RNA sequences encoding the L protein, P protein, C protein and NP protein are optimized for human cells, and have a GC content adjusted within a range of 50 to 60%.

[7] The stealth RNA gene expression system according to the [6], wherein the RNA virus belonging to a paramyxovirus family is an RNA virus selected from the group consisting of Sendai virus, human parainfluenza virus, and Newcastle disease virus.

[8] The stealth RNA gene expression system according to any one of the [1] to [7], wherein the transcription start signal sequences of the (3) are RNA sequences selected from the group of RNA sequences consisting of 3'-UCCCACUUUC-5' (SEQ ID NO: 1), 3'-UCCCUAUUUC-5' (SEQ ID NO: 2), 3'-UCCCACUUAC-5' (SEQ ID NO: 3), 3'-UCCUAAUUUC-5' (SEQ ID NO: 7), and 3'-UGCCCAUCUUC-5' (SEQ ID NO: 9), and the transcription termination signal sequences of the (4) are RNA sequences selected from the group of RNA sequences consisting of 3'-AAUUCUUUUU-5' (SEQ ID NO: 4), 3'-CAUUCUUUUU-5' (SEQ ID NO: 5), 3'-UAUUCUUUUU-5' (SEQ ID NO: 6), and 3'-UUAUUCUUUUU-5' (SEQ ID NO: 8).

[9] The stealth RNA gene expression system according to any one of the [4] to [8], wherein each of the transcription start signal sequences of the (3) having sequences identical to or

different from one another is placed adjacent to 3′ terminal site of each of the RNA sequences of the (2) that is placed adjacent to 3′ terminal site of each of gene sequences contained in the target RNA sequences of the (1), and each of the transcription termination signal sequences of the (4) is placed adjacent to 5′ terminal site of the RNA sequence that is placed adjacent to 5′ terminal site of each of gene sequences contained in the target RNA sequences of the (1).

[10] The stealth RNA gene expression system according to any one of the [7] to [9], wherein the RNA sequences containing a replication origins of the (5) contain the following sequences:

(a) an RNA sequence represented by 3′-UGGUCUGUUCUC-5′ (SEQ ID NO: 11) or 3′-UGGUUUGUUCUC-5′ (SEQ ID NO: 12),

(b) an RNA sequence represented by 3′-GAGAACAGACCA-5′ (SEQ ID NO: 13) or 3′-GAGAACAAACCA-5′ (SEQ ID NO: 14),

(c) an RNA sequence represented by $3'-(CNNNNN)_3-5'$ (SEQ ID NO: 15), and

(d) an RNA sequence represented by $3'-(NNNNNG)_3-5'$ (SEQ ID NO: 16).

[11] The stealth RNA gene expression system according to the [10], wherein the RNA sequence of the (a) is positioned at the 3′ terminus of the negative-sense single-stranded RNA (A), and the RNA sequence of the (b) is positioned at the 5′ terminus.

[12] The stealth RNA gene expression system according to the [10] or [11], wherein the RNA sequence of the (c) starts

at 79th nucleotide from the 3′ terminus of the negative-sense single-stranded RNA (A), and the RNA sequence of the (d) starts at 96th nucleotide from the 5′ terminus.

[13] The stealth RNA gene expression system according to any one of the [10] to [12], wherein the RNA sequences containing replication origins of the (5) further contain in a position of 97th to 116th nucleotides from the 3′ terminus of the negative-sense single-stranded RNA (A), an RNA sequence of (e) 3′-AAAGAAACGACGGUUUCA-5′ (SEQ ID NO: 17) or an RNA sequence having the same length of 18 nucleotides as the (e).

[14] A stealth RNA vector including a complex composed of the stealth RNA gene expression system according to any one of the [1] to [13], and having activity of introducing the complex into animal cells, that does not activate an innate immune system.

[15] The stealth RNA vector according to the [14], that forms a virus particle having ability to infect animal cells.

[16] An animal cell transfected with the stealth RNA vector according to the [14] or [15].

[17] A stealth RNA which is a negative-sense single-stranded RNA (A) having RNA sequences of (1) to (8) below, capable of forming a complex that does not activate an innate immune system together with a single-stranded RNA binding protein (B), and an RNA-dependent RNA polymerase (C):

(1) target RNA sequences encoding any given protein or functional RNA,

(2) RNA sequences constituting noncoding region(s) that is unrecognizable by an innate immune system,

(3) transcription start signal sequences recognized by an RNA-dependent RNA polymerase,

(4) transcription termination signal sequences recognized by the polymerase enzyme,

(5) RNA sequences containing replication origins recognized by the polymerase enzyme,

(6) RNA sequences encoding the polymerase enzyme and having a structure optimized to be unrecognizable by an innate immune system,

(7) an RNA sequence encoding a protein that regulates activity of the polymerase enzyme, and having a structure optimized to be unrecognizable by an innate immune system, and

(8) an RNA sequence encoding a single-stranded RNA binding protein and having a structure optimized to be unrecognizable by an innate immune system.

The present invention also includes the following modes.

[17'] A stealth RNA which is a negative-sense single-stranded RNA (A) having RNA sequences of (1) to (8) below, capable of forming a complex that does not activate an innate immune system together with a single-stranded RNA binding protein (B), and an RNA-dependent RNA polymerase (C):

(1) target RNA sequences encoding any given protein or functional RNA,

(2) RNA sequences constituting noncoding region(s) and derived from mRNA(s) expressed in animal cells,

(3) a transcription start signal sequence recognized by the RNA-dependent RNA polymerase,

(4) transcription termination signal sequences recognized by the polymerase enzyme,

(5) RNA sequences containing replication origins recognized by the polymerase enzyme,

(6) an RNA sequence encoding the polymerase enzyme with codons optimized for a biological species from which cells for transfection are derived,

(7) an RNA sequence encoding a protein that regulates activity of the polymerase enzyme with codons optimized for a biological species from which cells for transfection are derived, and

(8) an RNA sequence encoding the single-stranded RNA binding protein with codons optimized for a biological species from which cells for transfection are derived.

[17''] A stealth RNA which is a negative-sense single-stranded RNA (A) having RNA sequences of (1) to (8) below, capable of forming a complex that does not activate an innate immune system together with a single-stranded RNA binding protein (B), and an RNA-dependent RNA polymerase (C):

(1) target RNA sequences encoding any given protein or functional RNA,

(2) human mRNA-derived RNA sequences constituting noncoding region(s),

(3) transcription start signal sequences recognized by the RNA-dependent RNA polymerase,

(4) transcription termination signal sequences recognized by the polymerase enzyme,

(5) RNA sequences containing replication origins recognized by the polymerase enzyme,

(6) RNA sequences encoding the polymerase enzyme with codons optimized for human cells,

(7) an RNA sequence encoding a protein that regulates activity of the polymerase enzyme with codons optimized for human cells, and

(8) an RNA sequence encoding the single-stranded RNA binding protein with codons optimized for human cells.

[18] The stealth RNA according to the [17], wherein RNA sequences containing replication origins recognized by the RNA-dependent RNA polymerase of the (5) are located at the 3′ terminal site and the 5′ terminal site of the negative-sense single-stranded RNA (A), and the RNA sequence located at the 3' terminal site and the RNA sequence located at the 5' terminal site include RNA sequences complementary to each other.

[19] The stealth RNA according to the [17] or [18], wherein each of the transcription start signal sequences of the (3) having sequences identical to or different from one another is placed

adjacent to 3′ terminal site of each of the RNA sequences of the (2) that is placed adjacent to 3′ terminal site of each of plural gene sequences contained in the target RNA sequences of the (1), and each of the transcription termination signal sequences of the (4) is placed adjacent to 5′ terminal site of the RNA sequence that is placed adjacent to 5′ terminal site of each of plural gene sequences contained in the target RNA sequences of the (1).

[20] The stealth RNA according to any one of the [17] to [19], wherein each of the transcription start signal sequences of the (3) having sequences identical to or different from on another is placed adjacent to 3′ terminal site of each of the RNA sequences of the (2) that is placed adjacent to 3′ terminal site of each of plural gene sequences contained in the target RNA sequences of the (1); each of the transcription termination signal sequences of (4) is placed adjacent to 5′ terminal site of the RNA sequence that is placed adjacent to 5′ terminal site of each of plural gene sequences contained in the target RNA sequences of the (1); and both of them constitute a cassette structure together with restriction sites located at both ends of the cassette that can be cleaved by plural restriction endonucleases, and plural cassette structures are bound to each other.

[21] A method for reconstituting a stealth RNA gene expression system, comprising the following processes (1) to

(5):

(1) preparing an Escherichia coli expressing T7 RNA polymerase;

(2) introducing into the Escherichia coli host of the (1), at least a vector for Escherichia coli carrying an RNA encoding an RNA-dependent RNA polymerase and an RNA binding protein, and a vector for Escherichia coli for expressing a DNA encoding RNA binding protein, together with the negative-sense single-stranded RNA (A) according to any one of the [1] to [13] to transform the host,

(3) forming a complex of the negative-sense single-stranded RNA containing exogenous gene RNA expressed by T7 RNA polymerase, and RNA binding protein in the transformed Escherichia coli of the (2),

(4) preparing animal cells in which an RNA-dependent RNA polymerase is expressed, and

(5) introducing the complex of the negative-sense single-stranded RNA and the RNA binding protein obtained in the (3) into an animal cell host of the (4) to reconstitute a stealth RNA gene expression system composed of the negative-sense single-stranded RNA, and the complex of the RNA binding protein and the RNA-dependent RNA polymerase.

[22] A DNA-based tandem cassette having two cloning sites A and B,

the tandem cassette being composed of (1) multimerization

site A, (2) transcription start signal A, (3) noncoding sequence A1, (4) cloning site A, (5) noncoding region A2, (6) transcription termination signal A, (7) transcription start signal B, (8) noncoding sequence B1, (9) cloning site B, (10) noncoding region B2, (11) transcription termination signal B, and (12) multimerization site B in order from the 5′ terminus,

the multimerization site A of the (1), and multimerization site B of the 812) being DNAs that are identical to or different from each other and each containing a recognition site by restriction endonuclease and/or a recognition site by site-specific recombinase,

the transcription start signal A of the (2), and transcription start signal B of the (7) being DNAs that are identical to or different from each other and each containing a transcription start signal recognized by the RNA-dependent RNA polymerase when transcribed to RNA,

the noncoding sequence A1 of the (3), noncoding region A2 of (5), noncoding sequence B1 of the (8), and noncoding region B2 of the (10) being DNAs that are identical to or different from one another and each becoming RNA that is not recognized by an innate immune system of a host cell when transcribed to RNA,

the cloning site A of the (4), and cloning site B of the (9) being DNAs that are identical to or different from each other and each containing one or more recognition site by restriction

endonuclease and/or recognition site by site-specific recombinase,

the transcription termination signal A of the (6), and transcription termination signal B of the (11) being DNAs that are identical to or different from each other and each containing a transcription termination signal recognized by the RNA-dependent RNA polymerase when transcribed to RNA.

[23] The tandem cassette according to the [22], wherein

the cloning site A of the (4) contains a recognition site by restriction endonuclease A, and a recognition site by restriction endonuclease C in order from 5′ terminal side, and

the cloning site B of the (9) contains a recognition site by restriction endonuclease D, and a recognition site by restriction endonuclease B in order from 5′ terminal side,

provided that the restriction endonuclease A and the restriction endonuclease D give single-stranded protruding ends of the same sequence, and the restriction endonuclease C and the restriction endonuclease B give single-stranded protruding ends of the same sequence.

[24] The tandem cassette according to the [22] or [23], wherein both of the multimerization site A of the (1), and multimerization site B of the (12) are DNAs containing a recognition site by a restriction endonuclease giving a single-stranded protruding end of any sequence represented by NN or NNN.

[25] The tandem cassette according to any one of the [22] to [24], wherein the noncoding sequence A1 of the (3), noncoding region A2 of the (5), noncoding sequence B1 of the (8), and noncoding region B2 of the (10) are identical to or different from one another and each of them is cDNA corresponding to a partial sequence of RNA sequence derived from mRNA expressed in animal cells, and

one of human-derived genes identical to or different from each other is inserted into the cloning site A of the (4), and cloning site B of the (9).

Effects of the Invention

[0034]

Since the stealth RNA gene expression system of the present invention is difficult to be captured by the innate immune system, it has a very low cytotoxicity, and is capable of carrying ten genes and introducing them into various tissue cells, and expressing them persistently for any required period. The wording of "capable of avoiding an innate immune system" or "not recognized by an immune system" used herein means that the introduced gene or the vector or the like used for introduction does not substantially stimulate the innate immunity of the host. Specifically, it means that the interferon β inducibility as an index is 30 or less, preferably 20 or less, more preferably 10 or less, when the expression amount of IFN-β mRNA in normal

cells is 1.0.

Also, since the stealth RNA gene expression system functions in cytoplasm, by using the stealth RNA vector including the gene expression system, it is possible to introduce and express the installed genes into cells of peripheral blood not having proliferating ability and have not undergone cell division. Furthermore, a gene expression system with various expression intensity within a maximum of 80 times can be selected, and easy removal is allowed by suppressing activity of the RNA-dependent RNA polymerase if no longer necessary. Therefore, this technique is suited for the object of efficiently reprogramming characteristics of animal cells including human cells by using six or more genes, that has been impossible heretofore.

For example, application of efficiently preparing iPS cells having high quality for clinical use in regenerative medicine under such a severe condition not containing animal derived components (Xeno-free) and not using feeder cells (Feeder-free) using human peripheral blood cells as a material can be conceived. Also, application to the technology called direct reprogramming for creating useful cells such as nerve cells, neural stem cells, stem cells, pancreatic beta cells and the like from human tissue cells (blood, skin, placenta, etc.) using six or more genes is enabled. Further, since the possibility of causing cell death or inflammation is low, application to gene therapy by various genes including giant genes, and application to regenerative

medicine by in vivo reprogramming are expected.

[0035]

Since the stealth RNA gene expression system can carry plural genes simultaneously, and express them in a certain ratio, it is also effective in production of biopharmaceuticals made up of plural subunits. For example, in production of human immunoglobulin G, it is necessary that each subunit is expressed simultaneously in the same cell. It is also required that H-chain and L-chain are expressed simultaneously in a ratio of 1 : 1 in the same cell in production of human immunoglobulin G, and H-chain, L-chain, and μ-chain are expressed simultaneously in a ratio of 1 : 1 : 0.2 in the same cell in production of human immunoglobulin M. The stealth RNA gene expression system can easily satisfy such a requirement.

Further, since the level of gene expression can be varied in the stealth RNA gene expression system, strong gene expression required for production of biopharmaceuticals can be easily realized. Conventional manufacturing process of biopharmaceuticals using animal cells requires the process of establishing a stable cell strain in which the number of copies of the gene integrated into chromosome is amplified, which requires large amounts of time and labor. However, by employing the stealth RNA gene expression system, such labor is no longer required.

Also, the stealth RNA gene expression system is effective

for suppressing gene mutation which is problematic in production of biopharmaceuticals. Recently, it has been reported that the primary cause of occurrence of mutation in genome of an RNA virus is cytosolic adenosine deaminase (Adenosine deaminase acting on RNA, ADAR1) (Non-Patent Document 39). Since ADAR1 is induced by activation of the innate immune system, it is possible to suppress mutation of genes in the stealth RNA gene expression system by controlling induction of ADAR1 as low as possible.

[0036]

The stealth RNA gene expression system is also suited for expression of a drug-discovery target protein made up of plural subunits. For example, for expression of NADPH oxidase (Nox2) which is a drug-discovery target enzyme, it is necessary to simultaneously express six subunits, gp91phox, p22phox, Rac, p47phox, p67phox, and p40phox, and this can be easily realized by the stealth RNA gene expression system. Further, by using the stealth RNA vector, it is possible to express the drug-discovery target protein in target cells such as primary culture vascular endothelial cells and nerve cells for which gene introduction and expression has been difficult because they do not undergo cell division, and it is possible to achieve the object easily.

[0037]

Further, since the stealth RNA gene expression system and the stealth RNA vector are less likely to cause cell injury or

inflammation, they can be applied as a platform of gene therapy for obtaining a therapeutic effect by in vivo gene expression. In particular, since the stealth RNA gene expression system and the stealth RNA vector can carry and persistently express a giant gene such as cDNA of blood coagulation factor VIII which is a product of a gene responsible for hemophilia A (7053 nucleotides) and cDNA of dystrophin which is a product of a gene responsible for Duchenne muscular dystrophy (11058 nucleotides) unlike conventional gene introduction/expression vectors, application as vectors for gene therapy of these diseases is expected.

Further, since the tandem cassette used in a tandem cassette linking method developed for carrying six or more, preferably eight or more exogenous genes on the vector of the present invention is constructed on a DNA basis, the present technique can be widely applied to common DNA expression vectors besides the stealth RNA vector of the present invention.

Brief Description of Drawings

[0038]

FIG. 1 illustrates a structure of a negative-sense single-stranded RNA molecule prepared by combining RNA derived from mRNA expressed in animal cells, and transcription start signals, transcription termination signals, and replication origins recognized by an RNA-dependent RNA polymerase.

FIG. 2 illustrates structures of 3′ terminus and 5′ terminus

of a nucleic acid required for replication of a negative-sense single-stranded RNA molecule.

FIG. 3 illustrates a structure of 3′ terminus of a nucleic acid required for replication of a negative-sense single-stranded RNA molecule.

FIG. 4 illustrates a structure of 5′ terminus of nucleic acid required for replication of a negative-sense single-stranded RNA molecule.

FIG. 5 illustrates analysis of codon adaptation index in mRNAs derived from RNA viruses.

FIG. 6 illustrates analysis of GC contents in mRNAs derived from RNA viruses.

FIG. 7 illustrates a method of designing an exogenous gene cDNA to be installed on a stealth RNA gene expression system.

FIG. 8 illustrates a method of connecting two exogenous gene cDNAs.

FIG. 9 illustrates a method of connecting ten exogenous gene cDNAs.

FIG. 10 illustrates a method of constructing a template cDNA for preparing a stealth RNA gene expression system into which ten exogenous genes are incorporated.

FIG. 11 illustrates a first method for reconstituting a stealth RNA gene expression system from a template cDNA.

