DESCRIPTION

SYSTEM FOR INCREASING GENE EXPRESSION AND VECTOR COMPRISING THE SYSTEM

TECHNICAL FIELD

The present invention relates to a method for increasing gene expression using promoters, enhancers, and the like, and an expression cassette for increasing gene expression, comprising promoters, enhancers, and the like.

BACKGROUND ART

Various gene expression promoters, such as CMV promoters and CAG promoters, have been developed to increase gene expression efficiency (patent documents 1 to 4). However, the use of these conventional techniques causes daily problems in the field of biotechnology, such as situations in which almost no gene expression takes place or the amount of the thus-expressed protein is extremely low, depending on cell type or gene type. Furthermore, these problems serve as significant barriers to the development of medical science in which gene expression is used for diagnosis or treatment.

Patent document 1 JP Patent Publication No. 2814433

Patent document 2 JP Patent Publication No. 2814434

Patent document 3 U.S. Patent No. 5168062

Patent document 4 U.S. Patent No. 5385839

DISCLOSURE OF THE INVENTION

An object of the present invention is to provide a method for increasing gene expression using promoters, enhancers, and the like, and an expression cassette capable of increasing gene expression, comprising promoters, enhancers, and the like.

The present inventors have attempted to develop a new gene expression system using promoters that allow genes to be expressed with higher efficiency. Specifically, they have compared and examined promoter activity resulting from combinations of promoters and enhancers for various genes. As a result, the present inventors have discovered that a gene can be expressed with high efficiency by causing such gene to be flanked by two promoters or a promoter and an enhancer with the use of a gene expression cassette. The gene expression cassette comprises a DNA construct containing a gene to be expressed and a poly A addition sequence that are located downstream of a 1^{st} promoter, as well as an enhancer or a 2^{nd} promoter ligated downstream of the DNA construct. The present inventors have further discovered that a gene can be expressed with even higher efficiency by causing the above expression cassette to further contain an element such as a poly A addition sequence, RU5', UAS, or SV40-ori.

Through the development of "a system for increasing gene expression and the vector comprising the system" of the present invention, protein expression can be strongly increased in almost all cells and genes.

The present invention is as follows.

In one aspect, there is provided a gene expression cassette, which comprises:

(i) a DNA construct in which a 1st promoter, a gene to be expressed, and a polyA addition sequence are linked in such order; and

(ii) an enhancer or an enhancer with UAS ligated to the upstream portion thereof, in such order,

wherein the enhancer or the enhancer with UAS ligated to the upstream portion thereof is ligated immediately downstream of the polyA addition sequence, and

can enhance the protein expression from the gene compared with a case in which an enhancer is inserted upstream of the 1st promoter.

In another aspect, there is provided a vector, containing the expression cassette for a foreign gene as defined herein.

In yet another aspect, there is provided a host cell, containing the vector as defined herein.

In yet another aspect, there is provided a preparation for disease detection or treatment, comprising the vector as defined herein.

In yet another aspect, there is provided an in vitro method for expressing a gene to be expressed using the expression cassette as defined herein or the vector as defined herein.

In yet another aspect, there is provided an in vitro method for producing a protein encoded by a gene to be expressed, comprising introducing the expression cassette as defined herein or the vector as defined herein into a cell, and then culturing the cell.

In yet another aspect, there is provided an in vitro method for enhancing protein expression from a gene compared with a case in which an enhancer is inserted upstream of a 1st promoter by: ligating an enhancer or an enhancer with UAS ligated to the upstream portion thereof to a site immediately downstream of a polyA addition sequence of a DNA construct in which a 1st promoter, a gene to be expressed, and the polyA addition sequence are linked in such order, so as to insert the enhancer to a site immediately downstream of the DNA construct comprising the gene to be expressed, in an expression cassette for gene expression comprising the 1st promoter, the gene to be expressed, the polyA addition sequence, and the enhancer.

Also disclosed are:

[1] A gene expression cassette, comprising

a DNA construct containing a gene to be expressed and a poly A addition sequence that are located downstream of a 1st promoter, and further comprising an enhancer or a 2nd promoter downstream of the DNA construct.

[2] The expression cassette according to [1], which does not have another mechanism for gene expression downstream of the ligated enhancer or the 2nd promoter, but has a structure in which a gene to be expressed is flanked by one 1st promoter and one enhancer, or one 1st promoter and one 2nd promoter.

[3] The expression cassette according to [1], wherein the promoter is selected

from the group consisting of a CMV i promoter, an SV40 promoter, an hTERT promoter, a β actin promoter, and a CAG promoter.

[4] The expression cassette according to any one of [1] to [3], wherein the enhancer is at least one enhancer selected from the group consisting of a CMV enhancer, an SV40 enhancer, and an hTERT enhancer.

[5] The expression cassette according to any one of [1] to [4], wherein 1 to 4 CMV enhancers are ligated upstream of the DNA construct containing DNA encoding a protein to be expressed and the poly A addition sequence that are located downstream of the promoter.

[6] The expression cassette according to any one of [1] to [5], comprising at least any one of the following elements:

(i) RU5' ligated immediately upstream of DNA encoding a foreign protein;

(ii) UAS ligated immediately upstream of an enhancer and/or a promoter; and

(iii) SV40-ori ligated to the most upstream portion of the expression cassette.

[7] The expression cassette according to any one of [1] to [6], wherein the gene to be expressed is a therapeutic gene that can be used for the treatment of diseases, or the gene of a protein that can be used for a drug, a diagnostic agent, or a reagent.

[8] The expression cassette according to [7], wherein the therapeutic gene is a cancer suppressor gene that can be used for the treatment of tumors.

[9] The expression cassette according to [8], wherein the cancer suppressor gene is a REIC/Dkk-3 gene.

[10] The expression cassette according to [9], wherein the gene to be expressed is a DNA fragment of the REIC/Dkk-3 gene.

[11] The expression cassette according to [10], wherein the DNA fragment of the REIC/Dkk-3 gene is DNA encoding amino acids 1 to 78 of the amino acid sequence of SEQ ID NO: 18.

[12] The expression cassette for a foreign gene according to [1], having the structure of constructs No.2 (Fig. 8), No.4 (Fig. 10), No.6 (Fig. 12), No.8 (Fig. 14), No.10 (Fig. 16), No.12 (Fig. 18), and No.14 (Fig. 20).

[13] The expression cassette for a foreign gene according to [1], having the structure of constructs No.15 (Fig. 16), No.16 (Fig. 37), No.17 (Fig. 49), No.20 (Fig. 57), and No.21 (Fig. 58).

[14] A vector, comprising the expression cassette for a foreign gene according to any one of [1] to [13].

[15] The vector according to [14], which is an adenovirus vector or an adeno-associated virus vector.

[16] A host cell, containing the vector according to [14] or [15].

[17] A preparation for disease detection or treatment, comprising the vector according to [14] or [15].

[18] A method for expressing a gene to be expressed using the expression cassette according to any one of [1] to [13] or the vector according to [14] or [15].

[19] A method for gene expression, comprising:

ligating an enhancer or a 2^{nd} promoter to a site downstream of a DNA construct containing a gene to be expressed and a poly A addition sequence that are located downstream of a 1^{st} promoter;

introducing the resultant into a vector, and then expressing the gene using the vector.

[20] A method for producing a protein encoded by a gene to be expressed, comprising introducing the expression cassette according to any one of [1] to [13] or the vector according to [14] or [15] into a cell, and then culturing the cell.

This description includes part or all of the contents as disclosed in the specification and/or drawings of Japanese Patent Application No. 2009-264299, which is a priority document of the present application.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the expression of various foreign genes transfected for 36 hours into a HEK293 cell line using FuGENE (trademark)-HD.

Fig. 2 shows the expression of a KLF gene transfected for 36 hours into various cell lines using FuGENE (trademark)-HD.

Fig. 3 shows the expression of a KLF gene transfected for 36 hours into a HEK293 cell line using various reagents for transfection.

Fig. 4 shows the expression of a full-length REIC gene and an N78-REIC-coding gene transfected for 36 hours into a HEK293 cell line using FuGENE (trademark)-HD.