FIG. 12 illustrates a second method for reconstituting a stealth RNA gene expression system from a template cDNA.

FIG. 13 illustrates a genome structure of a stealth RNA gene expression system carrying ten exogenous gene cDNAs.

FIG. 14 illustrates gene expression activity of a stealth RNA gene expression system carrying ten exogenous gene cDNAs.

FIG. 15 illustrates a genome structure of a stealth RNA gene expression system carrying ten exogenous gene cDNAs, prepared while the base sequences of the nucleic acid are optimized in a different manner from FIG. 13.

FIG. 16 illustrates a genome structure of a stealth RNA gene expression system carrying ten exogenous gene cDNAs, prepared while the arrangement of N, C, PolS (P) genes is changed.

FIG. 17 illustrates interferon induction activity of a stealth RNA vector.

FIG. 18 illustrates a genome structure of a stealth RNA gene expression system carrying an additional factor for completely avoiding an innate immunity inducibility.

FIG. 19 illustrates interferon inducibility by a stealth RNA vector carrying an additional factor.

FIG. 20 illustrates structures of stealth RNA gene expression systems having different gene expression levels (indicated by positive-sense RNA sequences).

FIG. 21 illustrates a genome structure and gene expression of a stealth RNA gene expression system in which C gene is deleted or translation of C gene is suppressed.

FIG. 22 illustrates activity of packaging signal of a stealth

RNA gene expression system.

FIG. 23 illustrates removal of a stealth RNA gene expression system from cells.

FIG. 24 illustrates genome structures of stealth RNA vectors prepared heretofore.

FIG. 25 illustrates a preparation efficiency of induced pluripotent stem cells (iPS cells) by a stealth RNA vector carrying six reprogramming genes.

FIG. 26 illustrates expression of immunoglobulin M by a stealth RNA gene expression system.

FIG. 27 illustrates expression of a bi-specific antibody molecule by a stealth RNA gene expression system.

Description of Embodiments

[0039]

1. Constituents of "stealth RNA gene expression system" of the present invention

The RNA molecule used in the present invention is "stealthy", namely it is difficult to be captured by the innate immune system. Therefore, in the present invention, the RNA molecule is referred to as "stealthy RNA", the gene expression system using the RNA as a material is referred to as "stealth RNA gene expression system", the structure including the gene expression system and having the activity of introducing the gene expression system into animal cells is referred to as "stealth RNA vector".

A stealth RNA gene expression system in the present invention is a complex that includes a negative-sense single-stranded RNA (A) containing RNA sequences (1) to (8) below, a single-stranded RNA binding protein (B), and an RNA-dependent RNA polymerase (C), and does not activate an innate immune system. A stealth RNA vector is a particle that contains the complex and has activity of introducing the complex into animal cells. In the present invention, a sequence encoding protein means an RNA sequence of the antisense strand in describing an RNA sequence of negative-sense single-stranded RNA.

(1) target RNA sequences encoding any given protein or functional RNA,

(2) RNA sequences constituting noncoding region(s) that is unrecognizable by an innate immune system,

(3) a transcription start signal sequences recognized by the RNA-dependent RNA polymerase,

(4) transcription termination signal sequences recognized by the polymerase enzyme,

(5) RNA sequences containing replication origins recognized by the polymerase enzyme,

(6) RNA sequences encoding the polymerase enzyme and having a structure optimized to be unrecognizable by an innate immune system,

(7) an RNA sequence encoding a protein that regulates activity of the polymerase enzyme and having a structure

optimized to be unrecognizable by an innate immune system, and

(8) an RNA sequence encoding the single-stranded RNA binding protein and having a structure optimized to be unrecognizable by an innate immune system.

(Hereinafter, also referred to as gene RNA or simply referred to as gene.)

Here, each of the RNA sequences of (2) preferably has a length of 5 to 49 nucleotides, and is placed as a noncoding region on 3' terminal side and 5' terminal side of each of the introduced exogenous gene RNAs (1).

While the stealth RNA gene expression system functions even when the introduced exogenous gene RNA of (1) contains less than six genes, for example, one to five genes, or contains a total nucleotide length of less than 5,000 nucleotides, the RNA gene expression system of the present invention exerts a significant effect in particular, when the exogenous gene RNA contains six or more, preferably eight or more, more preferably ten or more genes, or contains RNA of a total nucleotide length of 5,000 nucleotides, preferably 8,000 nucleotides, and more preferably 10,000 nucleotides.

In this description, the wording "gene or gene material" includes a negative-sense RNA or cDNA, and a positive-sense RNA or cDNA that is complementary to the same. In other words, those capable of synthesizing any of the gene or gene material by transcription or reverse transcription are also included in the

present invention.

[0040]

2. Constituents of stealth RNA expression system of the present invention

2-1. Preparation of tandem cassette for introduction of exogenous gene RNA

The exogenous gene RNA in the stealth RNA gene expression system of the present invention have "(2) RNA sequences that are not recognized by an innate immune system" within 3' terminal and 5' terminal noncoding regions thereof, wherein each of the RNA sequences is identical to or different from each other and having a length of 5 to 49 nucleotide, and can be prepared as a cassette by providing a "transcription start signal" of (3) and a "transcription termination signal" of (4) on further outer 3' terminal and 5' terminal site respectively, and providing multimerization sites at both outermost terminals.

[0041]

The negative-sense single-stranded RNA used in the stealth RNA gene expression system of the present invention can be easily constructed by using the DNA-based tandem cassette shown below.

The tandem cassette of the present invention is composed of (1) multimerization site A, (2) transcription start signal A, (3) noncoding sequence A1, (4) cloning site A, (5) noncoding region A2, (6) transcription termination signal A, (7) transcription start signal B, (8) noncoding sequence B1, (9)

cloning site B, (10) noncoding region B2, (11) transcription termination signal B, and (12) multimerization site B in order from the 5' terminus. The tandem cassette is schematically shown in the lower diagram of FIG. 7.

The multimerization sites A and B may be identical to or different from each other, and any sequence can be used as long as it can be used for multimerization of the cassette or for binding with other nucleic acid. Preferred examples of the multimerization site include a restriction site by a restriction endonuclease, and a recognition site by a site-specific recombinase. Examples of preferred restriction endonucleases include SapI, BbsI, BbvI, BcoDI, BfuAI, BsaI, BsmBI, BsmFI, BtgZI, EarI, FokI, HgaI, and SfaNI having a characteristic of generating a single-stranded protruding end structure having any sequence indicated, for example, by NN or NNN on the terminus generated by digestion. As other preferred examples, AlwNI, BglI, BstAPI, BstXI, DraIII, SfiI and so on having an indefinite sequence within the recognition site are recited. When homologous recombination is utilized, sequences such as attB1 and attB2 can be recited as a recognition site by a recombinase. Further, when Gibson Assembly System (New England Biolabs, Inc) is utilized, any sequence of 15 or more nucleotides can be used as a multimerization site providing that it has the same sequence as the overlapping sequence at an end of other tandem cassette which is to be a counter part of linkage.

The transcription start signals A and B can be identical to or different from each other, and can be any sequence as long as they are functional as transcription start signals recognized by an RNA-dependent RNA polymerase when they are transcribed to RNA. Examples of the transcription start signals recognized by an RNA-dependent RNA polymerase will be specifically described in the following paragraphs. Preferably, the sequences represented by SEQ ID NOs: 1 to 3 can be recited.

The noncoding sequences A1, A2, B1 and B2 can be identical to or different from one another. Any sequence is acceptable as long as the sequence gives "RNA that is not recognized by an innate immune system" defined in the above when transcribed to RNA, and as a preferred example, sequences having a length of 5 to 49 nucleotides and shown in Table 1 can be recited.

The cloning sites A and B can be any sequence that allows insertion of a desired exogenous gene. Preferably, one cloning site contains one or two or more recognition sites by restriction endonucleases, or contains one or two or more recognition sites by site-specific recombinases. Preferred examples of the cloning site include a sequence containing Acc65I recognition site and SalI recognition site, a sequence containing Acc65I recognition site and XhoI recognition site, a sequence containing BsiWI recognition site and SalI recognition site, and a sequence containing BsiWI recognition site and XhoI recognition site.

The transcription termination signals A and B can be

identical to or different from each other, and can be any sequences as long as they are functional as transcription termination signals recognized by an RNA-dependent RNA polymerase when they are transcribed into RNA. Examples of the transcription termination signals recognized by an RNA-dependent RNA polymerase will be specifically described in the following paragraphs. Preferably, the sequences represented by SEQ ID NOs: 4 to 6 can be recited.

In the one tandem cassette, at least two exogenous genes can be inserted. A cassette multimer formed of five linked cassettes each having insertion of two exogenous genes carries ten exogenous genes. In the following Examples, DNA fragments carrying four, six, or ten exogenous genes are prepared by utilizing multimerization of tandem cassettes as described above, and sub-cloned into plasmids. Further, by combining with RNA-dependent RNA polymerase genes derived from virus, and an RNA binding protein gene, an RNA expression system desired in the present invention is constructed.

[0042]

2-2. RNA-dependent RNA polymerase, and transcription start signal and transcription termination signal recognized by the polymerase enzyme

Preferably, the "RNA-dependent RNA polymerase", and the transcription start signal and the transcription termination signal recognized by the polymerase enzyme are selected from

sequences derived from the same negative-sense RNA virus, and are typically sequences derived from genome of a virus belonging to the paramyxovirus family. Since the combination of "RNA-dependent RNA polymerase" of genome of a virus belonging to the paramyxovirus family, "transcription start signal recognized by the polymerase enzyme", and "transcription termination signal recognized by the polymerase enzyme" has the same basic structure, combinations of sequences derived from any virus can be used.

In Examples of the present invention, a combination of L protein (large subunit of RNA polymerase, PolL) and P protein (small subunit of RNA polymerase, PolS) derived from Sendai virus was selected as "RNA-dependent RNA polymerase", "3'-UCCCACUUUC-5' (SEQ ID NO: 1)" was selected as "RNA which is a transcription start signal recognized by an RNA-dependent RNA polymerase", "3'-AAUUCUUUUU-5' (SEQ ID NO: 4)" was selected as "RNA which is a transcription termination signal recognized by an RNA-dependent RNA polymerase", and the transcription start signal and the transcription termination signal were placed respectively on 3' side and 5' side of each gene (FIG. 1). Then C protein of Sendai virus (C) is used as "protein that regulates activity of RNA polymerase", and NP protein of Sendai virus (N) is used as "single-stranded RNA binding protein".

In the technique disclosed in the present description, since RNA is used mainly in the form of a negative-sense single-stranded

RNA, an RNA sequence is disclosed from 3′ terminal side as sequence information of a negative strand unless otherwise noted. However, sequence information in the sequencing listings that forms part of the present description is described from 5′ terminal side according to the guidelines.

[0043]

When a combination of L protein and P protein of Sendai virus is selected as "RNA-dependent RNA polymerase", as a transcription start signal, besides "3′-UCCCACUUUC-5′ (SEQ ID NO: 1)", "3′-UCCCUAUUUC-5′ (SEQ ID NO: 2)", and "3′-UCCCACUUAC-5′ (SEQ ID NO: 3)", RNA having an equivalent function as these sequences can be used. Similarly, also as a transcription termination signal, besides "3′-AAUUCUUUUU-5′ (SEQ ID NO: 4)" "3′-CAUUCUUUUU-5′ (SEQ ID NO: 5)", and "3′-UAUUCUUUUU-5′ (SEQ ID NO: 6)", RNA having an equivalent function as these sequences can be used. When a combination of L protein and P protein of human parainfluenza viruses type 3 is selected as "RNA-dependent RNA polymerase", "3′-UCCUAAUUUC-5′ (SEQ ID NO: 7)" or an RNA having an equivalent function can be used as a transcription start signal, and "3′-UUAUUCUUUUU-5′ (SEQ ID NO: 8)" or an RNA having an equivalent function can be used as a transcription termination signal. Further, when a combination of L protein and P protein of Newcastle disease virus is selected as "RNA-dependent RNA polymerase", "3′-UGCCCAUCUUC-5′ (SEQ ID NO: 9)" or an RNA having an equivalent function can be used as a

transcription start signal, and "3′-AAUCUUUUUU-5′ (SEQ ID NO: 10)" or an RNA having an equivalent function can be used as a transcription termination signal.

[0044]

2-3. Elements for replication function of stealth RNA gene expression system of the present invention

Essential elements for the replication function of the stealth RNA gene expression system of the present invention include replication origins recognized by an RNA-dependent RNA polymerase, and sequences having the structure of $(CNNNNN)_3$- on 3′ terminal site, and $(NNNNNG)_3$- on 5′ terminal site.

In Examples of the present invention, since a combination of L protein and P protein of Sendai virus was selected as "RNA-dependent RNA polymerase", RNA of 114 nucleotides existing at 3′ terminus of the genome of Sendai virus, and RNA of 96 nucleotides existing at 5′ terminus of the genome of Sendai virus were selected as "RNA containing a replication origin recognized by an RNA-dependent RNA polymerase". Among these structures, those essential for the replication function of the stealth RNA gene expression system are as follows (FIG. 2, FIG. 3, FIG. 4).

(1) "3′-UGGUCUGUUCUC-5′ (SEQ ID NO: 11)" existing at 3′ terminus of the genome or an RNA sequence of 12 nucleotides having an equivalent function (for example, "3′-UGGUUUGUUCUC-5′ (SEQ ID NO: 12)"),

(2) "3′-GAGAACAGACCA-5′ (SEQ ID NO: 13)" existing at 5′

terminus of the genome or an RNA sequence of 12 nucleotides having an equivalent function (for example, "3′-GAGAACAAACCA-5′ (SEQ ID NO: 14)"),

(3) an RNA sequence of 18 nucleotides having a structure of "3′-$(CNNNNN)_3$-5′ (SEQ ID NO: 15)" starting from the 79th nucleotide from 3′ terminus of the genome, and

(4) an RNA sequence of 18 nucleotides having a structure of "3′-$(NNNNNG)_3$-5′ (SEQ ID NO: 16)" starting from the 96th nucleotide from 5′ terminus of the genome.

Among these, (1) and (2) are considered as replication origins recognized by an RNA-dependent RNA polymerase because they are mutually complementary sequences, and then 3′ terminus of the genome RNA and 3′ terminus of the antigenome RNA (RNA complementary to the genome RNA) are identical. While the functions of (3) and (4) are unknown, it is known that they are the sequences essential for replication of single-stranded RNA by an RNA-dependent RNA polymerase (Non-Patent Document 42).

[0045]

2-4. Packaging signal region essential for particulation in negative-sense single-stranded RNA

In the present invention, the present inventors first identified the region spanning from the 97th nucleotide to the 114th nucleotide of 3′ terminus of the genome as a region that is a packaging signal for particle formation in the negative-sense single-stranded RNA.

As shown in Example 18 (FIG. 22), when the whole of the region (indicated as "sequence D") was deleted, the efficiency of particle formation of the stealth RNA vector was significantly deteriorated although gene expression in the packaging cell was not influenced.

This indicates that this sequence of 18 nucleotides, or the region having a length of 18 nucleotides or a part thereof is a sequence or region that is essential for incorporation into a virus-like particle.

Then the present inventors replaced this sequence of 18 nucleotides with a partial sequence that is arbitrarily selected from partial sequences of mRNA derived from House-keeping gene recited in (Table 1) ((5) of FIG. 3, SEQ ID NO: 75), and confirmed that the efficiency of particle formation was not changed.

On the basis of this result, it is considered that the region having a length of 18 nucleotides from the 97th to 114th nucleotides from 3' terminus of the genome or a region having a partial length thereof is essential for packaging for particle formation in the negative-sense single-stranded RNA. In other words, it can be concluded that the region is "packaging signal region" that is not essential for transcription and replication of the negative-sense single-stranded RNA as a template, but is essential for incorporation of the stealth RNA gene expression system into a virus-like particle.

(5) RNA having a length of 18 nucleotides, corresponding to

"3'-AAAGAAACGACGGUUUCA-5' (SEQ ID NO: 17)" from the 97th to 114th nucleotides from 3' terminus of the genome, or any RNA having a length of at least consecutive 8 or more nucleotides, preferably 10 or more nucleotides, more preferably 15 or more nucleotides thereof.

The possibility that the stealth RNA gene expression system lacking the length of 18 nucleotides or a partial region thereof of the above (5) leads production of a virus-like particle containing the stealth RNA gene expression system is very low even if the host cells are infected with a homogeneous or heterogeneous virus.

Thus, the region having a length of 18 nucleotides or a partial region thereof is an essential region when the stealth RNA gene expression system of the present invention is prepared as an infectious particle, and used as a stealth RNA gene expression vector, however, the region is contrarily a sequence that should be eliminated for biopharmaceutical production where it is desired to ultimately eliminate contamination with virus-like particles and to ensure the safety.

[0046]

2-5. Construction of template for gene expression of negative-sense single-stranded RNA

It is known that an RNA molecule carrying a combination of "RNA which is a transcription start signal recognized by an RNA-dependent RNA polymerase", "RNA which is a transcription

termination signal recognized by an RNA-dependent RNA polymerase" and "RNAs containing a replication origin recognized by an RNA-dependent RNA polymerase" existing at 3' terminus and 5' terminus of a negative-sense single-stranded RNA, together with any exogenous gene between the transcription start signal and the transcription termination signal serves as a template for transcription or replication in the presence of essential factors such as an RNA-dependent RNA polymerase derived from a virus supplied in trans (Non-Patent Document 43, Non-Patent Document 44, and Non-Patent Document 45). For example, it is demonstrated that a negative-sense single-stranded RNA having the aforementioned structure carrying a combination of a transcription start signal, a transcription termination signal and replication origins derived from Sendai virus, and Chloramphenicol acetyltransferase (CAT) gene of Escherichia coli as a exogenous gene serves as a template for transcription and replication in a cell infected with Sendai virus to produce CAT (Non-Patent Document 43, and Non-Patent Document 44). Also it is indicated that a negative-sense single-stranded RNA having an equivalent structure is persistently replicated in cells in which NP (single-stranded RNA binding protein), P (small subunit of RNA-dependent RNA polymerase) and L (large subunit of RNA-dependent RNA polymerase) proteins of Sendai virus are stably expressed (Non-Patent Document 45).

These reports indicate that the negative-sense

single-stranded RNA prepared in the present invention serves as a template for gene expression, however, when such a technique is used as it is, the activity of transcription or replication depends on the NP, P, and L proteins supplied in trans from the cells containing genes of the virus, so that a general gene expression system enabling gene expression in any cell is not obtained. Thus, the present inventors attempted to carry genes required for transcription and replication on an RNA molecule having the structure shown in the above section 2-3. (FIG. 1) and formed of components that are not recognized by an innate immune system.

[0047]

3. Findings regarding avoidance of activation of innate immune system (PAMP) in animal cells

3-1. Regarding PAMP of virus-derived RNA

An innate immune system possessed by an animal cell is activated by recognition of a "molecular pattern characteristic of pathogenic microorganism (Pathogen-associated molecular pattern, PAMP)" existing in genome RNA of a virus that has been entered inside the cell, or mRNA of a virus gene. The structure of PAMP has been identified in hepatitis C virus and human immunodeficiency virus. In hepatitis C virus, it has been reported that a uridine-rich sequence positioned in a noncoding region at 3' terminal of the genome is PAMP (Non-Patent Document 35). Meanwhile, in a human immunodeficiency virus, it has been

reported that the region having a high adenine content existing in mRNA transcribed from three genes of Gag, Pol, and Env is PAMP (Non-Patent Document 36). Besides the above, in Sendai virus, strong PAMP activity is detected in a long-chain RNA fraction exceeding 600 nucleotides existing in infected cells (Non-Patent Document 37), and the existence of a high secondary structure that potentially functions as PAMP is known also in a noncoding region of each gene of F, HN and L (Non-Patent Document 38). Thus, it is expected that most of virus-derived RNAs contain PAMP.

[0048]

3-2. Investigation of optimization of virus-derived RNA

The attempt to disrupt the PAMP structure by optimizing codons of the region encoding a protein in the RNA virus genome for human cells, and thus to avoid the activation of the innate immune system has been often conducted heretofore. For example, it has been reported that since PAMP exists in each mRNA transcribed from each of Gag, Pol, and Env genes of human immunodeficiency virus (HIV), each gene induces interferon when it is expressed as it is in animal cells, whereas interferon induction is suppressed in each of Gag, Pol, and Env proteins that are optimized for human cells and expressed (Non-Patent Document 36). Also in a simian immunodeficiency virus (SIV), likewise in HIV, it is known that PAMP exists in each mRNA of Gag, Pol, and Env, and by optimizing codons in the region

containing PAMP in each gene for human cells, the interferon inducibility decreases (Non-Patent Document 48). However, the interferon inducibility of SIV little changes only by optimization of codons in the region containing PAMP in Pol gene in the SIV genome sequence. In light of this, codons of the region containing PAMP of Gag gene were also optimized in addition to optimization of Pol gene, and this resulted in reduction in the replicability of the virus to 1% or less, and significant impairment in functions of transcription and replication of the virus (Non-Patent Document 48). This result not only reveals that Pol gene or Gag gene of SIV encodes Pol protein or Gag protein, but also reveals that the information required for the function of transcription or replication of the virus exists in the nucleic acid sequence itself that encodes the protein.

Also for the region containing PAMP in a noncoding region of 3' terminus of the genome of hepatitis C virus, there is a report that the virus replicability is impaired when the region is disrupted (Non-Patent Document 82, and Non-Patent Document 83).

These results indicate that the "region containing PAMP" in RNA virus genome is very likely to be also a region essential for the functions such as replication of the virus.

Thus, since a universal method for removing the structure having a function of PAMP from genome nucleic acid without impairing the function of the RNA virus is not known, application

of the technique for optimizing codons of the region containing PAMP in the virus RNA for human cells to an RNA virus vector contrarily leads a negative result.

[0049]

3-3. Utilization of virus derived innate immunity inhibitory factor

In conventional techniques using genome of an RNA virus or a synthetic RNA as a platform for gene expression, the cytotoxicity is weakened by inhibiting activation of the innate immune system by PAMP by the action of the factor competing the innate immune system possessed by various viruses, rather than by elucidating the structure recognized as PAMP and removing the structure. For example, B18R protein, which is used as an essential constituent in Non-Patent Document 26 and Non-Patent Document 27, is an interferon binding protein encoded by genomic DNA of vaccinia virus, and has a function of inhibiting activation of the innate immune system by inhibiting the activity of interferon.

Further, in the vectors based on Sendai virus described in Patent Document 3, Patent Document 4, and Non-Patent Document 7, mutation of an RNA-dependent RNA polymerase (L protein and P protein), and expression of V protein derived from Sendai virus serve to suppress the innate immune system. V protein is one of proteins produced from mRNA transcribed from P gene region of Sendai virus, and has an N-terminal region (317 amino acid

residues) common to that of P protein, and a basic C-terminal region (67 amino acid residues) having a structure peculiar to V protein (Non-Patent Document 39). V protein inhibits activation of the innate immune system through inhibition of a transcription factor IRF-3 (Non-Patent Document 40). It is known that in a V protein-defective Sendai virus prepared by artificially introducing mutation into a base sequence of P gene, the function of suppressing activation of the innate immune system is lost, and the virus is easily eliminated from the infected individual (Non-Patent Document 40, and Non-Patent Document 41).

[0050]

In the case of using a virus derived innate immunity inhibitory factor together as described above, there arises a concern in safety that the innate immune system cannot be activated even when the cells into which the exogenous gene is introduced are infected with other species of pathogenic microorganism. For example, in cells stably retaining genome of the Sendai virus vector, V protein is constantly expressed. Thus, when this vector is used in tissue cells of a living body, there is a possibility that the innate immune system cannot be activated even when the cells are infected with other virus. Therefore, a technique of avoiding activation of an innate immune system by a method not relying on suppression of the innate immune system by a virus derived factor is desired.

[0051]

4. Techniques for avoiding PAMP in RNA gene expression system of the present invention

4-1. "RNA that is not recognized by an innate immune system" found within noncoding region sequence

The key of the present invention is selection of RNA capable of avoiding activation of an innate immune system possessed by animal cells. As described above, the wording "avoiding activation of an innate immune system" used in the present invention means that the interferon β inducibility as an index is 30 or less, preferably 20 or less, more preferably 10 or less, when the expression amount of IFN-β mRNA in normal cells is 1.0.

Thus, in the present invention, as a material for "RNA that is not recognized by an innate immune system", the present inventors decided to use RNA sequences derived from mRNA expressed in animal cells such as human cells, and selected mRNA derived from House-keeping genes that are expressed in a wide variety of human cells. The mRNA is expressed in most of human cells in relatively large quantity, and does not contain a motif recognized by the human innate immune system. Further, from noncoding regions in the mRNAs that do not encode protein, RNAs each having a length of 5 nucleotides to 49 nucleotides that do not form a complicated secondary structure were selected (Table 1), and placed in a noncoding region on 5′ side and in a noncoding region of 3′ side of each gene installed on the vector

(FIG. 1).

All of the partial sequences of mRNA derived from House-keeping gene recited below (Table 1) can be used as particularly preferred sequences among "RNA sequences derived from mRNA expressed in animal cells" of "RNA that is not recognized by an innate immune system" in the noncoding region sequence of the present invention. As other examples of such preferred sequences, partial sequences of RNA sequences derived from mRNA of genes that are expressed in large quantity in a living body such as albumin gene can also be preferably used.

As described above, in Examples of the present invention, noncoding region sequences derived from mRNA expressed in human cells are selected, and partial sequences thereof were used in consideration of application to regenerative medicine, however, "RNA that is not recognized by an innate immune system" is not limited to the sequences derived from noncoding region sequences derived from mRNA recited in Examples or (Table 1). For example, in OptimumGen Gene Design System (Patent Document 7, GenScript USA Inc.), partial sequences of a noncoding sequence possessed by human mRNA appropriately selected from a group of human mRNAs that are highly expressed in human, employed for determining a standard CAI value can be used. Besides these, human RNAs other than mRNA, RNAs expressed in cells of other animal species, and non-native synthetic RNAs can also be selected as long as they are not recognized by the innate immune system of the host

cells in which the vector is used.

[0052]

[Table 1]

Position of cassette	Position in cassette	Animal species from which sequence is derived	Name (abbreviated name) of gene from which sequence is derived	Length (nucleotides)	GenBank accession No.	Sequence ID No.
#1	3'	Human	glyceraldehyde-3-phosphate dehydrogenase	5	NM_002046	18
#1	5'	Human	eukaryotic translation elongation factor 1 alpha 1	27	NM_001402	19
#2	3'	Human	hydroxymethylbilane synthase	24	NM_000190	20
#2	5'	Human	glyceraldehyde-3-phosphate dehydrogenase	30	NM_002046	21
#3	3'	Human	glyceraldehyde-3-phosphate dehydrogenase	15	NM_002046	22
#3	5'	Human	mitochondrial ribosomal protein L32	29	NM_031903	23
#4	3'	Human	β-actin	30	NM_001101	24
#4	5'	Human	β-actin	29	NM_001101	25
#5	3'	Human	phosphoglycerate kinase 1	29	NM_000291	26
#5	5'	Human	phosphoglycerate kinase 1	29	NM_000291	27
#6	3'	Human	peptidylprolyl isomerase A	29	NM_021130	28
#6	5'	Human	peptidylprolyl isomerase A	29	NM_021130	29
#7	3'	Human	tubulin, α-1b	29	NM_006082	30
#7	5'	Human	tubulin, β-1	29	NM_030773	31
#8	3'	Human	transferrin receptor	29	NM_003234	32
#8	5'	Human	eukaryotic translation elongation factor 2	29	NM_001961	33
#9	3'	Human	ubiquitin C	29	NM_021009	34
#9	5'	Human	transferrin receptor	29	NM_003234	35
#10	3'	Human	TATA box binding protein	29	NM_003194	36
#10	5'	Human	lamin B2	29	NM_032737	37
#11	3'	Human	α-actin, cardiac muscle 1	29	NM_005159	38
#11	5'	Human	α-actin, cardiac muscle 1	29	NM_005159	39
#12	3'	Human	tubulin, β-1	29	NM_030773	40
#12	5'	Human	tubulin, β-1	29	NM_030773	41
#13	3'	Human	1-acylglycerol-3-phosphate O-acyltransferase 1	29	NM_006411	42
#13	5'	Human	1-acylglycerol-3-phosphate O-acyltransferase 1	21	NM_006411	43
#14	3'	Human	tubulin, α-1b	13	NM_006082	44
#14	5'	Human	glyceraldehyde-3-phosphate dehydrogenase	46	NM_002046	45
		Human	ATP synthase, mitochondrial Fo complex subunit B1	13	NM_001688	73
		Human	ATP synthase, mitochondrial Fo complex subunit B1	18	NM_001688	74
		Human	peptidylprolyl isomerase A (cyclophilin A)	18	NM_021130	75
		Human	ribosomal protein, large, P1 (RPLP1)	12	NM_001003	76

[0053]

4-2. Replacement with "RNA that is not recognized by an innate immune system" in 3′ terminal and 5′ terminal regions of RNA vector genome

As shown in FIG. 2, FIG. 3 and FIG. 4, among the genome RNA sequences constituting the stealth RNA vector of the present invention, the 3′ terminal region and the 5′ terminal region include sequence regions of which function is unknown besides the essential constituents involved in transcription, replication and the like shown in the above sections 2-2. to 2-3. and so on, and these sequences include regions that can be replaced with "RNA that is not recognized by an innate immune system" such as partial sequences of mRNA derived from House-keeping gene in plural sites.

For example, as shown in Example of FIG. 3, among the structures existing in 3' terminus of native virus genome, the regions of (1) to (6) can be replaced with other non-homologous base sequences including partial sequences of mRNA derived from House-keeping gene in Table 1, and all of 3′ Variant 1 to 3′ Variant 6 of the stealth RNA gene expression system are capable of achieving stable gene expression and production of vector particles. Also as shown in Example of FIG. 4, among the structures existing in 5' terminus of native virus genome, the regions of (1) to (4) can be replaced with or inserted by other non-homologous base sequence, and all of 5′ Variant 1 to 5′ Variant

5 of the stealth RNA gene expression system are capable of achieving stable gene expression.

It would be highly possible that the interferon inducibility is further suppressed by replacing sequences of these positions with "RNA that is not recognized by an innate immune system" such as partial sequences of mRNA derived from House-keeping gene of (Table 1).

[0054]

5. Techniques for avoiding activation of an innate immune system (PAMP) by proteins essential for transcription and replication

5-1. Investigation of value that provides index for PAMP structure in gene encoding protein essential for transcription and replication

In Examples of the present invention, L protein (large subunit of RNA polymerase, PolL) and P protein (small subunit of RNA polymerase, PolS) of Sendai virus were selected as "RNA-dependent RNA polymerase", C protein (C) of Sendai virus was selected as "protein that regulates activity of RNA polymerase", and NP protein(N) of Sendai virus was selected as "single-stranded RNA binding protein". Although these proteins are essential for transcription and replication from a negative-sense single-stranded RNA, it is highly possible that "pathogen-associated molecular pattern (PAMP)" exists in genome RNA or mRNA of Sendai virus encoding these proteins as shown in Non-Patent Document 37. Therefore, it is necessary to remove

a structure that is a potential PAMP from the RNA encoding these protein so as to construct a stealth RNA gene expression system that does not activate an innate immune system.

[0055]

Although it is sure that active PAMPs exist in genome RNA and mRNA constituting Sendai virus, the region where the active PAMP actually exists has not been elucidated. However, RNA having active PAMP must have a structure that is clearly different from that of RNA expressed in host cells. Thus, the present inventors first made comparison according to codon adaptation index (CAI) of coding region as an index in order to examine the difference in structure between mRNA derived from an RNA virus and mRNA of a human cell. CAI is an index for dissociation from the frequency of appearance of codons of mRNAs encoding 100 proteins that are most strongly expressed in cells of a certain biological species, and CAI = 1.0 indicates that the codon use frequency is the same as that of mRNAs of these 100 proteins (Non-Patent Document 46). As a result of analysis according to "OptimumGen Gene Design System (Patent Document 7, GenScript USA Inc.)", an average value of CAI of coding regions of arbitrarily selected 151 human mRNAs was 0.778, an average value of CAI of seven mRNAs of Sendai virus was 0.704, and an average value of CAI of seven mRNAs of measles virus belonging to the same paramyxovirus family was 0.697, revealing that the CAI of mRNA of paramyxovirus was significantly lower than the average

CAI of mRNAs of human cells (FIG. 5). An average value of CAI of arbitrarily selected eleven mRNAs expressed in Escherichia coli analyzed for reference was 0.698 (FIG. 5). This suggests the possibility that in use in human cells, mRNA of paramyxovirus has a structural deviation comparable to that of mRNA of Escherichia coli which is a prokaryote, and this is recognized as PAMP.

[0056]

For examining the difference in structure between mRNA derived from RNA virus and mRNA of human cells from other point of view, GC contents of coding regions were calculated. An average value of GC contents of native paramyxovirus-derived RNA was 47.7% to 48.5%, which was significantly lower than 56.3% which was an average value of GC contents of coding regions of human mRNA (Non-Patent Document 47) (FIG. 6). Considering that genome of an RNA virus has a relatively low GC content, and adenine-rich or uridine-rich sequences have high potential to become PAMP (Non-Patent Document 43), the GC content also has potential becomes an index suggesting the existence of PAMP.

[0057]

5-2. "Codon optimization" application experiment for genes involved in transcription and replication derived from Sendai virus

It has been confirmed that "codon optimization" for approximating such CAI values and GC contents to average values

of mRNA of human cells is effective for disrupting a PAMP structure in a virus-derived coding region and avoiding PAMP, as shown for HIV, SIV, hepatitis virus and the like in the above section 3-2..

However, the above section 3-2. also indicates the result that the replicability is largely impaired when "codon optimization" is conducted in a PAMP region in a sequence of gene essential for transcription and replication of these viruses. Therefore, it would be conventional common knowledge that PAMP structures in sequences of genes essential for transcription and replication highly possibly serve as secondary structures essential for transcription and replication.

Considering various functions are generally integrated compactly in virus genome, it would be highly possible that a PAMP structure in a gene sequence essential for transcription and replication is important for the function of the virus also in the case of Sendai virus as is the case with these virus genomes from the conventional findings as described above. That is, it was highly expected that when codons in coding regions of proteins involved in transcription and replication, such as Sendai virus-derived "RNA-dependent RNA polymerase" for use in the RNA gene expression system of the present invention are optimized for human cells, the original transcription and replication ability is also largely impaired although PAMP can be avoided.

Under such circumstances, the present inventors dared to optimize codons of all RNAs encoding proteins such as "RNA-dependent RNA polymerase" and "RNA binding protein" involved in transcription and replication for human cells.

[0058]

In the present invention, since L protein (large subunit of RNA polymerase, PolL) and P protein (small subunit of RNA polymerase, PolS) of Sendai virus are used as "RNA-dependent RNA polymerase", C protein (C) of Sendai virus is used as "protein that regulates activity of RNA polymerase", and NP protein (N) of Sendai virus is used as "single-stranded RNA binding protein", codon optimization was conducted according to "OptimumGen Gene Design System (Patent Document 7, GenScript USA Inc.)" which is one program generally used as a codon optimization method so as to remove PAMP from RNAs encoding these proteins. As a result of this, CAI values fall within the range from 0.86 to 0.88, and showed values approximate to those of mRNAs encoding proteins highly expressed in human cells.

Results of applying codon optimization to genes of L, P, C and N proteins of Sendai virus according to "OptimumGen Gene Design System (also referred to as OGGDS method)" are shown in the following (Table 2).