Fig. 5-1 is a graph showing suppressed proliferation of and induction of cell death in a human prostate cancer PC3 cell line by construct No. 14 into which N78-REIC-coding DNA was inserted (graph).

Fig. 5-2 shows photographs showing suppressed proliferation of and induction of cell death in a human prostate cancer PC3 cell line by construct No. 14 into which N78-REIC-coding DNA was inserted.

Fig. 6 shows the suppressed proliferation of a human prostate cancer PC3 cell line by construct No. 14 into which a full-length REIC gene was inserted.

Fig. 7 shows the structure of construct No. 1.

Fig. 8 shows the structure of construct No. 2.

Fig. 9 shows the structure of construct No. 3.

Fig. 10 shows the structure of construct No.4.

Fig. 11 shows the structure of construct No. 5.

Fig. 12 shows the structure of construct No. 6.

Fig. 13 shows the structure of construct No. 7.

Fig. 14 shows the structure of construct No. 8.

Fig. 15 shows the structure of construct No. 9.

Fig. 16 shows the structure of construct No. 10.

Fig. 17 shows the structure of construct No. 11.

Fig. 18 shows the structure of construct No. 12.

Fig. 19 shows the structure of construct No. 13.

Fig. 20 shows the structure of construct No. 14.

Fig. 21 shows of an adenovirus construct encoding a full-length REIC/Dkk-3 gene.

Fig. 22-1 shows the full nucleotide sequence of a pDNR-1r Donor vector.

Fig. 22-2 shows the full nucleotide sequence of the pDNR-1r Donor vector (a continuation from Fig. 22-1).

Fig. 23 shows the full nucleotide sequence of a pIDT-SMART vector.

Fig. 24 shows the nucleotide sequence of a CMV i promoter (hCMV + intron promoter) region.

Fig. 25 shows the nucleotide sequence of a BGH polyA (3 x stop + BGH polyA) region.

Fig. 26 shows the nucleotide sequence of a CMV enhancer region.

Fig. 27 shows the nucleotide sequence of a human β actin promoter region.

Fig. 28 shows the nucleotide sequence of RU5' forward {R segment of HTLV Type 1 long terminal repeat and a portion (R-U5') of U5 sequence} region.

Fig. 29 shows the nucleotide sequence of RU5'reverse {R segment of

HTLV Type 1 long terminal repeat and a portion (R-U5') of U5 sequence} region.

Fig. 30 shows the nucleotide sequence of a 4 x CMV enhancer region.

Fig. 31 shows the nucleotide sequence of a CAG promoter region.

Fig. 32 shows the nucleotide sequence of a 2IRES insert region.

Fig. 33 shows the nucleotide sequence of a SV40ori-UAS-CMVi-RU5' region.

Fig. 34 shows the nucleotide sequence of a 3 x stop-BGH-polyA-UAS-hTERT enhancer + SV40 enhancer + CMV enhancer region.

Fig. 35-1 shows the full nucleotide sequence of construct No. 14 vector.

Fig. 35-2 shows the full nucleotide sequence of construct No. 14 vector (a continuation from Fig. 35-1).

Fig. 36 shows construct No. 15.

Fig. 37 shows construct No. 16.

Fig. 38 shows the nucleotide sequence of a SV40ori-UAS-SV40 enh-intron A-RU5' region. Fig. 38 specifically shows the nucleotide sequence of an insertion portion on the left (upstream side) of the insertion gene (gene of interest) in construct No. 15.

Fig. 39 shows the nucleotide sequence of a SV40ori-UAS-hTERT enh-intron A-RU5' region. Fig. 39 specifically shows the nucleotide sequence of an insertion portion on the left (upstream side) of the insertion gene (gene of interest) in construct No. 16.

Fig. 40 shows the nucleotide sequence of the GFP region in a plasmid.

Fig. 41-1 shows the nucleotide sequence of a plasmid in which the DNA of a GFP gene was inserted into the insertion gene (gene of interest) region in construct No. 14.

Fig. 41-2 shows the nucleotide sequence of the plasmid in which the DNA of the GFP gene was inserted into the insertion gene (gene of interest)

region in construct No. 14 (a continuation from Fig. 41-1).

Fig. 42-1 shows the nucleotide sequence of the plasmid in which the DNA of the GFP gene was inserted into the insertion gene (gene of interest) region in construct No. 15.

Fig. 42-2 shows the nucleotide sequence of the plasmid in which the DNA of the GFP gene was inserted into the insertion gene (gene of interest) region in construct No. 15 (a continuation from Fig. 42-1).

Fig. 43-1 shows the nucleotide sequence of the plasmid in which the DNA of the GFP gene was inserted into the insertion gene (gene of interest) region in construct No. 16.

Fig. 43-2 shows the nucleotide sequence of the plasmid in which the DNA of the GFP gene was inserted into the insertion gene (gene of interest) region in construct No. 16 (a continuation from Fig. 43-1).

Fig. 44 shows the intensity of GFP gene expression when a plasmid containing an SV40 promoter (construct No. 15) and an hTERT promoter (construct No. 16) was used.

Fig. 45 shows the nucleotide sequence of a human erythropoietin region in a plasmid.

Fig. 46 shows the result of the expression of human erythropoietin using plasmid construct No. 14.

Fig. 47A shows the nucleotide sequence of a human IgG light chain region (Fig. 44A) in a plasmid.

Fig. 47B shows the nucleotide sequence of a human IgG heavy chain region (Fig. 44B) in a plasmid.

Fig. 48 shows the result of the expression of a human IgG light chain (Fig. 48A) and a human IgG heavy chain (Fig. 48B) using plasmid construct No. 14.

Fig. 49 shows the structure of construct No. 17.

Fig. 50 shows the nucleotide sequence of a plasmid in which the DNA of full-length human REIC was inserted into the insertion gene (gene of interest) region in construct No. 17.

Fig. 51 shows the result of the expression of full-length human REIC using plasmid construct No. 17.

Fig. 52 shows the sequence of a c-myc gene.

Fig. 53 shows the nucleotide sequence prepared by linking BGH polyA existing downstream of an expression gene in expression plasmid construct No. 14 and 3 enhancers.

Fig. 54 shows the result of the expression of a c-myc gene after incorporation of the nucleotide sequence (prepared by linking BGH polyA existing downstream of an expression gene of expression plasmid construct No. 14 and 3 enhancers) into a commercial plasmid.

Fig. 55 shows the structure of construct No. 18.

Fig. 56 shows the structure of construct No. 19.

Fig. 57 shows the structure of construct No. 20.

Fig. 58 shows the structure of construct No. 21.

Fig. 59-1 shows the nucleotide sequence of a plasmid in which the DNA of a GFP gene was inserted into the insertion gene (gene of interest) region of construct No. 21.

Fig. 59-2 shows the nucleotide sequence of the plasmid in which the DNA of the GFP gene was inserted into the insertion gene (gene of interest) region of construct No. 21 (a continuation from Fig. 59-1).

Fig. 60 shows the result of insertion of a gene of interest into a plasmid containing an hTERT promoter and the following expression thereof.

Fig. 61 shows the expression after simultaneous transfection of a

plurality of expression vectors into which different foreign genes were incorporated.

Fig. 62 shows the production of human erythropoietin using the expression vectors of the present invention.

Fig. 63 shows the purity of human erythropoietin produced using the expression vectors of the present invention.

Fig. 64 shows the amounts of human erythropoietin produced using the expression vectors of the present invention. Fig. 64A shows the amount of the same produced in 25 mL of each culture supernatant and Fig. 64B shows the amount of the same produced in terms of 1 L of the culture supernatant.

Fig. 65 shows human REIC protein production using the expression vector of the present invention.

Fig. 66 shows an ion exchange column chromatogram for a human REIC protein produced using the expression vector of the present invention.

Fig. 67 shows the amounts of a human REIC protein produced using the expression vector of the present invention (the amount of the protein produced in 520 mL of the culture supernatant and the amount of the protein produced in terms of 1 L of the culture supernatant).

BEST MODE OF CARRYING OUT THE INVENTION

The present invention will be described in detail as follows.

In the present invention, the term "an expression cassette for a protein to be expressed" refers to a DNA set for enabling the expression of a protein to be expressed.