[0059]

[Table 2]

Gene name	Function	Before optimization		After optimization	
		Codon Adaptation Index	GC content (%)	Codon Adaptation Index	GC content (%)
L	RNA-dependent RNA polymerase	0.68	44.0	0.88	52.5
P	Protein that regulates activity of RNA polymerase	0.73	49.6	0.86	54.4
C	Protein that regulates activity of RNA polymerase	0.73	50.1	0.88	53.5
N	RNA binding protein	0.71	49.4	0.88	55.5

[0060]

In the above (Table 2), GC contents as well as CAI values were calculated for RNAs after codon optimization so as to analyze the optimized RNAs from other point of view. While GC contents of RNAs before optimization were in the range of 44.0% to 50.1%, GC contents of RNAs after optimization increased to the range of 52.5% to 55.5%, and approximated 56.3% which is an average value of GC contents of coding regions of human mRNA (Non-Patent Document 47) (Table 2) (FIG. 6). In RNA viruses, it is known that adenine-rich or uridine-rich sequences have high potential to become PAMP (Non-Patent Document 36), and the experiment result strongly suggests the possibility that the structure of virus-derived RNA approximates the structure of human mRNA by the technique of codon optimization, and regions having activity of PAMP are removed at the same time.

[0061]

In the present invention, an RNA vector carrying RNAs encoding NP protein, P protein, C protein, L protein derived from Sendai virus that are optimized for human cells by the above technique together with ten exogenous genes was constructed (FIG. 13) (Example 8), and the vector was expressed in Hela cells, and investigated (Example 9). It was confirmed that all the ten exogenous genes were expressed in adequate quantities that can be observed. Also it was confirmed that the RNA vector is capable of avoiding INF-β induction in human fibroblasts (Example 13, FIG. 17).

This reveals that the RNA vector of the present invention carrying RNA of genes that are involved in transcription and replication derived from Sendai virus and are optimized for human cells functions as an excellent stealth RNA vector having the PAMP avoiding effect.

This result also shows that any PAMP structure existing in genes essential for transcription and replication was not essential for transcription and replication in the case of Sendai virus, and this was an unexpected surprising result for the present inventors who dared to made the experiment.

[0062]

5-3. Investigation of codon optimization method

The result of the above (Table 2) suggests that for "codon optimization" for suppressing induction of innate immune reaction by removing regions having active PAMP, the two

numerical ranges of "CAI value" and "GC content" are important requirements. Thus, the present inventors planned to conduct an experiment by applying other codon optimization method so as to investigate which one of the two requirements is more essential. As a method for codon optimization, since various methods have been proposed as represented by GeneOptimizer Process (Non-Patent Document 49) and GeneGPS Expression Optimization Technology (Patent Document 8, and Patent Document 9) besides the aforementioned OGGDS method, it is possible to confirm that the equivalent effect is achieved when a method other than the aforementioned OGGDS method is applied.

Thus, a codon optimization method based on GeneGPS Expression Optimization Technology (hereinafter, also referred to as a GGEOT method) which is a generally used "codon optimization" technique likewise the OGGDS method was applied to a template DNA encoding NP protein, P protein, C protein, and L protein of Sendai virus, and an RNA vector capable of carrying ten exogenous genes (FIG. 15) was prepared in the same manner. By the verification by the method of Example 9, it was confirmed that the stealth RNA vector was a stealth RNA vector capable of avoiding induction of the innate immune reaction as with the stealth RNA vector optimized by the OGGDS method (data not shown).

Optimization by the OGGDS method and optimization by the GGEOT method use completely different algorithms, and the identity between the base sequences of nucleic acid optimized

by these two methods was 77% to 80%, revealing that considerably different nucleotides were selected for codon optimization (Table 4).

The foregoing demonstrated that the method for optimizing the genes encoding "RNA-dependent RNA polymerase", "protein that regulates activity of RNA polymerase", and "single-stranded RNA binding protein" for preparing a stealth RNA gene expression system does not rely on a specific codon optimization method, and any codon optimization method based on any algorithm can be applied as a codon optimization method of the present invention.

The following (Table 3) shows values of GC contents and CAI values after codon optimization by the GGEOT method for L, P, C and N protein genes of Sendai virus, in comparison with the values by the OGGDS method shown in the above (Table 2). Since the GGEOT method lacks a calculation program for "CAI value", the calculation was conducted according to the calculation program for "CAI value" of the OGGDS method.

Also (Table 4) shows the original sequence, and a value of homology (identity) between the sequences after application of OGGDS and the sequences after application of GGEOT for each of L, P, C and N protein genes.

[0063]

[Table 3]

Gene name	Function	Before optimization		After optimization (OptimumGen Gene Design System)		After optimization (GeneGPS Expression Optimization Technology)	
		Codon Adaptation Index	GC content (%)	Codon Adaptation Index	GC content (%)	Codon Adaptation Index	GC content (%)
L	RNA-dependent RNA polymerase	0.68	44.0	0.88	52.5	(0.71)	51.1
P	Protein that regulates activity of RNA polymerase	0.73	49.6	0.86	54.4	(0.70)	59.9
C	Protein that regulates activity of RNA polymerase	0.73	50.1	0.88	53.5	(0.72)	51.4
N	RNA binding protein	0.71	49.4	0.88	55.5	(0.70)	59.4

[0064]

[Table 4]

N (NP) gene

Native Virus Genome	75.94%	Optimized with OGGDS Method
Native Virus Genome	76.13%	Optimized with GGEOT Method
Optimized with OGGDS Method	80.38%	Optimized with GGEOT Method

PolS (P) gene

Native Virus Genome	74.17%	Optimized with OGGDS Method
Native Virus Genome	74.99%	Optimized with GGEOT Method
Optimized with OGGDS Method	78.09%	Optimized with GGEOT Method

C gene

Native Virus Genome	77.24%	Optimized with OGGDS Method
Native Virus Genome	76.91%	Optimized with GGEOT Method
Optimized with OGGDS Method	77.56%	Optimized with GGEOT Method

GENE A	Homology between GENE A & B	GENE B

PolL (L) gene

Native Virus Genome	75.31%	Optimized with OGGDS Method
Native Virus Genome	74.92%	Optimized with GGEOT Method
Optimized with OGGDS Method	76.72%	Optimized with GGEOT Method

[0065]

5-4. Essential index for "codon optimization"

While CAI is used as an index for estimating the translation efficiency of mRNA in human cells (Non-Patent Document 46), codon optimization is used as a measure for eliminating a structure having active PAMP from a virus-derived RNA in the present invention, and elevation in the translation efficiency may not be necessarily obtained.

While CAI is an "index for dissociation from the frequency of appearance of codons of mRNAs encoding 100 proteins that are most strongly expressed in cells of a certain biological species", an objective standard for selecting 100 proteins which forms a standard has not been shown. Optimization by the OGGDS method and optimization by the GGEOT method that could achieve expression of ten genes while suppressing induction of the equivalent innate immune reaction in human-derived culture cells in the present invention were compared from each other, and the CAI value of the latter case (calculated by the OGGDS method) did not significantly vary before optimization and after optimization (Table 3).

Meanwhile, the GC content was 51% or more, and about 60% at most regardless of the employed optimization method. This shows that GC content is more effective than CAI value as an index of the possibility that a structure having active PAMP has been removed. In other words, as an index in "codon optimization" for "stealth RNA gene expression system" in which

induction of the innate immune reaction is suppressed, GC content is the most excellent index, and the possibility that the structure having active PAMP has been removed is estimated if the GC content after codon optimization of the virus-derived protein is at least 50.0% or more, desirably 52.0% or more.

From the above, the wording "codon optimization" for an RNA gene expression system used in the present invention means adjusting all of the base sequences encoding proteins required for the RNA gene expression system to have a GC content of 50 to 60%, preferably 52 to 56%.

As a result of modification of the base sequence by codon optimization, the sequences encoding C protein and V protein that had existed in P gene region disappeared, and neither C protein nor V protein was expressed from optimized P gene. The RNAs encoding C protein and V protein are not essential because gene expression is conducted even when the RNAs are completely removed, however, in the case of C protein, in particular, it is preferred to add C protein gene RNA that is optimized as described above into the sequence because C protein is important for properly regulating the expression amount of the RNA vector of the present invention. Also V protein RNA can be added into the sequence as necessary after it appropriately undergoes similar codon optimization.

[0066]

6. Method for carrying a large number of exogenous genes on stealth

RNA in the present invention (transcription cassette linking method)

6-1. Investigation of method for carrying six or more genes on one vector

Next, the method for carrying six or more, for example, ten exogenous genes on a stealth RNA in a simple manner was investigated. Since an RNA molecule itself cannot be engineered by gene recombination technique, every construction including carrying of an optimized virus-derived gene in 5-4. was conducted as a cDNA, and RNA was prepared by DNA-dependent RNA polymerase such as T7 RNA polymerase derived from T7 phage using the cDNA as a template.

For preparing a DNA molecule carrying ten genes in the simplest manner, a method of introducing restriction endonuclease cleavage sites peculiar to each gene upstream and downstream the respective cDNA of each gene, and inserting the cDNAs cleaved by restriction endonucleases into sequence is conceivable. However, this method is impractical because at least 20 different restriction endonucleases are required, and it is necessary to prepare every cDNA again for changing the combination of genes or for changing the position on the stealth RNA.

[0067]

6-2. Preparation of "tandem transcription cassette" carrying two genes

In the present invention, as described in the above section 2-1. (FIG. 1), the technique of preparing "tandem transcription cassette" carrying two genes, and linking plural tandem transcription cassettes is employed. In this technique, genes to be installed were designed to have the same structure, and designed so that they can be installed at any position in the stealth RNA (FIG. 7). In this designing method, restriction endonuclease cleavage sites are separately provided: restriction endonuclease A cleavage site on 5' upstream side of the gene to be installed, and restriction endonuclease B cleavage site on 3' downstream side of the gene, and cDNA cleaved at these sites is inserted into a DNA molecule which is to be a template. The template DNA into which the cDNA is to be inserted is provided with recognition sites by restriction endonuclease C and restriction endonuclease D, in addition to recognition sites by restriction endonuclease A and restriction endonuclease B. These combinations of restriction endonucleases are selected so that the DNA fragment cleaved by restriction endonuclease A can covalently bind with the DNA fragment cleaved by restriction endonuclease D, and the DNA fragment cleaved by restriction endonuclease B can covalently bind with the DNA fragment cleaved by restriction endonuclease C. There are a large number of such combinations, besides the combination Acc65I and BsiWI and the combination of XhoI and SalI recited in Examples, combination of XbaI, and SpeI or NheI, and combination of BamHI

and BglII are conceivable. In this case, any cDNA to be installed is structurally restricted to design so that recognition sites by restriction endonuclease A, restriction endonuclease B, restriction endonuclease C, and restriction endonuclease D are absent within its sequence. By using such combinations of restriction endonucleases, DNA fragments each carrying two cDNAs are first prepared (FIG. 8).

[0068]

6-3. "Transcription cassette" linking method

Next, five DNA fragments each carrying two cDNAs linked in this manner are connected to prepare a DNA carrying a total of ten cDNAs (FIG. 7, FIG. 8and FIG. 9). In Examples, a DNA fragment carrying two linked cDNAs prepared in the above section 6-2. was cleaved by a restriction endonuclease called SapI and isolated. The protruding end structure of the DNA cleaved by SapI has such a structure that three nucleotides are protruded on 5' side, and by setting the sequence of three nucleotides arbitrarily, $4 \times 4 \times 4 = 64$ patterns of protruding end structure can be selected (FIG. 7) (FIG. 9). Therefore, it is possible to bind five DNA fragments accurately as designed and to collect them as one DNA molecule (FIG. 9). In this case, cDNAs of genes to be installed are designed so that recognition site by SapI, in addition to recognition sites by restriction endonuclease A, restriction endonuclease B, restriction endonuclease C, and restriction endonuclease D are absent within its sequence(FIG.

7).

The restriction endonuclease having the characteristic of generating any given single-stranded protruding end structure in the sequence represented by NN or NNN on the terminus generated by digestion is not limited to SapI, and the equivalent results are obtained with various restriction endonucleases including BbsI, BbvI, BcoDI, BfuAI, BsaI, BsmBI, BsmFI, BtgZI, EarI, FokI, HgaI, and SfaNI. The equivalent effect can be obtained also with AlwNI, BglI, BstAPI, BstXI, DraIII, SfiI and the like having an indefinite sequence in the recognition site. This step is not necessarily cloning by a restriction endonuclease, and a method using homologous recombination (In-Fusion HD Cloning System (TAKARA-Bio, Inc) or Gibson Assembly System (New England Biolabs, Inc)) can also be employed. The step of incorporating into a circular plasmid DNA after connecting ten cDNAs can be achieved also by covalent bonding using ordinary T4 DNA ligase without using the method of incorporating into pDONR-221 or the like by homologous recombination (Gateway System (Life Technologies, Inc.)) shown in Examples. Also it is possible to prepare a DNA molecule carrying any of one to ten genes by the method shown in FIG. 9.

[0069]

6-4. Regulation of expression level of exogenous gene

Generally, in the case of inserting plural exogenous genes in a negative-sense single-stranded RNA gene expression system

containing genes respectively encoding a set of RNA-dependent RNA polymerase (PolS and PolL), single-stranded RNA binding protein (N), and RNA polymerase activity regulating protein (C), it is known that the expression level is higher as the position is closer to upstream 3′ terminal site. The stealth RNA gene expression system of the present invention also shows this trend. In the present invention, since a large number of exogenous genes can be incorporated in any order in the manner of integrating cassettes, the genes can be conveniently arranged in the order of the desired expression levels. Also the expression levels of the proteins generated from respective genes can be regulated by changing the translation efficiency (FIG. 20).

[0070]

7. Synthesis of stealth RNA

Next, the DNA fragment in which ten cDNAs are linked, prepared in the above section 6., and a gene encoding single-stranded RNA binding protein (hN), a gene encoding protein that regulates activity of RNA polymerase (hC), and genes encoding an RNA-dependent RNA polymerase (hPolL and hPolS) having codons optimized for human cells in the manner as described in the above section 5. (hereinafter, also referred to "humanized", and "h" is added to the abbreviated name of the protein) were linked to prepare a circular template cDNA for synthesizing a stealth RNA (FIG. 10). The structure of the stealth RNA can be selected from a negative strand and a positive strand depending on the

position of the promoter recognized by the RNA polymerase. Here, the RNA having the same orientation as the mRNA expressed from the gene installed on the stealth RNA is defined as a positive strand, and the RNA having the orientation complementary to the mRNA is defined as a negative strand, and FIG. 10 illustrates preparation of a template for synthesizing a negative-sense RNA by using T7 RNA polymerase. Downstream the T7 promoter (Non-Patent Document 50), a ribozyme derived from antigenome of human hepatitis D virus for cleaving RNA and creating an accurate end (Non-Patent Document 51) and a transcription termination signal of T7 RNA polymerase (Non-Patent Document 50) are arranged so that RNA corresponding to the entire length of the stealth RNA can be synthesized. The enzyme used for synthesis of RNA is not limited to T7 RNA polymerase, but any DNA-dependent RNA polymerase that can be used in Escherichia coli or animal cells can be used. For example, T3 RNA polymerase derived from Escherichia coli T3 phage (Non-Patent Document 52) and SP6 RNA polymerase derived from salmonella SP6 phage (Non-Patent Document 53) can also be used in combination with the promoter and the transcription termination point recognized by these enzymes. The ribozyme is used for accurately cleaving 3′ terminus of RNA, and not only the ribozyme derived from antigenome of human hepatitis D virus as used in Examples, but also a ribozyme derived from genome of human hepatitis D virus (Non-Patent Document 51), a hairpin ribozyme of tobacco ringspot

virus (Non-Patent Document 54), and a short inhibitory RNA (siRNA) capable of cleaving RNA in cells (Patent Document 55) can be used.

[0071]

The cDNA complementary to the entire length of the stealth RNA cDNA is cloned into a plasmid having a replication origin derived from p15A (Non-Patent Document 56). Since the plasmid having a replication origin derived from p15A is maintained in a low copy number state in Escherichia coli, not only it is advantageous for stably retaining a large DNA fragment in Escherichia coli, but also it can coexist in Escherichia coli with a plasmid for expressing N protein having a replication origin derived from ColE1 in the method 2 for reconstituting the stealth RNA gene expression system (Non-Patent Document 56). In Examples, the plasmid having a replication origin derived from p15A carries ampicillin resistance, and the plasmid having a replication origin derived from ColE1 carries kanamycin resistance, and the two plasmids are maintained in the same Escherichia coli by double selection of ampicillin and kanamycin, however, the combination of antibiotics is not limited to this example. Regarding the combination of plasmids, a plasmid having a replication origin derived from F factor in place of the replication origin derived from p15A, and a plasmid having a replication origin derived from pUC in place of the replication origin derived from ColE1 can be used.

[0072]

8. Reconstruction of stealth RNA gene expression system

8-1. Conventional reconstruction method

Reconstruction of a stealth RNA gene expression system composed of a negative-sense single-stranded RNA and a protein that binds to the RNA can be achieved in two methods. The first method is a technique using a virus having genome of a negative-sense single-stranded RNA, and known as a vector reconstituting method using the virus, wherein a positive-sense single-stranded RNA complementary to the negative-sense single-stranded RNA is expressed in animal cells by using T7 RNA polymerase, and simultaneously, three proteins, NP (N), P (PolS), and L (PolL) are expressed in the cells, and thus a stealth RNA gene expression system of a positive-sense RNA is reconstituted (FIG. 11)(Non-Patent Document 57, and Patent Document 3). The merit of this method lies in the convenience that by using animal cells expressing T7 RNA polymerase stably, reconstitution can be achieved only by introducing the plasmid DNA which is a material into the cells. On the other hand, it is also known that since a plasmid containing a template cDNA for synthesizing a positive-sense single-stranded RNA, and three plasmids carrying genes for expressing three proteins, NP, P, and L are simultaneously introduced into cells, gene recombination often occurs among these DNA molecules, and mutation is inserted into the structure of the negative-sense

single-stranded RNA to be prepared (Non-Patent Document 57). In Examples of Patent Document 3, plasmids for expressing M, F, and HN proteins are further added so as to increase the efficiency of reconstitution.

[0073]

8-2. Reconstruction method developed in the present invention

In the second method, first, a complex of a negative-sense single-stranded RNA and a NP protein (N) having a single-stranded RNA binding ability is prepared in Escherichia coli, and the complex is introduced into animal cells in which P (PolS) protein and L (PolL) protein are expressed, to reconstitute a stealth RNA gene expression system (FIG. 12). In this method, first, mRNAs encoding N protein and a stealth RNA are synthesized by T7 RNA polymerase respectively from two plasmids that can coexist in Escherichia coli, and the single-stranded RNA binding protein (N) and the stealth RNA are co-expressed in Escherichia coli to produce a complex. Although the method of reconstituting an RNA virus by using an RNA-protein complex isolated from a naturally occurring RNA virus as a material is disclosed in Non-Patent Document 58, the method developed in the present invention enables reconstitution using a stealth RNA synthesized by gene recombination techniques as a material. Although this method disadvantageously requires a larger number of processes and is more complicated as compared with the first method, this method makes it possible to reconstitute a stealth RNA gene

expression system without introducing mutation into genome RNA by using Escherichia coli in which a gene involved in homologous recombination (RecA) and a gene encoding RNase (RNaseE) are disrupted (Non-Patent Document 59).

That is, the method for reconstituting a stealth RNA gene expression system developed in the present invention is a method of preparing a complex of a negative-sense single-stranded RNA and a protein having a single-stranded RNA bindability (for example, NP protein (N)) in host cells expressing T7 RNA polymerase in advance, and introducing the complex into animal cells in which the RNA-dependent RNA polymerase (for example, P (PolS) protein and L (PolL) protein) is expressed to reconstitute a stealth RNA gene expression system. Preferably, as host cells, Escherichia coli in which RecA gene and RNaseE gene are disrupted and T7 RNA polymerase is expressed is used.

[0074]

Subsequently, using the stealth RNA carrying ten genes synthesized by the method shown in the above section 7., a stealth RNA gene expression system is constructed by any of the methods described in the above section 8. (FIG. 13). It was confirmed that the gene expression system having a negative-sense single-stranded RNA prepared in this manner can persistently express all the ten installed genes by stable expression of three drug resistance characters (puromycin resistance, Zeocin resistance, and hygromycin resistance), four fluorescent

proteins (EGFP, E2-Crimson, EBFP2, and Keima-Red), and three luciferases (firefly luciferase, Renilla luciferase, and Cypridina noctiluca luciferase) (FIG. 14).

[0075]

8-3. Order of linking RNA-binding protein (hN, hC, hPol) genes in stealth RNA gene expression system

In the stealth RNA gene expression system of the present invention, the positions on the stealth RNA of the gene encoding single-stranded RNA binding protein (hN), the gene encoding protein that regulates activity of RNA polymerase (hC), and the gene encoding an RNA-dependent RNA polymerase (hPolS) are not limited to the order of hN-hC-hPolS from 3′ terminal side shown in FIG. 13. For example, a stealth RNA gene expression system can be constructed while the order is changed as is hN-hPolS-hC or hPolS-hN-hC (FIG. 16).

[0076]

8-4. Need for mutation in virus-derived protein genes in stealth RNA gene expression system

In the stealth RNA gene expression system, there is no need of existence of specific mutation in the proteins expressed from the humanized gene encoding single-stranded RNA binding protein (hN), the humanized gene encoding protein that regulates activity of RNA polymerase (hC), and the humanized genes encoding an RNA-dependent RNA polymerase (hPolS, hPolL).

As described in the above section 3-3., in the conventional

technique, introduction of a mutation for suppressing PAMP activity into the virus-derived RNA-dependent RNA polymerase or the like has been used as the most effective means for avoiding the innate immune system.

However, in the present invention, since any active PAMP is removed from the virus-derived protein gene by codon optimization according to the method shown in the above section 5., it is not necessary to preliminarily introduce a mutation into a virus-derived protein in a protein level. For example, even when genes expressing NP, P, C and L proteins derived from Sendai virus Z strain which is a wild-type paramyxovirus known to have strong interferon inducibility are used, they can be used as a material for the stealth RNA gene expression system through optimization by the method shown in the above section 5.. For example, as the gene that expresses L protein shown as "hPol" in (FIG. 16), a gene sequence derived from Z strain is optimized for human cells and used.

[0077]

9. Verification of activity of inducing innate immune system

9-1. Comparison with innate immune system avoiding effect in conventional art

Next, for comparing the activity of inducing the innate immune system in gene introduction between the stealth RNA vector carrying the stealth RNA gene expression system and a conventional art, a stealth RNA vector carrying exactly the same

four genes (Keima-Red, Blasticidin S resistant gene, EGFP, and Kusabira-Orange) as the persistent expression type Sendai virus vector which is a conventional art described in FIG. 1B of Non-Patent Document 7 was prepared. Genes were introduced into human primary culture fibroblasts using these two vectors, and the amount of interferon beta mRNA after 24 hours was quantified by the Real-Time PCR method (FIG. 17). Induction by the stealth RNA vector was within five times the amount of interferon β mRNA in normal cells although it does not carry V gene that suppresses the innate immune system. On the other hand, in the conventional art, induction of 47 times compared with normal cells was observed although V gene is contained (FIG. 17). This results reveal that in the stealth RNA gene expression system, activation of the innate immune system could be avoided even under the condition where a factor that inhibits the innate immune system is absent.

[0078]

9-2. Further avoidance of activation of innate immune system

Activity of inducing the innate immune system is also influenced by the kind of the cells retaining the stealth RNA gene expression system, or the strength of gene expression from the stealth RNA gene expression system. For example, interferon beta is little induced in human-derived HeLa cells, while it is strongly induced in human-derived 293 cells. As the gene expression is strengthened for production of biopharmaceuticals, the induction of interferon beta is strengthened. In the case

of production of biopharmaceuticals using a stealth RNA gene expression system, since mutation of RNA genome (Non-Patent Document 39) by activity of cytoplasmic adenosine deaminase induced by interferon (Adenosine deaminase acting on RNA, ADAR1) is problematic, it is desired to further suppress the innate immune system inducing activity remaining in the stealth RNA gene expression system.

This object can be achieved by additionally carrying a factor that suppresses the innate immune system on the stealth RNA gene expression system (FIG. 18 and FIG. 19). As such a factor, a deletion mutant of "molecular pattern characteristic of pathogenic microorganism (Pathogen-associated molecular pattern, PAMP)" receptor RIG-I existing in cytoplasm (RIG-IC) (Non-Patent Document 71), C-terminal region of Sendai virus V protein (Non-Patent Document 72), and PSMA7 which is a constituent of proteasome (Non-Patent Document 73) can be recited.

[0079]

10. Regulation of gene expression level

Next, how the level of gene expression in the stealth RNA gene expression system varies by regulating the expression of factors involved in transcription and replication installed on the vector was examined (FIG. 20, FIG. 21). FIG. 20 indicates positive-sense RNA sequences. Expression of each factor can be regulated by altering the efficiency of translation from mRNA

to protein. The simplest means for modifying the translation efficiency is to change the 5′ noncoding sequence directly in front of the translation initiation codon (AUG). It is considered that the highest translation efficiency in animal cells is achieved when the sequence directly in front of AUG is 5′-CCACC-3′ (SEQ ID NO: 18) (Non-Patent Document 60). On the other hand, it is possible to reduce the translation efficiency by inserting a short coding region on 5′ upstream side (Non-Patent Document 61). In Examples, a vector in which expressions of single-stranded RNA binding protein (hN) and protein that regulates activity of RNA polymerase (hC) are suppressed to 40% and 23%, respectively while expression of RNA-dependent RNA polymerase (hPolS and hPolL) is kept constant was prepared, and expressions of firefly luciferase installed thereon were compared (FIG. 20). Expression of the installed luciferase gene increased by suppressing the expression of hN or hC, and increase in gene expression of up to 79 times was observed by combining expression suppression of hN and expression suppression of hC.

[0080]

Regulation of gene expression level as described above can be conducted only by regulation of the expression level of the protein that regulates activity of RNA polymerase (hC) (FIG. 21). In this case, the stealth RNA gene expression system can be reconstructed even when hC gene is deleted, and the gene

expression level is maximum, and hence, hC gene is not an essential element for the stealth RNA gene expression system. However, proliferation of cells will be strongly inhibited when the expression of the installed gene is too strong, and hence it is practical to realize gene expression adapted to the purpose by expressing hC protein at an appropriate level.

[0081]

As important characteristics in the gene expression system, selectivity of optimum expression level depending on the purpose is recited. For example, in cell-reprogramming, cell death is induced when the expression of the transcription factors is too strong. In production of biopharmaceuticals, the production efficiency is deteriorated when the expression is weak. Generally, it is difficult to alter the expression level of the vector in a gene expression system using an RNA virus. In contrast, in the stealth RNA gene expression system, it is possible to alter the strength of the expression freely depending on the use purpose by finely regulating the expression balance of the individual constituents.

[0082]

Next, an attempt was made to prepare a vector particle capable of introducing the stealth RNA gene expression system into various animal cells by enclosing therein the stealth RNA gene expression system completed through the processes described above. When three proteins M, F, and HN of paramyxovirus were

expressed in BHK cells having the stealth RNA gene expression system in cytoplasm by using a strong SRα promoter, vector particles having gene introduction activity were detected in the culture supernatant of the cells. The infectivity titer was about 10^7 infectious units/mL, and high activity comparable to that by a conventional persistent expression type Sendai virus vector was obtained. This vector particle adsorbs to the cell surface by the activities of F and HN proteins, and is capable of introducing the content, namely, the stealth RNA gene expression system into the cytoplasm by the fusion of membranes. Since this process does not require cell division, the gene can be introduced into nondividing cells.

While the cell specificity and the species specificity of the cells for which gene introduction can be made are determined by the origin of F and HN proteins, genes could be introduced into a very wide range of human cells and animal cells including blood cells of peripheral blood when F and HN proteins of Sendai virus were used.

[0083]

11. Removal of vector

In a persistent expression type Sendai virus vector which is a conventional art, rapid vector removal is successfully achieved by suppressing the activity of RNA-dependent RNA polymerase by siRNA (Patent Document 3, and Non-Patent Document 7). Thus, whether a vector in the stealth RNA gene expression

system can be removed by a similar method was examined (FIG. 23). Since the base sequence of humanized RNA-dependent RNA polymerase (hPolL) possessed by the stealth RNA gene expression system is different from that in the persistent expression type Sendai virus vector which is a conventional art, three siRNAs were newly synthesized, and the activities thereof were examined. Removal could be achieved in the same manner as in the conventional art by using one of the three siRNAs (target sequence is SEQ ID NO: 46) (FIG. 23). Thus, it was found that for the stealth RNA gene expression system of the present invention, the vector removing method by RNAi that has been used in a conventional persistent expression type Sendai virus vector can be applied. Likewise, the removing method using microRNA (miRNA) is also applicable, and as described, for example, in Patent Document 3, removal can be achieved by reaction with endogenous miRNA by inserting a target sequence of microRNA(miRNA) into the 3′ noncoding region or the 5′ noncoding region of the exogenous gene.

[0084]

12. Use of stealth RNA expression system of the present invention

The negative-sense single-stranded stealth RNA vector used in the stealth RNA expression system of the present invention can carry six or more, further up to ten any given genes such as human-derived genes, and can carry a length of 5,000 nucleotides, further a length of up to 15,000 nucleotides.

Since the system is stealthy, namely the system is capable of avoiding activation of an innate immune system in animal cells such as human cells, and removal of the vector can be easily conducted, a wide variety of uses including cell-reprogramming technology requiring simultaneous introduction of plural genes, gene therapy including giant gene, regenerative medicine, production of biopharmaceuticals and the like are conceivable.

Specifically, the following embodiments are conceivable.

(1) Application to the technique of preparing iPS cells of high quality for clinical use in regenerative medicine with high efficiency

When six or more genes for reprogramming animal cells such as human cells, for example, a total of six genes including four Yamanaka factors (KLF4, OCT4, SOX2 and c-Myc) + BRG1 + BAF155 for converting into iPS cells are installed, the length amounts to 13,132 nucleotides. When six genes, OCT4, KLF4, SOX2, c-MYC, NANOG, and LIN28 are installed, the length amounts to 7,000 nucleotides.

Actually, these six genes were installed on the stealth RNA vector of the present invention (FIG. 25), and expressed in human embryonic fibroblasts, and initialization efficiency exceeding 40% was achieved (Example 21). It has been confirmed that the order of four Yamanaka factors (KLF4, OCT4, SOX2 and c-Myc) to be installed on in this case can be appropriately changed (data not shown).

A similar experiment was conducted in the absence of animal components (Xeno-free) and feeder cells (Feeder-free) by using human peripheral blood cells as a material, and as a result, higher initialization than the conventional method could be conducted (data not shown).

Also by carrying the four genes, KLF4, OCT4, SOX2, and c-MYC, and CHD1 gene encoding a chromatin remodeling factor (a total of 9,907 nucleotide length), and further adding TET1 gene encoding DNA demethylase (a total of 11,203 nucleotide length), it is possible to increase the initialization efficiency.

As other possible combinations, by expressing a total of eight genes including further added two oocyte-specific histones in human somatic cells, it is possible to prepare human iPS cells efficiently.

[0085]

(2) Application to regenerative medicine utilizing direct reprogramming technology for creating useful cells of nerve cells, neural stem cells, stem cells, pancreatic beta cells and the like from human tissue cells (blood, skin, placenta and the like)

For example, in the technique of reprogramming human fibroblasts into motor nerves, three genes, HB9, ISL1, and NGN2 can be added to four genes, LHX3, ASCL1, BRN2, and MYT1L, and a total of seven genes (9,887 nucleotide length) can be installed.

[0086]

(3) Production of biopharmaceuticals made up of plural subunits

It is useful for producing immunoglobulins G, and M because the genes correspond thereto are giant, and the subunits are required to be expressed simultaneously in the same cell, and regulation of the expression amount of each subunit is required.

Actually, an H (μ) chain gene, an L (κ, λ) chain gene and a J gene of human immunoglobulin were installed on the stealth RNA vector of the present invention (FIG. 24), and human immunoglobulin M was produced by using BHK cells (Example 22). In that case, the present inventors also succeeded in expressing H chain, L chain, and μ chain in a ratio of roughly 1 : 1 : 0.2 by considering the order in which the genes are installed.

The present inventors also succeeded in expressing human bispecific antibody by carrying four cDNAs of human immunoglobulin (two H chains and two L chains) on the stealth RNA vector of the present invention (Example 23).

[0087]

(4) Application to expression of drug-discovery target protein made up of plural subunits

For example, by carrying six subunits, gp91phox, p22phox, Rac, p47phox, p67phox, and p40phox on the stealth RNA vector of the present invention, and expressing them simultaneously, it is possible to express NADPH oxidase of the drug-discovery target enzyme (Nox2).

[0088]

(5) Use as gene therapy vector for disease for which responsible

gene is giant gene, by carrying the giant gene on stealth RNA vector of the present invention and expressing it persistently

Specifically, cDNA of blood coagulation factor VIII which is a product of gene responsible for hemophilia A (7053 nucleotide length) and cDNA of dystrophin which is a product of gene responsible for Duchenne muscular dystrophy (11058 nucleotide length) can be used while they are installed on the stealth RNA vector of the present invention (FIG. 24).

Examples

[0089]

Hereinafter, the present invention will be described more specifically by way of Examples, however, it is to be noted that the present invention is not limited to these Examples.

Other terms and concepts in the present invention are based on the meanings of the terms that are commonly used in the art, and various techniques used for carrying out the present invention can be carried out easily and securely by a person skilled in the art according to known literature and the like except for the techniques of which source is particularly specified. Various analyses were conducted according to the methods described in the instruction manuals, catalogues and the like of the employed analytical instruments, reagents or kits.

The contents described in the conventional art literature,

patent publications, patent application specifications cited in the present description are referred to as if they were described in the present invention.

[0090]

(Example 1) Preparation of DNA fragments carrying ten exogenous genes (1)

The following genes were amplified by PCR to have a structure of Acc65I-cDNA-XhoI and sub-cloned (FIG. 7).

1) Firefly luciferase: (GenBank Accession Number AY738224)

2) Renilla luciferase: (GenBank Accession Number AY738228)

3) Enhanced Green Fluorescent Protein (EGFP): (GenBank Accession Number U55761)

4) Puromycin resistant gene (synthesized while codons were optimized for human cells): Non-Patent Document 62, SEQ ID NO: 47

5) Cypridina noctiluca luciferase: Non-Patent Document 63 (GenBank Accession Number AB177531)

6) E2-Crimson: derived from pE2-Crimson (Clontech Laboratories, Inc), SEQ ID NO: 48

7) Enhanced Blue Fluorescent Protein 2 (EBFP2): Non-Patent Document 64 (GenBank Accession Number EF517318)

8) Zeocin resistant gene (synthesized while codons were optimized for human cells): Non-Patent Document 65, SEQ ID NO: 49

9) dKeima-Red:Non-Patent Document 66 (GenBank Accession

Number AB209968)

10) Hygromycin B resistant gene (synthesized while codons were optimized for human cells): Non-Patent Document 67, SEQ ID NO: 50

[0091]

(Example 2) Preparation of DNA fragment carrying ten exogenous genes (2)

Next, the following plasmids were prepared.

All the nucleic acids used in the present Example are DNA fragments, and a sequence specified as a negative-sense RNA sequence in the sequencing listings such as SEQ ID NO: 1 or SEQ ID NO: 4 means a corresponding DNA sequence. This also applies to other Examples using a DNA fragment.