The expression cassette has a structure in which

a DNA construct contains the gene of a protein to be expressed (gene to be expressed) and a poly A addition sequence that are located downstream of at

least a 1st promoter, and

an enhancer or a 2nd promoter is ligated downstream of the DNA construct. Also, any gene can be used as a gene to be expressed. In the expression cassette of the present invention, a site into which a gene to be expressed is inserted may exist as a multicloning site. In this case, a gene to be expressed may be inserted into such a multicloning site (insertion site) using a sequence that is recognized by a restriction enzyme. Such an expression cassette that does not comprise the gene (DNA) itself to be expressed but comprises a site into which the DNA is inserted as a multicloning site is included in examples of the expression cassette of the present invention. In addition, a gene to be expressed may be referred to as a target gene or a gene of interest and a protein to be expressed may also be referred to as a target protein or a protein of interest. Also, in view of the construction of the expression cassette, these genes are also referred to as insertion genes, since they are inserted into regions of target genes in the expression cassette. Alternatively, these genes may also be referred to as foreign genes.

Furthermore, the above enhancer or the 2nd promoter is present at the most downstream site of the expression cassette of the present invention, and no mechanism for gene expression is present downstream thereof. Specifically, the expression cassette of the present invention has a structure in which at least a gene to be expressed is flanked by one 1st promoter and at least one enhancer, or a gene to be expressed is flanked by the one 1st promoter and the one 2nd promoter. Here, the term "(another) mechanism for gene expression" refers to a mechanism for expression of a gene other than the above gene to be expressed, which comprises a promoter, an enhancer, and the like for expression of such a gene other than the gene to be expressed. In the expression cassette of the present invention, promoters can be present downstream and upstream of a gene

to be expressed. These two promoters are used for enhancement of the expression efficiency of the gene to be expressed.

The expression cassette of the present invention is incorporated into an expression vector and then used. The present invention also encompasses such a vector containing the expression cassette of the present invention. As described above, the above enhancer or 2^{nd} promoter is present at the most downstream site of the expression cassette of the present invention, and a mechanism for gene expression does not exist downstream thereof. A vector containing the expression cassette of the present invention does not have any mechanism for gene expression at a site downstream of the expression cassette.

In the present invention, examples of a gene to be expressed include an artificially inserted gene (DNA) encoding a foreign protein. Examples of the same also include such a gene from an origin differing from that of a host cell and a gene from the same origin as that of a host cell. In this case, in the present invention, such a foreign gene is also referred to as an insertion gene. Types of gene to be expressed are not limited. DNAs encoding all proteins from which recombinants are produced and DNAs encoding proteins to be expressed *in vivo* so as to be used for the treatment of specific diseases can be used. Moreover, examples of therapeutic genes that can be used for the treatment of specific diseases include an REIC/Dkk-3 gene (the nucleotide sequence thereof is shown in SEQ ID NO: 17), tumor suppressor genes such as p53 and Rb, and genes encoding proteins that can be used as drugs such as biologically active substances (e.g., interleukin 1 (IL-1), IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, α-interferon, β-interferon, γ-interferon, angiostatin, thrombospondin, endostatin, METH-1, METH-2, GM-CSF, G-CSF, M-CSF, a tumor necrosis factor, a hepatocyte growth factor, erythropoietin, thrombopoietin, insulin, growth hormone,

antibody (IgG light chain or IgG heavy chain), protein G, and protein A), genes encoding proteins useful as diagnostic agents to be used for detection of diseases and the like or reagents to be used for experiments, studies, and the like at laboratories. A target protein to be expressed is preferably an extracellular secretory protein. Also, through artificial ligation of an enhancer or a promoter to a site downstream of an original gene of an organism, the expression of such an original gene of an organism can also be enhanced. Specifically, examples of a gene to be expressed in the present invention include original genes of organisms. The present invention further encompasses the insertion of an enhancer or a promoter to a site downstream of an original gene of an organism, so as to control the expression of the gene. The present invention further encompasses a cell into which an enhancer or a promoter is inserted downstream of an original gene (of the relevant organism). Further examples of a gene to be expressed include DNA and the like encoding siRNA, shRNA, miRNA, and the like having RNA interference action. When RNA having RNA interference action is used, such RNA is transcribed and produced using a transfer RNA promoter, so that the suppressed expression of a specific gene becomes possible.

For example, of these genes, a REIC/Dkk-3 gene and the like can be used for the treatment of tumors. Specifically, not only a full-length gene thereof, but also a fragment thereof can be used. An example thereof is DNA encoding a polypeptide that consists of the amino acid sequence starting from amino acid 1 and terminating at any one of amino acids 39 to 78 in the amino acid sequence of the REIC/Dkk-3 protein shown in SEQ ID NO: 18 or an amino acid sequence that has a substitution, a deletion, or an addition of 1 or several amino acids with respect to the amino acid sequence starting from amino acid 1 and terminating at any one of amino acids 39 to 78 in the amino acid sequence of the REIC/Dkk-3 protein shown in SEQ ID NO: 18, and has apoptotic activity.

Among such DNAs, DNA (N78-REIC DNA) encoding a peptide consisting of amino acids 1 to 78 of the amino acid sequence of the REIC/Dkk-3 protein shown in SEQ ID NO: 18 is preferably used.

A reporter gene may also be contained for detection or diagnosis of disease.

A promoter is a specific nucleotide sequence on DNA for initiation of transcription with the DNA as a template, and generally has a common sequence. For example, prokaryotes such as *Escherichia coli* generally has a TATAATG sequence at a 10-base-pair site that is a transcription initiation site, and a TTGACA sequence at a 35-base-pair site. Furthermore, eukaryotes generally have a TATA box at a 20-base-pair site. The expression cassette of the present invention may always have a 1st promoter at a site upstream of a gene to be expressed and may have a 2nd promoter at a site downstream of the gene to be expressed. These promoters to be used as the 1st promoter and the 2nd promoter are not limited and the 1st promoter and the 2nd promoter may be the same or different from each other. Non specific promoters that can accelerate the expression of foreign genes in all cells or tissues, tissue- or organ-specific promoters, tumor-specific promoters, and specific or selective promoters such as development- or differentiation-specific promoters can also be used herein. For example, a specific promoter can be used as the 1st promoter and a non specific promoter can be used as the 2nd promoter. Promoters to be used in the present invention are as follows. Examples of a cancer- or tumor-specific promoter include hTERT (human telomerase reverse transcriptase), PSA (prostate-specific antigen), c-myc, and a GLUT promoter. Examples of an ES cell- or cancer stem cell-specific promoter include OCT3/4 and NANOG promoters. An example of a neural stem cell-specific promoter is a Nestin promoter. Examples of a cell stress sensitive promoter include HSP70, HSP90, and p53

promoters. An example of a hepatocyte-specific promoter is an albumin promoter. An example of a radiosensitive promoter is a TNF-alpha promoter. An example of a promoter for increasing the number of copies of an infection plasmid is a SV40 promoter and the like. An example of a proliferative cell-specific promoter is an EF1-alpha promoter. Further specifically, for example, as the 1st promoter, a CMV-i promoter (hCMV + intron promoter) (SEQ ID NO: 5), a β actin promoter, a CAG promoter (SEQ ID NO: 12), a CMV promoter, or the like is used and as the 2nd promoter, a CMV promoter or the like is used. Animal species from which a β actin promoter is derived is not limited. Mammalian β actin promoters such as a human β actin promoter (SEQ ID NO: 8) and a chicken actin promoter are used. Furthermore, an artificial hybrid promoter such as the above CMV-i promoter can also be used. The CMV-i promoter can be synthesized based on the disclosure in the specification of U.S. Patent No. 5168062 or the specification of U.S. Patent No. 5385839. As such a promoter, a core promoter portion consisting of a minimum sequence having promoter activity may be used. The term "core promoter" refers to a promoter region capable of functioning to result in precise transcription initiation, which may contain a TATA box. Among the above promoters, a cancer- and/or tumor-specific promoter such as an hTERT promoter can be preferably used for cancer-targeting gene therapy or diagnosis of cancer with the use of gene expression. Construct No. 21 comprising the hTERT promoter can be used for such purposes.