1) Plasmid #1

Between the ApaI cleavage site and the StuI cleavage site of plasmid LITMUS38i (New England BioLab, Inc), a DNA having the following structure is cloned: SapI cleavage site-attB1 (SEQ ID NO: 51)-SEQ ID NO: 1-SEQ ID NO: 24-Acc65I cleavage site-SalI cleavage site-SEQ ID NO: 25-SEQ ID NO: 4-ctt-SEQ ID NO: 1-SEQ ID NO: 26-BsiWI cleavage site-XhoI cleavage site-SEQ ID NO: 27-SEQ ID NO: 4-SapI cleavage site

2) Plasmid #2

Between the ApaI cleavage site and the StuI cleavage site of plasmid LITMUS38i, a DNA having the following structure is cloned: SapI cleavage site-SEQ ID NO: 1-SEQ ID NO: 28-Acc65I

cleavage site-SalI cleavage site-SEQ ID NO: 29-SEQ ID NO: 4-ctt-SEQ ID NO: 1-SEQ ID NO: 30-BsiWI cleavage site-XhoI cleavage site-SEQ ID NO: 31-SEQ ID NO: 4-SapI cleavage site

3) Plasmid #3

Between the ApaI cleavage site and the StuI cleavage site of plasmid LITMUS38i, a DNA having the following structure is cloned: SapI cleavage site-SEQ ID NO: 1-SEQ ID NO: 32-Acc65I cleavage site-SalI cleavage site-SEQ ID NO: 33-SEQ ID NO: 4-ctt-SEQ ID NO: 1-SEQ ID NO: 34-BsiWI cleavage site-XhoI cleavage site-SEQ ID NO: 35-SEQ ID NO: 4-SapI cleavage site

4) Plasmid #4

Between the ApaI cleavage site and the StuI cleavage site of plasmid LITMUS38i, a DNA having the following structure is cloned: SapI cleavage site-SEQ ID NO: 1-SEQ ID NO: 36-Acc65I cleavage site-SalI cleavage site-SEQ ID NO: 37-SEQ ID NO: 4-ctt-SEQ ID NO: 1-SEQ ID NO: 38-BsiWI cleavage site-XhoI cleavage site-SEQ ID NO: 39-SEQ ID NO: 4-SapI cleavage site

5) Plasmid #5

Between the ApaI cleavage site and the StuI cleavage site of plasmid LITMUS38i, a DNA having the following structure is cloned: SapI cleavage site-SEQ ID NO: 1-SEQ ID NO: 36-Acc65I cleavage site-SalI cleavage site-SEQ ID NO: 37-SEQ ID NO: 4-ctt-SEQ ID NO: 1-SEQ ID NO: 38-BsiWI cleavage site-XhoI cleavage site-SEQ ID NO: 39-SEQ ID NO: 4-attB2 (SEQ ID NO: 52)-SapI cleavage site

[0092]

(Example 3) Preparation of DNA fragments carrying ten exogenous genes (3) (see FIG. 8)

Next, the following plasmids were prepared.

1) Plasmid #1C

Between Acc65I-SalI of plasmid #1, an Acc65I-XhoI fragment containing firefly luciferase gene was cloned to prepare plasmid #1B. Further, between BsiWI-XhoI of plasmid #1B, an Acc65I-XhoI fragment containing Renilla luciferase gene was cloned to prepare plasmid #1C.

2) Plasmid #2C

Between Acc65I-SalI of plasmid #2, an Acc65I-XhoI fragment containing EGFP gene was cloned to prepare plasmid #2B. Further, between BsiWI-XhoI of plasmid #2B, an Acc65I-XhoI fragment containing puromycin resistant gene was cloned to prepare plasmid #2C.

3) Plasmid #3C

Between Acc65I-SalI of plasmid #3, an Acc65I-XhoI fragment containing Cypridina noctiluca luciferase gene was cloned to prepare plasmid #3B. Further, between BsiWI-XhoI of plasmid #3B, an Acc65I-XhoI fragment containing E2-Crimson gene was cloned to prepare plasmid #3C.

4) Plasmid #4C

Between Acc65I-SalI of plasmid #4, an Acc65I-XhoI fragment containing EBFP2 gene was cloned to prepare plasmid #4B. Further,

between BsiWI-XhoI of plasmid #4B, an Acc65I-XhoI fragment containing Zeocin resistant gene was cloned to prepare plasmid #4C.

5) Plasmid #5C

Between Acc65I-SalI of plasmid #5, an Acc65I-XhoI fragment containing dKeima-Red gene was cloned to prepare plasmid #5B. Further, between BsiWI-XhoI of plasmid #5B, an Acc65I-XhoI fragment containing hygromycin B resistant gene was cloned to prepare plasmid #5C.

[0093]

(Example 4) Preparation of DNA fragments carrying ten exogenous genes (4) (see FIG. 9)

A total of 500 ng including 100 ng of a DNA fragment containing firefly luciferase gene and Renilla luciferase gene cut out from plasmid #1C with SapI, 100 ng of a DNA fragment containing EGFP gene and puromycin resistant gene cut out from plasmid #2C with SapI, 100 ng of a DNA fragment containing Cypridina noctiluca luciferase gene and E2-Crimson gene cut out from plasmid #3C with SapI, 100 ng of a DNA fragment containing EBFP2 gene and Zeocin resistant gene cut out from plasmid #4C with SapI, and 100 ng of a DNA fragment containing dKeima-Red gene and hygromycin B resistant gene cut out from plasmid #5C with SapI was dissolved in 5 μL of H_2O, and the solution was mixed with 5 μL of Ligation-Convenience Kit (NIPPON GENE Co., Ltd.) and allowed to react at 16°C for 60 minutes. After purification, the product

was dissolved in 7 μL of H_2O, and 1 μL of plasmid #6 (pDONR-221, Life Technologies, Inc.) (150 ng) and 2 μL of BP Clonase2 (Life Technologies, Inc.) were added and allowed to react at 25°C for 2 hours, and then the product was introduced into Escherichia coli DH-5α, and a kanamycin resistant colony was isolated to prepare plasmid #7.

[0094]

(Example 5) Preparation of template DNA for forming stealth RNA carrying ten exogenous genes (see FIG. 10)

Plasmid #8 is prepared by replacing the kanamycin resistant gene of plasmid pACYC177 having a replication origin of p15A (Non-Patent Document 56) with a DNA fragment containing attB1-chloramphenicol resistant gene-attB2 of pDONR-221. The DNA fragment in which attB1, T7 terminator, and HDV ribozyme are connected in sequence on 5′ side of a DNA containing hN-hC-hPolS optimized with OptimumGen Gene Design System (SEQ ID NO: 53) was synthesized by GenScript. Similarly, the DNA in which T7 promoter and attB2 are connected on 3′ side of a DNA containing hPolL optimized with OptimumGen Gene Design System (SEQ ID NO: 54) was synthesized. A total of 300 ng including 100 ng of a DNA fragment containing attB1-T7 terminator-HDV ribozyme- hN-hC-hPolS in this order cut out with BamHI and XmaI, 100 ng of a DNA fragment containing ten genes cut out from plasmid #7 with XmaI and NotI, and 100 ng of a DNA fragment containing hPolL-T7 promoter-attB2 in this order cut out with NotI and SalI

was dissolved in 5 μL of H_2O, and the solution was mixed with 5 μL of Ligation-Convenience Kit and allowed to react at 16°C for 60 minutes. After purification, the product was dissolved in 7 μL of H_2O, and 1 μL of plasmid #8 (150 ng) and 2 μL of BP Clonase2 were added and allowed to react at 25°C for 16 hours, and then the product was introduced into Escherichia coli HST-08 (Takara Bio Co.), and an ampicillin resistant colony was isolated to prepare plasmid #9B which is to be a template for synthesizing a negative-sense stealth RNA.

A template DNA for synthesizing a positive-sense stealth RNA is prepared by replacing T7 promoter with T7 terminator. Specifically, a total of 300 ng including 100 ng of a DNA fragment containing attB1-T7 promoter-hN-hC-hPolS in this order, 100 ng of a DNA fragment containing ten genes cut out from plasmid #7 with XmaI and NotI, and 100 ng of a DNA fragment containing hPolL-HDV ribozyme-T7 terminator-attB2 in this order cut out with NotI and SalI was dissolved in 5 μL of H_2O, and the solution was mixed with 5 μL of Ligation-Convenience Kit and allowed to react at 16°C for 60 minutes. After purification, the product was dissolved in 7 μL of H_2O, and 1 μL of plasmid #8 (150 ng) and 2 μL of BP Clonase2 were added and allowed to react at 25°C for 16 hours, and then the product was introduced into Escherichia coli HST-08, and an ampicillin resistant colony was isolated to prepare plasmid #9A which is to be a template for synthesizing a positive-sense stealth RNA.

[0095]

(Example 6) Reconstitution of stealth RNA gene expression system carrying ten exogenous genes (Method 1) (see FIG. 11)

Method 1 was conducted according to the method described in Patent Document 3 and Non-Patent Document 7.

Specifically, as BHK/T7/151M (SE) cells, BHK-21 cells derived from hamster in which T7 RNA polymerase and M protein are stably expressed were prepared in the following manner. BHK-21 cells were obtained from RIKEN BioResource Center. A cDNA synthesized by optimizing codons of T7 RNA polymerase gene (Non-Patent Document 74) for animal cells (Sequence information 77) was installed on a retrovirus vector pCX4neo (Non-Patent Document 75, GenBank Accession Number AB086385), and introduced into BHK-21 cells, and then selected in 10% FCS-containing DMEM culture medium containing 800 μg/mL of G-418 was conducted, and BHK/T7 cells were obtained. Next, M gene of Sendai virus temperature-sensitive mutant Clone 151 strain (GenBank Accession Number NM_011046) was installed on a retrovirus vector pCX4pur (Non-Patent Document 75, GenBank Accession Number AB086386), and introduced into BHK-21/T7 cells, and then selection in 10% FCS-containing DMEM culture medium containing 200 μg/mL of Puromycin was conducted, and thus BHK/T7/151M (SE) cells were obtained.

Expression vectors used in reconstitution were prepared in the following manner. A plasmid pCMV-NP for expressing NP

protein, a plasmid pCMV-P for expressing P protein, an plasmid pCMV-L for expressing L protein, and plasmid pCMV-Furin for expressing mouse Furin were prepared by respectively connecting NP gene, P gene, and L gene (GenBank Accession Number M30202.1) of Sendai virus Z strain, and mouse Furin cDNA (Non-Patent Document 76, GenBank Accession Number NM_011046) downstream the enhancer and the promoter of the Immediate Early gene of Cytomegalovirus (Non-Patent Document 77). A plasmid pSRD-HN-Fmut (Non-Patent Document 78) for expressing F and HN proteins is a plasmid in which F and HN genes of Sendai virus Z strain are connected downstream the SRα promoter (Non-Patent Document 79). pMKIT-151M was prepared by connecting M gene of Sendai virus temperature-sensitive mutant Clone 151 strain downstream the SRα promoter.

BHK/T7/151M (SE) cells stably expressing M protein were seeded on a 6-well plate at 5×10^5 cells/well, and cultured for 24 hours, and then washed. Plasmid #9A, a plasmid pCMV-NP for expressing NP protein, a plasmid pCMV-P for expressing P protein, a plasmid pCMV-L for expressing L protein, a plasmid pSRD-HN-Fmut for expressing F and HN protein, and a plasmid pCMV-Furin for expressing mouse Furin were suspended in 300 μL of OptiMEM (Life Technologies, Inc.) in a quantitative ratio of 2 μg, 1 μg, 1 μg, 1 μg, 2 μg, and 20 ng, respectively, and the suspension was mixed with 300 μL of OptiMEM containing 10 μL of Lipofectamine LTX (Life Technologies, Inc.) and left at

room temperature for 20 minutes. The culture medium thus prepared was added to cells and the cells were cultured for 4 hours. After washing the cells again, a 10% FCS-containing DMEM culture medium was further added, and the cells were further cultured at 32°C for 3 days. Then the cells were transferred to a 10% FCS-containing DMEM culture medium containing 300 μg/mL of hygromycin B, and cultivation was continued, and BHK/#9A cells were separated. Occurrence of the reconstitution of the stealth RNA gene expression system was confirmed by the expression of EGFP and Keima-Red.

[0096]

(Example 7) Reconstitution of stealth RNA gene expression system carrying ten exogenous genes (Method 2) (see FIG. 12)

Escherichia coli E-AIST7 strain which is a double deletion mutant of RecA and RNaseE was prepared by disrupting RNase E gene and Rec A gene of Escherichia coli BL21 (DE3) strain (Non-Patent Document 68) in this order. Deletion mutation of C-terminus of RNase E (rne131) was introduced into RNase E gene (Non-Patent Document 59), and complete deletion mutation was introduced into Rec A gene. The gene disruption was conducted by using Quick & Easy E. coli Gene Deletion Kit available from Gene Bridges GmbH according to the protocol of the kit. Plasmid #10 for expressing single-stranded RNA binding protein (N) in Escherichia coli was prepared by carrying N gene having codons optimized for Escherichia coli (eN) (SEQ ID NO: 55) on plasmid

pET-24a(+)(Merck KGaA).

Plasmid #9B and plasmid #10 were introduced into Escherichia coli E-AIST7 strain, and an E-AIST7/N/9B strain was prepared by selection with ampicillin and kanamycin. E-AIST7/N/9B strain was cultured at 30°C, and at OD_{600} = 0.3, 0.5 mM IPTG was added to induce expression of T7 RNA polymerase, and cultured for 3 hours and Escherichia coli was collected. The collected cells were suspended in 10 mL of 10% Sucrose, 50 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, and after addition of 150 kunits of rLysozome (Merck KGaA) and 25 units of Benzonase (Merck KGaA), the cells were treated at 30°C for 30 minutes, and protoplasts were collected. The protoplasts were broken with 50 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 50 mM CHAPS, and a supernatant from centrifugation at 4,500 rpm for 10 minutes was centrifuged at 25,000 rpm for 60 minutes with a Beckman SW41Ti rotor, and an RNA-N protein complex was collected as a precipitate. The RNA-N protein complex was further suspended in 28% calcium chloride, and centrifuged at 37,000 rpm for 45 hours with a Beckman SW41Ti rotor, and thus the RNA-N protein complex was purified.

BHK/T7/151M (SE) cells were seeded on a 6-well plate at 5 × 10^5 cells/well, and cultured for 24 hours, and then each 1 μg of plasmid pCMV-P for expressing P protein, and plasmid pCMV-L for expressing L protein were introduced by Lipofectamine LTX. After another 24 hours, 5 μg of the RNA-N protein complex was mixed with 10 μL of a Pro-DeliverIN reagent (OZ Biosciences)

and introduced into the cells. From after 24 hours, the cells were transferred to a 10% FCS-containing DMEM culture medium containing 300 μg/mL of hygromycin B and cultivation was continued, and BHK/#9A2 cells were separated. Occurrence of reconstitution of the stealth RNA gene expression system was confirmed by the expression of EGFP and Keima-Red.

[0097]

(Example 8) Preparation of stealth RNA vector #1 carrying ten exogenous genes

For 5.0×10^5 BHK/#9A cells (or BHK/#9A2 cells), pMKIT-151M, pSRD-HN-Fmut, and pCMV-Furin which are defective gene expression plasmids were introduced in a ratio of 2 μg, 2 μg, and 30 ng with Lipofectamine LTX, and after washing the cells after 4 hours, a 10% FCS-containing DMEM culture medium was added, and the cells were further cultured at 32°C for 4 days. Then the culture supernatant containing stealth RNA vector #1 (FIG. 13) was collected, and filtered through a 0.45 μm filter, and the vector was concentrated by ultracentrifugation as necessary. The vector suspension was rapidly frozen with liquid nitrogen, and stored at -80°C. The activity of the vector was assayed by an indirect fluorescent antibody method by an anti-NP protein antibody by using $LLCMK_2$ cells derived from monkey kidney (Non-Patent Document 7). The infectivity titer of the stealth RNA vector obtained by the present method was about 10^7 infectious units/mL, and equivalent or higher activity was obtained as

compared with a conventional persistent expression type Sendai virus vector.

[0098]

(Example 9) Gene expression by stealth RNA vector carrying ten exogenous genes (see FIG. 14)

HeLa cells were infected with stealth RNA vector #1 prepared in (Example 8) at MOI = 3, and HeLa/#9 cells were established by selection in a 10% FCS-containing DMEM culture medium containing 100 μg/mL of hygromycin B. The drug resistance of these cells was selected by puromycin (1.5 μg/mL), Zeocin (100 μg/mL), hygromycin B (100 μg/mL), G418 (800 μg/mL), and Blasticidin S (10 μg/mL), and the survival rate was measured by a colony assay. It was confirmed HeLa/#9 cells showed resistance selectively to puromycin, Zeocin, and hygromycin B and expressed the resistance characters to these three drugs unlike the negative control HeLa cells that are sensitive to all of these antibiotics (FIG. 14, upper stage).

Expression of fluorescent proteins in HeLa/#9 cells was measured with a flow cytometer (Gallios, Beckman Coulter). Observation conditions of individual fluorescent proteins are as follows. EBFP2: excitation 405nm, detection 450nm; Keima-Red: excitation 405 nm, detection 620 nm; EGFP: excitation 488 nm, detection 530 nm; E2-Crimson: excitation 638 nm, detection 660 nm. It was confirmed that HeLa/#9 cells significantly express four fluorescent proteins as compared with

HeLa cells not having a vector (FIG. 14, middle stage).

Expression of luciferase in HeLa/#9 cells was examined by detecting the emission with a luminometer (Promega, Corp.) by using the following reagents. Firefly luciferase and Renilla luciferase: Dual-Luciferase Reporter Assay System (Promega, Corp.); Cypridina noctiluca luciferase: BioLux Cypridina Luciferase Assay Kit (New England Biolabs, Inc.). Activity of any luciferase was not detected in HeLa cells not having a vector, but high activity was detected in HeLa/#9 cells (FIG. 14, lower stage).

[0099]

(Example 10) Preparation of stealth RNA vector #2 carrying ten exogenous genes (FIG. 15)

A vector was prepared in the same manner as described in (Example 8) and verified in the manner as described in (Example 9) except that hN, hC, hPolS, hPolL genes used for preparation of the template cDNA were optimized by GeneGPS Expression Optimization Technology, and three genes, hN, hC, and hPolS were installed in the order of hN-hPolS-hC. The DNA fragment in which attB1 and T7 promoter are connected in this order on 5′ side of the DNA containing hN-hPolS-hC (SEQ ID NO: 78) was synthesized by DNA 2.0. Similarly, the DNA fragment in which HDV ribozyme, T7 terminator, and attB2 are connected on 3′ side of the DNA containing hPolL (SEQ ID NO: 79) was synthesized by DNA 2.0.

[0100]

(Example 11) Preparation of stealth RNA vectors #3 and #4 (FIG. 16) carrying ten exogenous genes

A vector was prepared in the same manner as described in (Example 8) and verified in the manner as described in (Example 9) except that three genes, hN, hC, and hPolS optimized by OptimumGen Gene Design System was installed in the order of hN-hPolS-hC (#3) or in the order of hPolS-hN-hC (#4). The DNA fragment in which attB1 and T7 promoter are connected in this order on 5′ side of the DNA containing hN-hPolS-hC (SEQ ID NO: 80), the DNA fragment in which attB1 and T7 promoter are connected in this order on 5′ side of the DNA containing hPolS-hN-hC (SEQ ID NO: 81), and the DNA fragment in which HDV ribozyme, T7 terminator, and attB2 are connected on 3′ side of the DNA containing hPolL (SEQ ID NO: 82) were synthesized by GenScript.

[0101]

(Example 12) Preparation of stealth RNA vector #5 carrying four exogenous genes

Blasticidin S resistant gene (Non-Patent Document 69) (SEQ ID NO: 56) and Kusabira-Orange gene (Non-Patent Document 70) (GenBank Accession Number AB128819) were amplified to have a structure of Acc65I-cDNA-XhoI by PCR, and sub-cloned (FIG. 7). Plasmid #5D is obtained by cloning the DNA having the following structure between the ApaI cleavage site and the StuI cleavage site of LITMUS38i. However, the SapI digested end is different from that of plasmid #5: SapI cleavage site-SEQ ID NO: 1-SEQ

ID NO: 36-Acc65I cleavage site-SalI cleavage site-SEQ ID NO: 37-SEQ ID NO: 4-ctt-SEQ ID NO: 1-SEQ ID NO: 38-BsiWI cleavage site-XhoI cleavage site-SEQ ID NO: 39-SEQ ID NO: 4-attB2-SapI cleavage site.

Between Acc65I-SalI of plasmid #1, an Acc65I-XhoI fragment containing dKeima-Red gene was cloned to prepare plasmid #1D. Further, between BsiWI-XhoI of plasmid #1D, an Acc65I-XhoI fragment containing Blasticidin S resistant gene was cloned to prepare plasmid #1E. Further, between Acc65I-SalI of plasmid #5D, an Acc65I-XhoI fragment containing EGFP gene was cloned to prepare plasmid #5E. Further, between BsiWI-XhoI of plasmid #5E, an Acc65I-XhoI fragment containing Kusabira-Orange gene was cloned to prepare plasmid #5F.

A total of 200 ng including 100 ng of a DNA fragment containing dKeima-Red gene and Blasticidin S resistant gene cut out from plasmid #1E with SapI, and 100 ng of a DNA fragment containing EGFP gene and Kusabira-Orange gene cut out from plasmid #5F with SapI was dissolved in 5 μL of H_2O, and the solution was mixed with 5 μL of Ligation-Convenience Kit and allowed to react at 16°C for 60 minutes. After purification, the product was dissolved in 7 μL of H_2O, and 1 μL of plasmid #6 (150 ng) and 2 μL of BP Clonase2 were added and allowed to react at 25°C for 2 hours, and then the product was introduced into Escherichia coli DH-5α, and a kanamycin resistant colony was isolated to prepare plasmid #11. Preparation of stealth RNA vector #5 using

the DNA fragment containing four genes cut out from plasmid #11 with XmaI and NotI was conducted in the manner as described in (Example 5) to (Example 8).

[0102]

(Example 13) Induction of IFN-β gene by stealth RNA vector (FIG. 17)

Defective and persistent Sendai virus vector SeVdp (KR/Bsr/EGFP/KO) is described in Non-Patent Document 7. Primary culture human skin-derived fibroblasts were infected with stealth RNA vector #5 prepared in (Example 12), and SeVdp (KR/Bsr/EGFP/KO) vector at MOI = 3 each. Under this condition, both of the vectors could introduce genes into about 80% of the cells. At 24 hours after infection with the vectors, total RNA of cells was extracted by using ISOGEN Kit (NIPPON GENE Co., Ltd.), and genomic DNA was degraded by using Deoxyribonuclease (RT Grade) (NIPPON GENE Co., Ltd.). Next, using this RNA as a template, First strand cDNA synthesis was conducted by reverse transcription reaction by using SuperScriptIII First-Strand Synthesis System for RT-PCR (Life Technologies, Inc.) and oligo(dT)20. Further, by using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad), and using the first strand cDNA as a template, an expression amount of IFN-β mRNA was analyzed by the real-time PCR method using Gene Specific Primers (GSP) of a reference gene or interferon beta gene, and CFX96 Real-Time System (Bio-Rad).

[0103]

(Example 14) Preparation of stealth RNA gene expression systems #6, #7, #8, #9 and #10 carrying six exogenous genes (FIG. 20 and FIG. 22)

Plasmid #2D was obtained by cloning the DNA having the following structure between the ApaI cleavage site and the StuI cleavage site of plasmid LITMUS38i. However, the SapI digested end is different from that of plasmid #2: SapI cleavage site-SEQ ID NO: 1-SEQ ID NO: 28-Acc65I cleavage site-SalI cleavage site-SEQ ID NO: 29-SEQ ID NO: 4-ctt-SEQ ID NO: 1-SEQ ID NO: 30-BsiWI cleavage site-XhoI cleavage site-SEQ ID NO: 31-SEQ ID NO: 4-SapI cleavage site.

Between Acc65I-SalI of plasmid #2D, an Acc65I-XhoI fragment containing EGFP gene was cloned to prepare plasmid #2E. Further, between BsiWI-XhoI of plasmid #2E, an Acc65I-XhoI fragment containing puromycin resistant gene was cloned to prepare plasmid #2F.

A total of 300 ng including 100 ng of a DNA fragment containing firefly luciferase gene and Renilla luciferase gene cut out from plasmid #1C with SapI, 100 ng of a DNA fragment containing EGFP gene and puromycin resistant gene cut out from plasmid #2F with SapI, and 100 ng of a DNA fragment containing dKeima-Red gene and hygromycin Bresistant gene cut out from plasmid #5C with SapI was dissolved in 5 μL of H_2O, and the solution was mixed with 5 μL of Ligation-Convenience Kit and allowed to react at

16°C for 60 minutes. After purification, the product was dissolved in 7 μL of H_2O, and 1 μL of plasmid #6 (150 ng) and 2 μL of BP Clonase2 were added and allowed to react at 25°C for 2 hours, and then the product was introduced into Escherichia coli DH-5α, and a kanamycin resistant colony was isolated to prepare plasmid #12. Preparation of stealth RNA gene expression systems #6, #7 and #8 (FIG. 20) (Example 16) and #9 and #10 (FIG. 22) (Example 18) using the DNA fragment containing six genes cut out from plasmid #12 with XmaI and NotI was conducted in the manner as described in (Example 5), (Example 6) and (Example 8).

[0104]

(Example 15) Preparation of stealth RNA gene expression systems #11, #12, #13, #14 and #15 carrying five exogenous genes (FIG. 18)

Plasmid #13 carrying five genes was prepared in the manner as described in (Example 14) except that among the genes installed on plasmid #12 in (Example 14), firefly luciferase gene was deleted, and puromycin resistant gene was replaced by tetracycline resistant gene derived from plasmid pBR322 (GenBank Accession Number J01749.1).

These five exogenous genes were installed on stealth RNA vector #3 (FIG. 16) carrying three genes, hN, hC, and hPolS in the order of hN-hPolS-hC, and stealth RNA gene expression system #11 carrying five exogenous genes were prepared. Further, in

the XmaI site of this stealth RNA gene expression system, a gene cassette containing codon-optimized RIG-IC (SEQ ID NO: 83), a gene cassette containing codon-optimized C-terminal region of Sendai virus V protein (SEQ ID NO: 84), or a gene cassette containing codon-optimized PSMA7 which is a constituent of proteasome (SEQ ID NO: 85) was inserted, and thus stealth RNA gene expression systems #12, #13 and #14 carrying five exogenous genes were prepared. Stealth RNA gene expression system #15 carrying five exogenous genes is designed to express V protein by replacing part of hPolS gene of hN-hPolS-hC gene with P gene of non-optimized Sendai virus Z strain (SEQ ID NO: 86).

From cells containing these stealth RNA gene expression systems, stealth RNA vectors were prepared according to Example 8, and introduced into human-derived 293 cells, and interferon inducibility was measured. In FIG. 19, stealth RNA vectors #11 and #12 were compared as representative, and it was shown that by adding RIG-IC gene, induction of interferon beta remaining in the stealth RNA vector is almost completely suppressed.

[0105]

(Example 16) Analysis of influence of variation in expression efficiency of N protein and C protein on expression of exogenous genes installed on stealth RNA gene expression system (see FIG. 20)

In front of the translation initiation codon (AUG) of firefly luciferase cDNA encoded by pGL4.12 (Promega Corporation)

(GenBank Accession Number AY738224), an RNA sequence corresponding to SEQ ID NO: 56, SEQ ID NO: 57 or SEQ ID NO: 58 was inserted, and firefly luciferase was expressed in HeLa cells by using a CMV promoter, and activity of luciferase was examined by using Dual-Luciferase Reporter Assay System (FIG. 20). It was demonstrated that when an another initiation codon is placed at out-frame position and upstream to the authentic translation initiation codon, the translating frame is shifted from the original translating frame of the protein, and the translation efficiency is deteriorated.

Next, stealth RNA gene expression systems #6, #7 and #8 in which a base sequence on 5′ upstream side of the translation initiation codon of hN mRNA and hC mRNA was modified were examined for their gene expression ability. In stealth RNA gene expression system #6, a so-called "Kozak sequence (SEQ ID NO: 57)" which is believed to provide the highest translation efficiency is positioned on 5′ upstream side of the translation initiation codon (AUG) of hN mRNA and hC mRNA. In stealth RNA gene expression system #7, 5′ upstream side of the translation initiation codon of hN mRNA is replaced by "Kozak sequence (SEQ ID NO: 57)" (Non-Patent Document 60), and 5′ upstream side of the translation initiation codon of hC mRNA is replaced by the base sequence of SEQ ID NO: 59 that lowers the translation efficiency to 23%. In stealth RNA gene expression system #8, 5′ upstream side of the translation initiation codon of hN mRNA

is replaced by the base sequence of SEQ ID NO: 58 that lowers the translation efficiency to 40%, and 5' upstream side of the translation initiation codon of hC mRNA is replaced by the base sequence of SEQ ID NO: 59 that lowers the translation efficiency to 23%. In this experiment, activity of luciferase was examined in BHK/T7/151M (SE) cells that stably retain stealth RNA gene expression systems #6, #7 or #8, by using Dual-Luciferase Reporter Assay System (FIG. 20).

[0106]

(Example 17) Preparation of stealth RNA gene expression systems #16 and #17 carrying five exogenous genes (FIG. 21)

Stealth RNA gene expression system #11 in which three genes, hN, hC, and hPolS are installed in the order of hN-hPolS-hC, and five exogenous genes are installed has been described in (Example 15). In hC gene of this vector, the translation efficiency is lowered to 23% by modification of the sequence of 5' untranslated region (FIG. 21). Stealth RNA gene expression system #16 is a system in which hC gene is removed from #11. The stealth RNA gene expression system #17 is a system in which the 5' untranslated sequence of hC gene in #11 is replaced by the Kozak sequence to achieve 100% of the translation efficiency.

As can be realized from the expression of EGFP shown in FIG. 21, the gene expression level of the stealth RNA gene expression system can be regulated by changing the translation efficiency of hC gene. Although hC gene is not an essential element for

reconstitution of a stealth RNA gene expression system, lack of hC gene results in very strong expression of exogenous genes, and thus proliferation of cells is suppressed. Therefore, it is realistic to obtain gene expression of the practical level by allowing a certain degree of expression of hC gene.

[0107]

(Example 18) Analysis of influence of packaging signal on genome 3′ side of stealth RNA gene expression system on production of vector particle (see FIG. 22)

Stealth RNA gene expression system #9 is a system in which sequence D on 3' side of genome RNA (SEQ ID NO: 17) is deleted from stealth RNA gene expression system #6 (FIG. 20). Stealth RNA gene expression system #10 is a system in which sequence D on 3' side of genome RNA (SEQ ID NO: 17) is deleted from stealth RNA gene expression system #7 (FIG. 20). In BHK/T7/151M (SE) cells stably retaining these stealth RNA gene expression systems, proteins M, F, and HN were expressed in the manner as described in (Example 5), and the gene introduction ability of the stealth RNA vector collected in the supernatant was assayed by an indirect fluorescent antibody method using an anti-NP protein antibody and $LLCMK_2$ cells (Non-Patent Document 7).

Further, this sequence of 18 nucleotides was replaced by an arbitrarily selected partial sequence of mRNA derived from House-keeping gene recited in (Table 1) ((5) of FIG. 2 (SEQ ID NO: 75)), and no variation was observed in the particle formation

efficiency (data not shown).

From the above, it can be considered that the region having a length of 18 nucleotides from the 97th to 114th nucleotides from 3' terminus of the genome or a region having a partial length thereof is an essential for packaging for particle formation in the negative-sense single-stranded RNA. In any case, it can be concluded that the region having a length of 18 nucleotides or a region having a partial length thereof at this position is "packaging signal region" that is essential for the stealth RNA gene expression system to be incorporated into the virus-like particle, although it is not essential for transcription and replication from the negative-sense single-stranded RNA as a template.

[0108]

(Example 19) Variation with time of luciferase activity when HeLa cells retaining stealth RNA gene expression system are treated with siRNA (see FIG. 23)

Stealth RNA vector #6 was prepared from stealth RNA gene expression system #6 (FIG. 20), and gene introduction into HeLa cells and selection with hygromycin B were conducted, and thus HeLa/#3 cell strain was established. HeLa/#3 cells were seeded on a 48-well plate at 1.0×10^4 /well, and on the next day, siRNA targeting a target sequence of PolL gene (SEQ ID NO: 46) was mixed with an introducing reagent RNAiMAX (Life Technologies, Inc.) in a final concentration 100 nM and introduced into the

cells. Luciferase activity was measured over time, and in all the four independent experiments, luciferase activity was suppressed to about 0.1% in 10 days. This reveals that the stealth RNA gene expression system was removed from cells efficiently.

[0109]

(Example 20) Preparation of stealth RNA vector carrying giant gene (see FIG. 24)

Stealth RNA vectors carrying various exogenous genes can be prepared by producing "transcription cassettes" each consisting of two genes in the same manner as in (Example 1) to (Example 2), sequentially linking the "transcription cassettes" in the same manner as in (Example 3) to (Example 5), and preparing in the same manner as in (Example 6) or (Example 7) and (Example 8). Names and base sequences of exogenous genes that can be installed as such a giant exogenous gene are as follows. Human KLF4: SEQ ID NO: 60, human OCT4: SEQ ID NO: 61, human SOX2: SEQ ID NO: 62, human c-Myc: SEQ ID NO: 63, human BRG1: SEQ ID NO: 64, human BAF155: SEQ ID NO: 65, human immunoglobulin G H chain: SEQ ID NO: 66, human immunoglobulin G L chain: SEQ ID NO: 67, human immunoglobulin M clone 2G9 H chain: SEQ ID NO: 68, human immunoglobulin M clone 2G9 L chain: SEQ ID NO: 69, human immunoglobulin M J chain: SEQ ID NO: 70, human blood coagulation factor VIII: SEQ ID NO: 71, and human dystrophin: SEQ ID NO: 72.

RNA expression systems carrying these genes as exogenous genes can be introduced into target cells in the technique corresponding to the procedure described in the foregoing Examples. By expressing plural exogenous genes simultaneously in the same cell, it becomes possible to add a desired modification such as cell-reprogramming to the introduced cells.

[0110]

(Example 21) Induction of induced pluripotent stem cells (iPS cells) by stealth RNA vector carrying six reprogramming genes (FIG. 25)

The capability of carrying six or more genes and expressing them securely, which is a feature of the stealth RNA vector would be particularly effective for cell-reprogramming in which human somatic cells are initialized and converted to iPS cells. Thus, the present inventors prepared a stealth RNA vector simultaneously expressing a total of six reprogramming genes by adding reprogramming genes NANOG and LIN28 (Patent Document 2, and Non-Patent Document 2) having complementary functions to the combination of four reprogramming genes, KLF4, OCT4, SOX2, and c-MYC that was first reported as a method for making human induced pluripotent stem cells (Patent Document 1, and Non-Patent Document 1), and compared the cell-reprogramming activity between the stealth RNA vector and the "persistent expression type Sendai virus vector simultaneously carrying the four reprogramming genes (KLF4, OCT4, SOX2 and c-MYC)" having the

highest reprogramming efficiency among the iPS cell preparation techniques that have been reported heretofore (Patent Document 3, Patent Document 4, and Non-Patent Document 7)(FIG. 25A).

Stealth RNA vector #23 carrying six reprogramming genes (FIG. 25B) was prepared according to Example 6 and Example 8 by binding human KLF4 (SEQ ID NO: 60), human OCT4 (SEQ ID NO: 61), human SOX2 (SEQ ID NO: 62), human c-MYC (SEQ ID NO: 63), human NANOG (SEQ ID NO: 87), and human LIN28 (SEQ ID NO: 88) in this order by the method shown in Example 14, and incorporating the genes into stealth RNA vector #3 of FIG. 16.

Preparation of iPS cells was conducted according to Patent Document 3. To be more specific, TIG3 cells derived from human embryonic fibroblasts were seeded on a 12-well plate at 1.0×10^5 cells/well, and on the next day, a Sendai virus vector for persistent expression carrying KLF4, OCT4, SOX2, and c-MYC (FIG. 25A), and a stealth RNA vector carrying KLF4, OCT4, SOX2, c-MYC, NANOG, and LIN28 (FIG. 25B) were added into the culture medium in the condition of MOI (Multiplicity of Infection) = 3, and left still for 2 hours at room temperature, and then cultured overnight at 37°C to infect the cells. MEF treated with mitomycin C was prepared as feeder cells on a gelatin-coated dish, and the aforementioned cells transfected with the vector were seeded thereon, and cultured in a culture medium for human multipotent stem cells StemFit AK03 (Ajinomoto, Co., Inc.). At 11 days after gene introduction, cells were stained with

AlexaFluor488-labeled anti-TRA-1-60 antigen antibody (Merck-Millipore), and the number of clones of TRA-1-60 positive iPS cells appeared from 1×10^4 TIG-3 cells was counted (FIG. 25C). While 85 clones of iPS cell clones appeared by the four-factor-carrying vector (reprogrammingefficiency 0.85%), 4290 clones of iPS cell clones appeared by the six-factor-carrying vector (reprogrammingefficiency 42.9%), revealing the effectiveness of the stealth RNA vector carrying six genes. The total nucleotide length of genes used in the present Example is 7.0 kb, and this size cannot be realized by a method using a conventional RNA vector.