The origin of a poly A addition sequence (polyadenylation sequence, polyA) is not limited. Examples of such a poly A addition sequence include a growth hormone gene-derived poly A addition sequence such as a bovine growth hormone gene-derived poly A addition sequence (contained in the nucleotide sequence shown in SEQ ID NO: 6 (a sequence following the 13th base)) and a

human growth hormone gene-derived poly A addition sequence, an SV40 virus-derived poly A addition sequence, and a human or rabbit β globin gene-derived poly A addition sequence. Containment of such a poly A addition sequence in the expression cassette results in increased transcription efficiency.

Examples of an enhancer are not limited, as long as it results in an increased amount of messenger RNA (mRNA) generated by transcription. An enhancer is a nucleotide sequence having an effect of accelerating the action of a promoter and generally has a length of around 100 bp in most cases. An enhancer can accelerate transcription regardless of the direction of the relevant sequence. One type of enhancer can be used in the present invention. Specifically, two or more (a plurality of) same enhancers may be used or a plurality of different enhancers may be used in combination. Also, when a plurality of different enhancers are used, the order thereof is not limited. For example, a CMV enhancer (SEQ ID NO: 7), an SV40 enhancer, an hTERT (Telomerase Reverse Transcriptase) enhancer, and the like can be used. An example thereof is a product resulting from linking of the hTERT enhancer, the SV40 enhancer, and the CMV enhancer in such order.

Moreover, a plurality of enhancers (e.g., 1 to 4 enhancers) may be ligated upstream of a DNA construct comprising DNA encoding a protein to be expressed and a poly A addition sequence. Enhancers to be ligated upstream thereof are not limited, and a CMV enhancer is preferable. An example thereof is 4 x CMV enhancer (SEQ ID NO: 11) prepared by linking four CMV enhancers.

When an enhancer is inserted immediately downstream of a DNA construct consisting of "promoter - gene to be expressed - poly A addition sequence," the protein of the gene (to be expressed more strongly) can be expressed than that in the case of a general conventional gene expression system.

In particular, through the use of a combination of a CMV i promoter

and a CMV enhancer, in almost all cells (host cells), strong protein expression of a gene to be expressed becomes possible regardless of the type of transfection reagent used herein, although any gene is inserted.

Insertion of one or more CMV enhancers into sites upstream of a promoter results in further enhanced expression of a specific gene (e.g., a REIC/Dkk-3 gene or a CD133 gene in Examples below) in specific cells (e.g., a HEK293 cell line or a MCF7 cell line in Examples below). Also, four CMV enhancers are inserted upstream of a promoter, so that the expression is further enhanced depending on specific cells (e.g., a HepG2 cell line or a HeLa cell line in Examples below).

In addition, insertion alone of a CMV enhancer to a site upstream of a promoter results in only slightly increased gene expression or a slight change therein (Fig. 1 and Fig. 2). It is important for the expression cassette of the present invention that a CMV enhancer is inserted and ligated immediately downstream of a poly A addition sequence.

Furthermore, RU5' (SEQ ID NO: 9) may be ligated immediately upstream of DNA encoding a protein to be expressed. The expression "..(to a site) immediately upstream of" means that the relevant sequence is directly ligated via no other elements having specific functions. However, a short sequence may be contained between them, as a linker. RU5' is HTLV-derived LTR and is an element that increases protein expression through insertion thereof (Mol. Cell. Biol., Vol. 8 (1), p. 466-472, 1988). Insertion of RU5' in a direction opposite to that reported previously may cancel the promoter's effect of enhancing expression due to enhancer insertion.

Furthermore, UAS may be ligated to a site immediately upstream of an enhancer and/or a promoter. UAS is a binding region for a GAL4 gene. Insertion of a GAL4 gene into a site downstream of UAS can result in increased

protein expression.

Moreover, SV40-ori may be ligated to the most upstream portion of the expression cassette. SV40-ori is a binding region for an SV40 gene. Insertion of an SV40 gene into a site downstream of SV40-ori results in increased protein expression.

Each of the above elements should be functionally ligated. Here, the term "functionally ligated" means that each element is ligated so that it can exhibit its functions and thus the expression of a gene to be expressed is enhanced.

Fig. 7 to Fig. 21, Fig. 36, Fig. 37, Fig. 49, and Fig. 55 to Fig. 58 show various expression cassette constructs. In the present invention, such a construct may also be referred to as the backbone of a plasmid. Examples of the constructs of the present invention for enhancing the expression of genes to be expressed include construct No. 2 (Fig. 8), construct No. 4 (Fig.10), construct No. 6 (Fig.12), construct No. 8 (Fig. 14), construct No. 10 (Fig. 16), construct No. 12 (Fig. 18), construct No. 14 (Fig. 20), construct No. 15 (Fig. 36), construct No. 16 (Fig. 37), construct No. 17 (Fig. 49), construct No. 20 (Fig. 57), and construct No.21 (Fig. 58). Another example thereof is a construct shown in Fig. 21.

Among these constructs, with the object of increasing gene expression, construct No.14, construct No.15, and construct No.16 are the best, in which 3 enhancers are ligated downstream of each gene of interest. For example, as a result of the use of a full-length REIC gene as a gene of interest in construct No.14, the protein expression level is equivalent to that in the case of an adenovirus vector (100 MOI, encoding the full-length REIC/Dkk-3 gene). Also, the plasmid backbone of construct No. 14 exhibits an effect of increasing gene expression even in the case of a gene fragment such as DNA encoding N78-

REIC.

For example, through transfection of human prostate cancer cells (PC3) with a preparation in which construct No.14 contains a DNA fragment encoding N78-REIC, significant effects of suppressing the proliferation of cancer cells and significant effects of inducing cancer cell death are observed. Through transfection of human prostate cancer cells (PC3) with a preparation in which a full-length REIC gene is encoded in construct No.14, effects of suppressing the proliferation of cancer cells are observed.

Furthermore, DNA encoding a protein such as human erythropoietin is inserted into construct No.14, an expression vector is constructed, and then cells are transfected with the expression vector, and thus the protein can be produced in a large amount using the cells.

In the present invention, DNAs represented by the nucleotide sequences shown in the above SEQ ID NOs may have a mutation(s) in the nucleotide sequences, as long as they retain the activity of each DNA or the activity of the protein or the polypeptide encoded by each DNA. Examples of such a DNA that can be used for construction of the DNA construct of the present invention include DNA hybridizing under stringent conditions to DNA having a nucleotide sequence complementary to the nucleotide sequence shown in each SEQ ID NO, DNA having at least 85%, preferably at least 90%, further preferably at least 95%, and particularly preferably at least 97% homology with the nucleotide sequence shown in each SEQ ID NO as calculated using BLAST (Basic Local Alignment Search Tool at the National Center for Biological Information (NCBI)) or the like (for example, using default; that is, initially set parameters), and DNA encoding a protein or a polypeptide comprising an amino acid sequence having a substitution, a deletion and/or an addition of one or a plurality of or several (1 to 10, preferably 1 to 5, further preferably 1 or 2 amino

acids) amino acids with respect to the amino acid sequence of the protein or the polypeptide encoded by the DNA. The term "stringent conditions" used herein refers to, for example, stringent conditions of about 1 x SSC, 0.1% SDS, and 37°C, more stringent conditions of about 0.5 x SSC, 0.1% SDS, and 42°C, or even more stringent conditions of about 0.2 x SSC, 0.1% SDS, and 65°C. Under more stringent hybridization conditions, accordingly, isolation of DNA having higher homology to a probe sequence can be expected. It should be noted that the above combinations of SSC, SDS, and temperature conditions are examples, and necessary stringency can be realized by adequately combining a DNA concentration, a DNA length, a hybridization reaction time, and other conditions.

Examples of a vector for insertion of the expression cassette of the present invention include viral vectors such as a plasmid, an adenovirus vector, an adeno-associated virus vector, a lentivirus vector, a retrovirus vector, a herpes virus vector, and Sendai virus vector and non-viral vectors such as a biodegradable polymer. A vector into which the above expression cassette has been introduced may be introduced into cells by a known technique such as infection or electroporation.

At this time, the vector may also be introduced using a known transfection reagent.

A vector constructed by insertion of the expression cassette of the present invention is introduced into cells for transfection, so that a gene of interest can be expressed in the cells and the protein of interest can be produced. For introduction of the expression cassette of the present invention and production of a target protein, eukaryotic cell lines or prokaryotic cell lines can be used. Examples of eukaryotic cells include cells of established mammalian cell lines, cells of insect cell lines, fungal cells or filamentous bacterial cells,

and yeast cells. Examples of prokaryotic cells include cells of bacteria such as *Escherichia coli*, *Bacillus subtilis*, and *Brevibacillus brevis*. Preferably, mammalian cells such as Hela cells, HEK193 cells, CHO cells, COS cells, BHK cells, or Vero cells are used. The above transformed host cells are cultured *in vitro* or *in vivo*, so that a protein of interest can be produced. Host cells are cultured by a known technique. For example, a known culture medium such as DMEM, MEM, RPMI1640, or IMDM can be used as a culture solution. The thus expressed protein can be purified by a known technique from a culture solution when it is a secretory protein or from a cell extract when it is a non-secretory protein. When a protein of interest is expressed and produced, the protein can also be produced by simultaneous transfection of cells with a plurality of vectors containing different genes of interest. With the use of such a technique, a plurality of proteins can be produced simultaneously.

Furthermore, a commercial vector may be modified to contain the expression cassette of the present invention. For example, an enhancer is incorporated into a region downstream of the expression gene cassette in a commercial vector such as pShuttle vector and then the vector can be used. Examples of the vector of the present invention include commercial cassettes that have been modified.

The present invention further encompasses an adenovirus (Ad) vector and an adeno-associated virus (AAV) vector containing the above expression cassettes for genes to be expressed. The vectors enable specific diagnosis or treatment of disease such as cancer. The vectors can be constructed by insertion of the above expression cassette for genes to be expressed into an adenovirus or an adeno-associated virus.

Adenovirus vectors are characterized in that: (1) transfection can be performed in a variety of cells; (2) transfection can be efficiently performed

even for cells at the stationary phase; (3) they can be concentrated by centrifugation such that a virus with a high titer (10 to 11 PFU/ml or more) can be obtained; and (4) they are appropriate for use in direct transfection into *in vivo* tissue cells.

As an adenovirus vector to be used for gene therapy, the following vectors have been developed: a second generation adenovirus vector (Lieber, A., et al., J. Virol., 70, 8944, 1996; Mizuguchi, H. & Kay, M. A., Hum. Gene Ther., 10, 2013, 1999) obtained by deleting the E2 or E4 domain in addition to the E1/E3 domain from a first generation adenovirus vector (Miyake, S., et al., Proc. Natl. Acad. Sci. U.S.A., 93, 1320, 1996) lacking the E1/E3 domain; and a third generation adenovirus vector (Steinwaerder, D. S. et al., J. Virol., 73, 9303, 1999) almost completely lacking the adenovirus genome (GUTLESS). However, for transfection of the gene according to the present invention, any adenovirus vector may be used without particular limitation.

Adeno-associated virus is a single-stranded DNA virus of the genus *Parvovirus*, which is characterized in that: (1) it undergoes long-term gene expression; (2) it exhibits low toxicity; and (3) it enables transfection of dividing and non-dividing cells. Concatamer (complex prepared by ligation of single-stranded DNAs) formation is thought to allow long-term gene expression.

An adenovirus (Ad) vector and an adeno-associated virus (AAV) vector containing the expression cassette of the present invention for a gene to be expressed and enables expression of the target gene to be expressed can be used for detection or treatment of disease. An example of disease is cancer. With the use of a peptide (inserted into the outer shell of an adenovirus vector or an adeno-associated virus vector) such as RGD selectively binding to a protein expressed in specific cells, the vector is directed to the cells. The cells are infected and the gene to be expressed is expressed. When a gene to be

expressed is a reporter gene such as a luciferase gene, specific cells can be detected with light emission or the like. When specific cells are cancer cells, through administration of the vector to a subject, cancer cells in the subject can be detected and then the subject can be diagnosed as having or not having cancer. A single cell can be detected by the adenovirus vector and the adeno-associated virus vector of the present invention, and thus they can be used for detection of micro cancer, for example.

Furthermore, when a gene to be expressed is a therapeutic gene, such a target gene to be expressed is expressed in specific cells, so that it can exhibit therapeutic effects. For example, when specific cells are cancer cells and a cancer suppressor gene such as a REIC/Dkk-3 gene is used, through administration thereof to a subject, such a cancer therapeutic gene is delivered to cancer cells of the subject, the gene is expressed in cancer cells, and thus it exhibits therapeutic effects. The present invention encompasses a diagnostic or therapeutic viral preparation containing such an adenovirus vector or adeno-associated virus vector. Examples of cancer to be treated in the present invention when a cancer suppressor gene such as a human REIC/Dkk-3 gene is used as a therapeutic gene include cranial nerve tumor, skin cancer, gastric cancer, lung cancer, hepatic cancer, lymphoma/leukemia, colon cancer, pancreatic cancer, anal/rectal cancer, esophageal cancer, uterine cancer, breast cancer, adrenal cancer, kidney cancer, renal pelvic and ureteral cancer, bladder cancer, prostate cancer, urethral cancer, penile cancer, testicular cancer, osteoma/osteosarcoma, leiomyoma, rhabdomyoma, and mesothelioma. The adenovirus vector and the adeno-associated virus vector of the present invention can also be used for treatment of primary cancer and metastatic cancer.

The adenovirus vector and the adeno-associated virus vector of the present invention can be administered by methods that can be employed in the

field of gene therapy, such as intravascular administration (e.g., intravenous administration and intraarterial administration), peroral administration, intraperitoneal administration, intratracheal administration, intrabronchial administration, subcutaneous administration, or transdermal administration. In particular, the adenovirus vector and the adeno-associated virus vector of the present invention have strong directivity to specific tissues or cells, which allows them to efficiently deliver a target gene to specific tissues or cells. Hence, efficient diagnosis and treatment are possible by intravenous administration of the adenovirus vector and the adeno-associated virus vector.

The adenovirus vector or the adeno-associated virus vector may be administered in a therapeutically effective dose. The therapeutically effective dose thereof can be easily determined by persons skilled in the field of gene therapy. Furthermore, dosage can be adequately varied depending on the severity of the pathological conditions, gender, age, body weight, common practice, and the like of a subject. For example, the adenovirus vector or the adeno-associated virus vector may be administered at 0.5×10^{11} to 2.0×10^{12} viral genome/kg body weight, preferably at 1.0×10^{11} to 1.0×10^{12} viral genome/kg body weight, and further preferably at 1.0×10^{11} to 5.0×10^{11} viral genome/kg body weight. A viral genome is represented by the molecularity (number of viral particles) of the adenovirus or the adeno-associated virus genome, and may also be referred to as "particle(s)." The vector contains a carrier, a diluent, and an excipient that are generally used in the field of pharmaceutical preparation. For example, as carriers for tablets and as excipients, lactose, magnesium stearate, and the like are used. As an aqueous liquid for injection, saline or an isotonic solution containing dextrose and other adjuvants is used, for example. Such a solution may be used in combination with an appropriate solubilizing agent, such as alcohol, polyalcohol (e.g.,

propylene glycol), or a nonionic surfactant. As an oily fluid, sesame oil, soybean oil, or the like is used. As a solubilizing agent, such as benzyl benzoate or benzyl alcohol may be used in combination.

Moreover, an expression vector that contains the expression cassette of the present invention comprising a gene of interest can also be used as a vaccine or a DNA vaccine for cancer and the like (Kaufman, H. L. et al., J. Clin. Oncol. 22, 2122-2132 (2004); McNeel, D. G. et al., J. Clin. Oncol. 27, 425-430 (2009)). For example, an expression vector that contains the expression cassette of the present invention in which a gene encoding a target cancer-specific antigen protein has been incorporated is administered *in vivo* via subcutaneous injection or the like, the antigen is expressed *in vivo*, and thus the activation of a host's cellular immunity or antibody immunity against the antigen can be induced. The expression cassette of the present invention can be used for prevention or treatment of disease such as cancer through the use thereof as a vaccine as described above.

The present invention is hereafter described in detail with reference to the following examples, although the present invention is not limited thereto.