[0111]

(Example 22) Production of human immunoglobulin M by simultaneous expression of H chain, L chain, and J chain of human immunoglobulin M (IgM) (FIG. 26)

As a representative product for which simultaneous expression of plural polypeptides is required in the field of production of biopharmaceuticals, antibody drugs are recited. While the commercial production technology of immunoglobulin G (IgG) capable of expressing and producing H chain and L chain has been already established, production of IgM for which simultaneous expression of three genes encoding H chain, L chain, and J chain are required is not still easy today (Non-Patent Document 84). It is known that in IgM, there is an antibody having strong antitumor activity that is not present in IgG

(Non-Patent Document 85), and establishment of a production method of IgM is industrially very significant. Thus, the present inventors attempted to produce an IgM having a molecular weight of 950 k Dalton by carrying three genes that encode H chain, L chain and J chain of human IgM on a stealth RNA vector and expressing them simultaneously.

In Example 22, human monoclonal IgM antibodies 9F11 and 2G9 that react with the cells infected with human immunodeficiency virus (HIV) (Non-Patent Document 86) were selected as a material, and a set of H chain gene (SEQ ID NO: 89) and L chain gene (SEQ ID NO: 90) of 9F11 antibody, J chain gene (SEQ ID NO: 70), and hygromycin B resistant gene (SEQ ID NO: 50), or a set of H chain gene (SEQ ID NO: 68) and L chain gene (SEQ ID NO: 69) of 2G9 antibody, J chain gene (SEQ ID NO: 70), and hygromycin B resistant gene (SEQ ID NO: 50) were linked in this order according to Example 12, and installed on stealth RNA vector #8 of FIG. 20, to obtain stealth RNA vectors #23 and #20. Then gene introduction into BHK cells derived from hamster acclimated to a serum-free culture medium, Opti-Pro SFM (Life Technologies, Inc.) for protein production was conducted in the condition of MOI = 3, and selection was conducted by adding 100 μg/mL hygromycin B. After renewing the culture medium, cells were collected after 24 hours of culture, and the culture supernatant was collected.

The amount of human IgM in the culture supernatant was quantified by an anti-human IgM ELISA kit (Bethyl Laboratories,

Inc.), and 9.17 μg/mL of IgM was detected when the gene set of 2G9 was introduced, and 11.15 μg/mL of IgM was detected when the gene set of 9F11 was introduced. IgM in the culture supernatant of BHK cells into which genes were not introduced was under or equal to the detection limit. Expression efficiency per cell per day (pg/cell/day) converted from the above amount was 16.38 pg/cell/day for 2G9, and 19.91 pg/cell/day for 9F11 (FIG. 26).

Then, the culture supernatants containing 300 ng and 100 ng of IgM were analyzed by SDS polyacrylamide gel electrophoresis using 4–20% Gradient Gel (Bio-Rad), and stained with BioSafe Coomasie G250 stain (Bio-Rad). Under a non-reduced condition, a band was detected at the position of 970 kDa as is the same with native human IgM, and under a reduced condition, bands were detected at the positions of H chain of molecular weight of 75 kDa and L chain of 25 kDa. This reveals that an IgM molecule in which 21 polypeptides are bound, that is the same with the native one is generated.

In Non-Patent Document 84, analytical results in four clones of cells stably expressing IgM obtained as a result of gene amplification with methotrexate by using CHO-DG44 cells and HEK293 cells are described, and the expression efficiency was 25.00, 3.59, 4.60, and 0.21 pg/cell/day, respectively. This reveals that by using a stealth RNA vector, it is possible to easily realize production at an equivalent or higher level

compared with expression of IgM achieved by gene amplification that requires several months.

[0112]

(Example 23) Production of human bispecific antibody by simultaneous expression of four cDNAs (FIG. 27)

Recently, bispecific antibodies capable of recognizing two different antigens attract attentions in the field of biopharmaceuticals as a molecule that greatly extends the possibility of the existing antibody drugs. A bispecific antibody is a tetramer made up of H chain (A) and L chain (A) that recognize antigen A, and H chain (B) and L chain (B) that recognize antigen B, and is prepared by introducing a mutation so that H chain (A) and L chain (B), or H chain (B) and L chain (A) are difficult to bind each other, and introducing a mutation so that binding between H chain (A) and H chain (B) is stronger than binding between H chains (A) or binding between H chains (B), and then expressing four genes encoding H chain (A), L chain (A), H chain (B), and L chain (B) simultaneously (Non-Patent Document 87). Since it is very difficult to obtain a cell strain that simultaneously expresses four polypeptides by gene amplification after simultaneous introduction of these four genes into cells, it is normally produced by transient gene expression. In the present Example, the present inventors attempted to prepare HEDesignLK that simultaneously recognizes HER2 and an epithelial growth factor receptor (EGFR) among the

bispecific antibodies described in Non-Patent Document 87.

H chain HC1 ($VH_{VRD1}CH1_{CRD2}$) gene (SEQ ID NO: 91) and L chain LC1 ($VL_{VRD1}C\lambda_{CRD2}$) gene (SEQ ID NO: 92) of anti-HER2 antibody, and H chain HC2 ($VH_{VRD2}CH1_{WT}$) gene (SEQ ID NO: 93) and L chain LC2 ($VL_{VRD2}C\kappa_{WT}$) gene (SEQ ID NO: 94) of anti-EGFR antibody disclosed in Non-Patent Document 87 were linked together with EGFP gene and hygromycin B resistant gene according to Example 14, and installed on stealth RNA vector #8 (FIG. 20) to prepare stealth RNA vector #24. For comparison, vector #25 for expressing only H chain and L chain of anti-HER2 antibody (FIG. 27B) and vector #26 for expressing only H chain and L chain of anti-EGFR antibody (FIG. 27C) were prepared according to Example 12.

Using these vectors, genes were introduced into BHK cells derived from hamster acclimated to Opti-Pro SFM (Life Technologies, Inc.) by the method of Example 22, and an amount of human IgG in the culture supernatant of stably expressing cells was quantified by an anti-human IgG ELISA kit (Bethyl Laboratories, Inc.). In contrast with the combination of only HC1 and LC1 (12.93 pg/cell/day), or the combination of HC2 and LC2 (14.02 pg/cell/day) that is poor in activity of forming a tetramer, significantly high (37.45 pg/cell/day) antibody production was observed when four genes, HC1, LC1, HC2, and LC2 were installed. This suggests that the bispecific antibody is produced efficiently. This expression level is comparable to

the gene expression level in a general cell strain established by CHO cells using gene amplification (about 90 pg/cell/day at maximum) (Non-Patent Document 88). This suggests that as a method for stably producing a bispecific antibody for which a stably expressing cell strain has been difficult to be obtained by conventional methods, the stealth RNA vector is very useful. The total nucleotide length of the genes used in the present Example is 6.7 k nucleotides, and this size cannot be realized by a method using a conventional RNA vector.

Industrial Applicability

[0113]

The present invention is useful in various industrial fields including reprogramming of human cells including preparation of induced pluripotent stem cells (iPS cells), production of protein drugs, gene therapy by various genes including giant genes, and expression of drug-discovery target molecules.

CLAIMS

1. A stealth RNA gene expression system comprising:

a negative-sense single-stranded RNA (A),

a single-stranded RNA binding protein (B), and

an RNA-dependent RNA polymerase (C),

wherein the stealth RNA gene expression system is a complex that does not activate an innate immune system,

wherein the negative-sense single-stranded RNA (A) comprises RNA sequences (1) to (8):

(1) RNA sequences encoding any given protein or functional RNA,

(2) RNA sequences constituting noncoding region(s), the RNA sequences (2) having a length of 5 nucleotides to 49 nucleotides and being fragments of mRNA(s) expressed in animal cells,

(3) transcription start signal sequences recognized by the RNA-dependent RNA polymerase,

(4) transcription termination signal sequences recognized by the RNA-dependent RNA polymerase,

(5) RNA sequences containing replication origins recognized by the RNA-dependent RNA polymerase,

(6) RNA sequences encoding the RNA-dependent RNA polymerase with codons optimized for a biological species of cells for transfection,

(7) an RNA sequence encoding a protein that regulates

activity of the RNA-dependent RNA polymerase with codons optimized for the biological species of the cells for transfection, and

(8) an RNA sequence encoding the single-stranded RNA binding protein with codons optimized for the biological species of cells for transfection,

wherein the RNA-dependent RNA polymerase encoded by the RNA sequences of the (6) consists of L protein and P protein of an RNA virus belonging to a paramyxovirus family,

wherein the protein that regulates activity of the RNA-dependent RNA polymerase encoded by the RNA sequence of the (7) is C protein of the RNA virus belonging to the paramyxovirus family,

wherein the single-stranded RNA binding protein encoded by the RNA sequence of the (8) is NP protein of the RNA virus belonging to the paramyxovirus family,

wherein all of the RNA sequences of the (3) to (5) are RNA sequences comprising a transcription start signal, a transcription termination signal, or a replication origin sequence isolated from a genome of the same virus as the RNA virus, and

wherein the RNA sequences encoding the L protein, P protein, C protein and NP protein are optimized for human cells, and have a GC content adjusted within a range of 50

to 60%.

2. The stealth RNA gene expression system according to claim 1, wherein the RNA sequences of the (1) comprise at least six genes, or are RNA sequences having a total length of 5000 or more nucleotides.

3. The stealth RNA gene expression system according to claim 1, wherein the RNA sequences of the (2) are fragments of mRNA(s) expressed in human cells.

4. The stealth RNA gene expression system according to claim 1, wherein each of the RNA sequences of the (2) has sequences identical to or different from one another and is adjacent to the 3′ terminal site and/or the 5′ terminal site of each of gene sequences comprised in the RNA sequences of the (1).

5. The stealth RNA gene expression system according to claim 1, wherein the RNA virus belonging to a paramyxovirus family is an RNA virus selected from the group consisting of Sendai virus, human parainfluenza virus, and Newcastle disease virus.

6. The stealth RNA gene expression system according to

claim 1,

wherein the transcription start signal sequences of the (3) are RNA sequences selected from the group of RNA sequences consisting of 3′-UCCCACUUUC-5′ (SEQ ID NO: 1), 3′-UCCCUAUUUC-5′ (SEQ ID NO: 2), 3′-UCCCACUUAC-5′ (SEQ ID NO: 3), 3′-UCCUAAUUUC-5′ (SEQ ID NO: 7), and 3′-UGCCCAUCUUC-5′ (SEQ ID NO: 9), and

wherein the transcription termination signal sequences of the (4) are RNA sequences selected from the group of RNA sequences consisting of 3′-AAUUCUUUUU-5′ (SEQ ID NO: 4), 3′-CAUUCUUUUU-5′ (SEQ ID NO: 5), 3′-UAUUCUUUUU-5′ (SEQ ID NO: 6), and 3′-UUAUUCUUUUU-5′ (SEQ ID NO: 8).

7. The stealth RNA gene expression system according to claim 4,

wherein each of the transcription start signal sequences of the (3) having sequence identical to or different from one another is adjacent to the 3′ terminal site of each of the RNA sequences of the (2) that is adjacent to the 3′ terminal site of each of gene sequences comprised in the RNA sequences of the (1), and

wherein each of the transcription termination signal sequences of the (4) is adjacent to the 5′ terminal site of the RNA sequence that is adjacent to the 5′ terminal site of each of gene sequences comprised in the RNA sequences of

the (1).

8. The stealth RNA gene expression system according to claim 5, wherein the RNA sequences comprising replication origins of the (5) comprise the following sequences (a), (b), (c) and (d):

(a) either the RNA sequence of 3′-UGGUCUGUUCUC-5′ (SEQ ID NO: 11) or 3′-UGGUUUGUUCUC-5′ (SEQ ID NO: 12),

(b) either the RNA sequence represented by 3′-GAGAACAGACCA-5′ (SEQ ID NO: 13) or 3′-GAGAACAAACCA-5′ (SEQ ID NO: 14),

(c) the RNA sequence represented by 3′-$(CNNNNN)_3$-5′ (SEQ ID NO: 15), and

(d) the RNA sequence represented by 3′-$(NNNNNG)_3$-5′ (SEQ ID NO: 16).

9. The stealth RNA gene expression system according to claim 8, wherein the RNA sequence of the (a) is positioned at the 3′ terminus of the negative-sense single-stranded RNA (A), and the RNA sequence of the (b) is positioned at the 5′ terminus.

10. The stealth RNA gene expression system according to claim 8, wherein the RNA sequence of the (c) starts at 79th nucleotide from the 3′ terminus of the negative-sense single-stranded RNA (A), and the RNA sequence of the (d)

starts at 96th nucleotide from 5′ terminus.

11. The stealth RNA gene expression system according to claim 8, wherein the RNA sequences comprising replication origins of the (5) further comprise, in a position of 97th to 116th nucleotides from the 3′ terminus of the negative-sense single-stranded RNA (A), the RNA sequence of (e) 3′-AAAGAAACGACGGUUUCA-5′ (SEQ ID NO: 17) or an RNA sequence having the same length of 18 nucleotides as the (e).

12. A stealth RNA vector comprising:

a complex comprising the stealth RNA gene expression system according to claim 1,

wherein the stealth RNA vector is capable of introducing the complex into animal cells, and

wherein the stealth RNA vector does not activate an innate immune system.

13. The stealth RNA vector according to claim 12, wherein the stealth RNA vector forms a virus particle capable of infecting animal cells.

14. An isolated animal cell transfected with the stealth RNA vector according to claim 12.

15. A stealth RNA which is a negative-sense single-stranded RNA (A) comprising:

(1) RNA sequences encoding any given protein or functional RNA,

(2) RNA sequences constituting noncoding region(s) that are unrecognizable by an innate immune system,

(3) transcription start signal sequences recognized by an RNA-dependent RNA polymerase,

(4) transcription termination signal sequences recognized by the RNA-dependent RNA polymerase,

(5) RNA sequences comprising replication origins recognized by the RNA-dependent RNA polymerase,

(6) RNA sequences encoding the RNA-dependent RNA polymerase and having a structure optimized to be unrecognizable by an innate immune system,

(7) an RNA sequence encoding a protein that regulates activity of the RNA-dependent RNA polymerase, and having a structure optimized to be unrecognizable by an innate immune system, and

(8) an RNA sequence encoding a single-stranded RNA binding protein and having a structure optimized to be unrecognizable by an innate immune system;

wherein the stealth RNA is capable of forming a complex that does not activate an innate immune system together with the single-stranded RNA binding protein (B), and the RNA-

dependent RNA polymerase (C),

wherein the RNA sequences of the (2) are RNA sequences having a length of 5 nucleotides to 49 nucleotides, and are fragments of mRNA(s) expressed in animal cells,

wherein the RNA-dependent RNA polymerase encoded by the RNA sequences of the (6) consists of L protein and P protein of an RNA virus belonging to a paramyxovirus family,

wherein the protein that regulates activity of the RNA-dependent RNA polymerase encoded by the RNA sequence of the (7) is C protein of the RNA virus belonging to the paramyxovirus family,

wherein the single-stranded RNA binding protein encoded by the RNA sequence of the (8) is NP protein of the RNA virus belonging to the paramyxovirus family from the same virus as the RNA virus,

wherein all of the RNA sequences of the (3) to (5) are RNA sequences comprising a transcription start signal, a transcription termination signal, or a replication origin sequence isolated from a genome of the same virus as the RNA virus, and

wherein the RNA sequences encoding the L protein, P protein, C protein and NP protein are optimized for human cells, and have a GC content adjusted within a range of 50 to 60%.

16. The stealth RNA according to claim 15,

wherein RNA sequences containing replication origins recognized by the RNA-dependent RNA polymerase of the (5) are located at the 3′ terminal site and the 5′ terminal site of the negative-sense single-stranded RNA (A), and

wherein the RNA sequence located at the 3' terminal site and the RNA sequence located at the 5' terminal site comprise RNA sequences complementary to each other.

17. The stealth RNA according to claim 15,

wherein each of the transcription start signal sequences of the (3) having sequences identical to or different from one another is placed adjacent to the 3′ terminal site of each of the RNA sequences of the (2) that is adjacent to the 3′ terminal site of each of plural gene sequences comprised in the RNA sequences of the (1), and

wherein each of the transcription termination signal sequences of the (4) is adjacent to the 5′ terminal site of the RNA sequence that is adjacent to the 5′ terminal site of each of plural gene sequences comprised in the RNA sequence of the (1).

18. The stealth RNA according to claim 15,

wherein each of the transcription start signal sequences of the (3) having sequences identical to or

different from one another is adjacent to the 3′ terminal site of each of the RNA sequences of the (2) that is adjacent to the 3′ terminal site of each of plural gene sequences comprised in the RNA sequences of the (1),

wherein each of the transcription termination signal sequences of the (4) is adjacent to the 5′ terminal site of the RNA sequence that is adjacent to the 5′ terminal site of each of plural gene sequences comprised in the RNA sequences of the (1),

wherein both of the transcription start signal sequences of the (3) and the transcription start signal sequences of the (4) constitute a cassette structure, together with restriction sites located at both ends of the cassette that can be cleaved by plural restriction endonucleases, and

wherein plural cassette structures are bound to each other.

19. A method for reconstituting a stealth RNA gene expression system, comprising:

(1) preparing an Escherichia coli expressing T7 RNA polymerase;

(2) introducing into the Escherichia coli host of the (1), at least a vector for Escherichia coli carrying an RNA encoding an RNA-dependent RNA polymerase and an RNA binding

protein, and a vector for Escherichia coli for expressing a DNA encoding RNA binding protein, together with the negative-sense single-stranded RNA (A) according to claim 1 to transform the host,

(3) forming a complex of the negative-sense single-stranded RNA comprising exogenous gene RNA expressed by T7 RNA polymerase, and RNA binding protein in the transformed Escherichia coli of the (2),

(4) preparing animal cells in which an RNA-dependent RNA polymerase is expressed, and

(5) introducing the complex of the negative-sense single-stranded RNA and the RNA binding protein obtained in the (3) into an animal cell host of the (4) to reconstitute a stealth RNA gene expression system comprising the negative-sense single-stranded RNA, and the complex of the RNA binding protein and the RNA-dependent RNA polymerase.