Example 1 Expression cassette comprising CMV promoter

The expression cassettes of the present invention were prepared and then transfected. The thus expressed proteins were analyzed by Western blotting.

(1) Expression cassette constructs

Construct No.1 (Fig. 7)

Construct No. 1 is a gene expression plasmid loaded with a CMV i promoter upstream of a gene to be expressed. Furthermore, BGH polyA (bovine growth hormone poly A addition sequence) was ligated downstream of

the gene. Specifically, construct No. 1 was thought to have protein expression capacity equivalent to that of a generally employed expression plasmid containing a CMV i promoter.

Construct No. 2 (Fig. 8)

Construct No. 2 was constructed by inserting a CMV enhancer into a site immediately downstream of BGH poly A of construct No. 1.

Construct No. 3 (Fig. 9)

Construct No. 3 was constructed by substituting the CMV i promoter of construct No.1 with a human β actin promoter.

Construct No. 4 (Fig. 10)

Construct No. 4 was constructed by inserting a CMV enhancer into a site immediately downstream of BGH poly A of construct No. 3.

Construct No. 5 (Fig. 11)

Construct No. 5 was constructed by inserting an RU5' region into a site immediately downstream of the human β actin promoter of construct No. 3.

Construct No.6 (Fig. 12)

Construct No. 6 was constructed by inserting a CMV enhancer into a site immediately downstream of BGH poly A of construct No. 5.

Construct No. 7 (Fig. 13)

Construct No. 7 was constructed by inserting a CMV enhancer into a site immediately upstream of the human β actin promoter of construct No. 5.

Construct No. 8 (Fig. 14)

Construct No. 8 was constructed by inserting a CMV enhancer into a site immediately downstream of BGH poly A of construct No. 7.

Construct No. 9 (Fig. 15)

Construct No. 9 was constructed by reversing the direction of the nucleotide sequence of the RU5' region of construct No. 7.

Construct No. 10 (Fig. 16)

Construct No. 10 was constructed by inserting a CMV enhancer into a site immediately downstream of BGH poly A of construct No. 9.

Construct No. 11 (Fig. 17)

Construct No. 11 was constructed by inserting 4×CMV enhancer (four CMV enhancers) into a site immediately upstream of the human β actin promoter of construct No. 5.

Construct No. 12 (Fig. 18)

Construct No. 12 was constructed by inserting a CMV enhancer into a site immediately downstream of BGH poly A of construct No. 11.

Construct No. 13 (Fig. 19)

Construct No. 13 is a gene expression plasmid loaded with a CAG promoter. Specifically, construct No. 13 was thought to have protein expression capacity equivalent to that of a generally employed expression plasmid containing a CAG promoter.

Construct No. 14 (Fig. 20)

The results of examining the above various constructs revealed that gene expression was significantly enhanced via insertion of a CMV enhancer into a site immediately downstream of BGH poly A. Construct No. 14 was constructed by further ligating other enhancers (an hTERT enhancer and a SV40 enhancer). As a result, construct No. 14 was thought to be better than construct No. 2.

Adenovirus construct encoding full-length REIC/Dkk-3 gene (Fig. 21)

The full-length cDNA of REIC/Dkk-3 (Ad-REIC) was incorporated into a pAxCAwt cosmid vector and then transferred to an adenovirus vector by a COS-TPC method (TAKARA Bio). At this time, an adenovirus vector (Ad-LacZ) retaining a LacZ gene was used as a control vector. Adenovirus vectors

used herein were the same as those described in JP Patent No. 3813872.

Fig. 22 to Fig. 35 show the nucleotide sequences of the following elements or constructs.

Fig. 22-1 and Fig. 22-2 (a continuation from Fig. 22-1) show the full nucleotide sequence of a pDNR-1r Donor vector (Plasmid cloning vector having no promoter (Clontech, Product No. PT3616-5)).

Fig. 23 shows the full nucleotide sequence of a pIDT-SMART vector (Plasmid cloning vector having no promoter (IDT)).

Fig. 24 shows the nucleotide sequence of a CMV i promoter (hCMV + intron promoter) region. The region was artificially synthesized based on the known information of the nucleotide sequence of the CMV i promoter region.

Fig. 25 shows the nucleotide sequence of a BGH polyA (3 x stop + BGH polyA) region.

The "TAATAAA" portion in the nucleotide sequence is a very important portion in the BGH polyA sequence. This sequence was artificially synthesized based on the known information of the nucleotide sequence of the BGH polyA region.

Fig. 26 shows the nucleotide sequence of a CMV enhancer region. This sequence was artificially synthesized based on the known information of the nucleotide sequence of the CMV enhancer region.

Fig. 27 shows the nucleotide sequence of a human β actin promoter region. This sequence was prepared by obtaining a pDRIVE-h β Actin-RU5' plasmid (InvivoGen) and then amplifying the nucleotide sequence of the relevant portion by PCR.

Fig. 28 shows the nucleotide sequence of a RU5' forward {R segment of HTLV Type 1 long terminal repeat and a portion (R-U5') of U5 sequence} region. This sequence was artificially synthesized based on the known

information of the nucleotide sequence of the {R segment of HTLV Type 1 long terminal repeat and a portion (R-U5') of U5 sequence} region.

Fig. 29 shows the nucleotide sequence of a RU5'reverse {R segment of HTLV Type 1 long terminal repeat and a portion (R-U5') of U5 sequence} region. This sequence was artificially synthesized based on the known information of the nucleotide sequence of the {R segment of HTLV Type 1 long terminal repeat and a portion (R-U5') of U5 sequence} region.

Fig. 30 shows the nucleotide sequence of a 4 x CMV enhancer region. In the nucleotide sequence shown in Fig. 30, each underlined portion is referred to as namely a "short CMV enhancer." Because of the presence of the four underlined portions, the region is designated as "4 x CMV enhancer region." In addition, the term "CMV enhancer" above is meant to include a "short CMV enhancer." The term "CMV enhancer" generally indicates a long CMV enhancer. The sequence was artificially synthesized based on the known information of the nucleotide sequence of a CMV enhancer region.

Fig. 31 shows the nucleotide sequence of a CAG promoter region, which is the nucleotide sequence of a known CAG promoter region. This sequence was prepared by obtaining a pCAGGS plasmid that was a gene expression vector loaded with a CAG promoter provided by Oriental Yeast Co., ltd., and then amplifying by PCR the nucleotide sequence of the relevant portion based on the thus obtained plasmid.

Fig. 32 shows the nucleotide sequence of a 2IRES insert region, which is the nucleotide sequence of an IRES control gene in Fig. 5 and Fig. 6 (described later). The sequence was prepared by linking the two nucleotide sequences of normal human DNA, a BiP-IRES region (the lower-case letters surrounded by frame (1) in the nucleotide sequence in Fig. 32) and a Myc-IRES region (the lower-case letters surrounded by frame (2) in the nucleotide

sequence in Fig. 32) in such order. This is a gene to be used as a control gene for an insertion gene. This sequence was artificially synthesized based on the known information of the nucleotide sequences of the BiP-IRES region and the Myc-IRES region.

Fig. 33 shows the nucleotide sequence of a SV40ori-UAS-CMVi-RU5' region, which is the nucleotide sequence of an insertion portion on the left of an insertion gene (foreign gene) in construct No. 14 (best construct). This sequence was artificially synthesized based on the known information of the nucleotide sequence of each region contained in the relevant portion. The portion surrounded by frame (1) in the nucleotide sequence in Fig. 33 indicates the SV40 ori region, the portion surrounded by frame (2) in the same indicates the CMV i promoter region, and the portion surrounded by frame (3) in the same indicates the RU5' region.

Fig. 34 shows the nucleotide sequence of a 3 x stop-BGH-polyA-UAS-hTERT enhancer + SV40 enhancer + CMV enhancer region, which is the nucleotide sequence of an insertion portion on the right (downstream side) of an insertion gene (foreign gene) in construct No. 14 (best construct). This sequence was artificially synthesized based on the known information of the nucleotide sequence of each region contained in the relevant portion. In the nucleotide sequence in Fig. 34, the uppercase letters surrounded by frame (1) indicate the BGH polyA region, the lowercase letters surrounded by frame (2) indicate the hTERT enhancer region, and the uppercase letters surrounded by frame (3) indicate the SV40 enhancer region, and the lowercase letters surrounded by frame (4) indicate the CMV enhancer region.

Fig. 35-1 and Fig. 35-2 show the full nucleotide sequence of construct No.14 vector and specifically show the nucleotide sequence of construct No.14 (best construct) shown in Fig. 20. The full nucleotide sequence also contains a

pIDT-SMART vector portion (the region terminates at the bold portion on line 10 of the nucleotide sequence in Fig. 35-1 and the region following GCGCGC surrounded by the frame on line 24 from the bottom in the nucleotide sequence of Fig. 35-2). This sequence was prepared by incorporating the insertion portion on the left and the same on the right of the insertion gene (foreign gene in construct No. 14) shown in former two figures into the above-mentioned pIDT-SMART vector. In Fig. 35-1 and Fig. 35-2, (1) indicates the SV-40 ori region, (2) indicates the CMV i promoter region, (3) indicates the RU5' region, (4) indicates the BiP IRES region, (5) indicates the Myc IRES region, (6) indicates the hTERT enhancer region, and (7) indicates the SV40 enhancer region.

(2) Foreign genes (insertion genes)

6His-S100A11-HA

6His-S100A11-HA was prepared by PCR using the cDNA of a normal human fibroblast as a template, a primer with a 6-His linker added to the 5' side, and a primer with an HA linker added to the 3' side.

GFP (Green fluorescent protein)

GFP was prepared by PCR using pEGFP-N2 (GFP expression vector (the product of Clontech)) as a template and primers designed on the basis of the template.

REIC/Dkk-3 (full-length)

REIC/Dkk-3 was prepared by PCR using the cDNA of a normal human fibroblast as a template and primers designed on the basis of the template.

N78-REIC-6His

N78 - REIC-6His was prepared by PCR using primers designed so that 6His was added to the 3' side of the above-obtained full-length REIC/Dkk-3 gene.

Control gene (IRES)

The term "IRES control gene" used herein refers to a gene prepared by linking the known nucleotide sequences of two normal human DNAs, the BiP-IRES gene and the Myc-IRES gene, in such order. This gene was artificially synthesized. This gene was inserted into the insertion gene (foreign gene) portion of construct No. 14, thereby preparing a control gene for the REIC/Dkk-3 (full-length) gene and N78-REIC-6His-coding DNA in Fig. 5 and Fig. 6 described later.

CD133-6His

CD133-6His was prepared by PCR using the cDNA of a normal human fibroblast as a template and primers designed on the basis of the template.

LGR5-HA

LGR5-HA was prepared by PCR using the cDNA of a normal human fibroblast as a template and primers designed on the basis of the template.

Telomerase-6His

Telomerase-6His was prepared by PCR using the cDNA of a normal human fibroblast as a template and primers designed on the basis of the template.

KLF16

KLF16 was prepared by PCR using the cDNA of a normal human fibroblast as a template and primers designed on the basis of the template.

(3) Transfection and western blot analysis (WB method)

Various cells (cultured in 6-well plates to 70% to 80% confluency) were transfected with various genes using transfection reagents, FuGENE (trademark)-HD (Roche)), Lipofectamine (trademark) 2000 (Invitrogen), and Trans-IT-Keratinocyte (TAKARA Bio). Procedures therefor were carried out as described in manuals for these products.

Western blot analysis of the thus expressed foreign genes (insertion

genes) was conducted by the following methods.

Cells were washed twice using PBS (phosphate buffered saline) and then lysed with lysis buffer (50 mM HEPES, pH 7. 4, 250 mM NaCl, 1 mM EDTA, 1% NP-40, 1 mM DTT, 1 mM PMSF, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 2 mM Na_3VO_4, 1 mM NaF, 10 mM β-GP), so that protein extraction was carried out. After centrifugation, the protein amounts in supernatants to be used for each experiment were adjusted to the same concentration, diluted with the same amount of 2 x SDS sample buffer, and then subjected to 5 minutes of heat treatment at 95°C. Each sample (10 μg protein) was separated by 7.5% or gradient SDS-PAGE gel (Bio Rad) and then transferred to a polyvinylidene difluoride (PVDF) membrane. The blot was subjected to 1 hour of blocking at room temperature with TBS (Tris buffered saline) containing 10% fat-free milk powder, 6% glycine, and 0.1% Tween-20. Subsequently, the proteins were identified using the following primary antibodies (1 : 1000 dilution).

Anti-HA antibody (Cell Signaling Technology)

Anti-GFP antibody (Clontech)

Anti-REIC antibody (rabbit anti-human REIC/Dkk-3 polyclonal antibody)

Anti-6His antibody (MBL)

Anti-KLF16 antibody (Abcam)

The resultants were each sufficiently washed with 0.1% Tween-20-containing TBS (T-TBS), the blot was caused to react with a horseradish peroxidase conjugate secondary antibody. After sufficient washing with T-TBS, detection was carried out by an enhanced chemiluminescence detection method (ECL kit, Amersham Pharmacia Biotech).

Cells were cultured by the following method.

HEK293 (derived from normal human kidney), MCF7 (derived from human breast cancer), PC3 (derived from human prostate cancer), HeLa (derived

from human uterine cancer), HepG2 (derived from human hepatic cancer) cell lines were obtained from ATCC (the American Type Culture Collection (Rockville, MD)). Media used herein are as follows.

HEK293: DMEM high glucose medium (Invitrogen)

MCF7: DMEM high glucose medium (Invitrogen)

PC3: F12 medium (Invitrogen)

HeLa: DMEM high glucose medium (Invitrogen)

HepG2: DMEM high glucose medium (Invitrogen)

The above cell lines were each grown in the above medium supplemented with 10% (v/v) fetal bovine serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml) and then incubated under 5% CO_2 conditions.

Fig. 1 to Fig. 6 show the results.

Fig. 1 shows the expression of various foreign genes that were transfected to the HEK293 cell line for 36 hours using FuGENE (trademark)-HD. The protein expression capacity of construct No. 1 was equivalent to that of a generally used (marketed) expression plasmid containing a CMV i promoter. Specifically, it can be said that the protein expression capacity of construct No. 2 was significantly stronger than that of such expression plasmids. Also, similar results were obtained for the following genes. It can be said that construct No. 2 was superior to gene expression systems that are currently broadly employed, in terms of the expression of all types of gene.

S100A11: possible involvement in cancer growth; cytoplasmic-nuclear import protein

GFP: fluorescent protein; cytoplasmic protein

REIC/Dkk-3 (full length): cancer suppressor protein; secretory protein

N78-REIC: protein fragment prepared based on the above gene fragment (peptide fragment consisting of amino acids 1 to 78 of the amino acid sequence

of the REIC/Dkk-3 protein shown in SEQ ID NO: 2)

CD133: cancer stem cell marker; expressed also on cell surface

LGR5: stem cell marker for normal cells and cancer cells; transmembrane protein

Telomerase: involvement in aging or anti-aging of cells; cytoplasmic protein

KLF16: involvement in protein transcription; nuclear protein

Fig. 2 shows the expression of a KLF gene after 36 hours of transfection of various cell lines using FuGENE (trademark)-HD. It is said that in all cells (HEK293 cells, MCF7 cells, PC3 cells, HeLa cells, and HepG2 cells) shown in Fig. 2, the protein expression capacity of construct No. 2 (KLF16 gene expression) was significantly stronger than that of construct No. 1. Specifically, it can be said that construct No.2 is superior to gene expression systems that are currently broadly employed, in terms of gene expression in all types of cell.

Fig. 3 shows the expression of a KLF gene transfected to the HEK293 cell line for 36 hours using various transfection reagents. With the use of any transfection reagent, in the case of construct No. 2, the KLF16 protein was expressed at the highest level with the WB method. Construct No. 1 is a construct containing a CMV i promoter that is generally available and often used. Construct No. 13 is a construct similarly containing a CAG promoter that is generally available and often used. Specifically, the use of the construct No. 2 of the present invention realized a protein expression level significantly higher (significantly improved efficiency throughout the gene expression processes) than that obtained with the use of plasmid constructs that are currently in widespread use throughout the world for gene expression.

Fig. 4 shows expression after 36 hours of transfection of the HEK293 cell line with a full-length REIC gene and a N78-REIC-coding gene FuGENE

(trademark)-HD. The left panel shows that the REIC/Dkk-3 protein (full-length) was expressed to a significantly higher extent in the case of construct No.14 than that in the case of construct-No. 2, as revealed by the WB method. Specifically, based on the results for the WB method in Fig. 1 (construct No. 2 exhibited the highest protein expression level among constructs No. 1 to No. 12) and the results for the WB method in Fig. 3 (construct No. 2 exhibited the highest protein expression level among constructs No. 1, No. 2, No. 11, No. 12, and No. 13), among the plasmid constructs examined herein, the useful construct capable of exhibiting the highest protein expression level was construct No. 14. The protein expression capacity of construct No.14 was strong and equivalent to that possible with the administration of Ad-REIC (full-length REIC/Dkk-3 gene-coding adenovirus) at 100 MOI. The right panel shows that results similar to those in the left panel were obtained when a gene fragment, N78-REIC-6His, was inserted into construct No. 14. Specifically, the useful construct capable of exhibiting the highest protein expression level among the plasmid constructs presented herein was construct No. 14.

Fig. 5 shows suppressed proliferation of and induction of cell death in a human prostate cancer PC3 cell line by construct No. 14 into which N78-REIC-coding DNA was inserted.

Cell survival assay

The PC3 cell line was seeded in complete medium at density of 50,000 cells per well of 6-well plates. After 24 hours of incubation, cells were transfected with a predetermined plasmid in complete medium using a FuGENE (trademark)-HD reagent and then incubated for predetermined days. After incubation, cell survival rate was measured using CellTiter 96 (registered trademark) Aqueous One Solution Cell Proliferation Assay (Promega).

Statistical analysis

Mann-Whitney U test was conducted between two groups. Significant difference was determined to be present when the result was $p < 0.05$.

As shown in Fig. 5, the proliferation of the PC3 cell line was significantly suppressed on days 2 and 5 after administration of construct No.14 into which N78-REIC-coding DNA had been inserted, compared with a group to which construct No.14 containing a control gene (IRES) inserted therein had been administered (it can be said that significantly many cell deaths were induced since the number of PC3 cells had decreased because of administration of N78-REIC-coding DNA-construct No. 14).

Fig. 6 shows suppressed proliferation of the human prostate cancer PC3 cell line by construct No. 14 into which a full-length REIC gene was inserted.

Cell survival assay

The PC3 cell line was seeded in complete medium at density of 50,000 per well of 6-well plates. After 24 hours of incubation, cells were transfected with predetermined plasmids in Hank's Balanced Salt Solutions using a FuGENE (trademark)-HD reagent and then incubated for predetermined days. After incubation, cell survival rate was measured using CellTiter 96 (registered trademark) Aqueous One Solution Cell Proliferation Assay (Promega).

Statistical analysis

Mann-Whitney U test was conducted between two groups. A significant difference was determined to be present when the result was $p < 0.05$.

As shown in Fig. 6, on day 2 after administration of construct No. 14 into which the full-length REIC gene had been inserted, the proliferation of PC3 cells was significantly suppressed compared with a group to which construct No. 14 containing a control gene (IRES) inserted therein had been administered.

Example 2 Expression cassette containing SV40 promoter or hTERT promoter

The expression cassettes of the present invention containing GFP (Green fluorescent protein) as a target gene (insertion gene) were prepared. Hela cells were transfected with the expression cassettes and then GFP expression was observed, so that the expression intensity was analyzed. Transfection was carried out using Lipofectamine 2000. After 48 hours, GFP expression was observed under a fluorescence microscope.

The constructs of the expression cassettes used herein were: construct No. 14 (Fig. 20) used in Example 1; construct No. 15 (Fig. 36) wherein the CMV i promoter of construct No. 14 had been substituted with an SV40 promoter; and construct No. 16 (Fig. 37) wherein the CMV i promoter of construct No. 14 had been substituted with an hTERT promoter. A GFP gene was prepared by PCR using primers designed with the use of a GFP expression vector (a product of Clontech), pEGFP-N2, as a template. The GFP gene was inserted into the target gene insertion part of each construct and then used. A commercial pEGFP-N1 GFP expression plasmid (Clontech) was used as a control.

Fig. 38 shows the nucleotide structure of a partial fragment comprising the SV40 ori+UAS+SV40 enh+intron A+RU5' region of expression vector construct No. 15. The nucleotide sequence thereof is shown in SEQ ID NO: 19. Furthermore, Fig. 39 shows the structure of a partial fragment comprising the SV40 ori+UAS+hTERT enh+intron A+RU5' region of expression vector construct No. 16. The nucleotide sequence thereof is shown in SEQ ID NO: 20. Furthermore, Fig. 40 shows the nucleotide sequence (SEQ ID NO: 21) of GFP-coding DNA to which a restriction enzyme site was ligated. Portions surrounded by frames (1), (2), (3), and (4) shown in the nucleotide sequence in Fig. 38 indicate SV40 ori, SV40 promoter, intron A, and RU5', respectively. Portions surrounded by frames (1), (2), (3), and (4) shown in the nucleotide sequence in Fig. 39 indicate SV40 ori, hTERT promoter, intron A, and RU5',

respectively. The full-length nucleotide sequence of expression vector construct No. 14 into which a GFP gene was inserted is shown in Fig. 41-1 and Fig. 41-2 (a continuation from Fig. 41-1) (SEQ ID NO: 22). Also, the full-length nucleotide sequence of expression vector construct No. 15 into which a GFP gene was inserted is shown in Fig. 42-1 and Fig. 42-2 (a continuation from Fig. 41-1) (SEQ ID NO: 23). Furthermore, the full-length nucleotide sequence of expression vector construct No.16 into which a GFP gene was inserted is shown in Fig. 43-1 and Fig. 43-2 (a continuation from Fig. 43-1) (SEQ ID NO: 24). Portions surrounded by frames (1), (2), (3), and (4) in the nucleotide sequence in Fig. 41-1 and Fig. 41-2 indicate CMV i promoter (P-CMViRU), GFP gene, Myc IRES, and 3 enhancers (pA-3enh), respectively. Portions surrounded by frames (1), (2), (3), and (4) in the nucleotide sequence in Fig. 42 indicate SV40 promoter (P-SViRU), GFP gene, Myc IRES, and 3 enhancers (pA-3enh), respectively. Portions surrounded by frames (1), (2), (3), and (4) in the nucleotide sequence in Fig. 43 indicate hTERT promoter (P-TiRU), GFP gene, Myc IRES, and 3 enhancers (pA-3enh), respectively.

Construct No. 15 is a plasmid vector that is advantageous in strong gene expression in an environment where the SV40 protein is expressed at a high level. Construct No. 16 is a plasmid vector that is advantageous in strong gene expression in an environment where the hTERT protein is expressed at a high level in cancer cells or the like.

Fig. 44 shows the results. Fig. 44 A, B, C and D show the results of a commercial GFP expression plasmid (pEGFP-N1), a GFP expression plasmid construct No. 14, GFP expression plasmid construct No. 15, and GFP expression plasmid construct No. 16, respectively. Panels in the upper row in Fig. 44 indicate the bright field. As shown in Fig. 44, the CMV promoter, the SV40 promoter, and the hTERT promoter were all observed to exhibit strong GFP gene

expression compared with the commercial plasmid. This example demonstrates that this system (the backbone of construct No. 14) can be used for gene enhancement even when promoters were varied in this manner.

Example 3 Expression of human erythropoietin

The expression cassette of the present invention containing human erythropoietin as a foreign gene (insertion gene) was prepared. HEK293 cells were transfected with the expression cassette and then the thus expressed protein was analyzed by Western blot. Transfection was carried out using FuGENE (trademark)-HD. After 24 hours, human erythropoietin in a cell culture solution was detected by Western blotting.

The construct of the expression cassette used herein was construct No. 14 (Fig. 20) used in Example 1, to which DNA encoding human erythropoietin was inserted as a target gene (insertion gene). Fig. 45 shows the nucleotide sequence (SEQ ID NO: 25) of DNA encoding human erythropoietin to which a restriction enzyme site had been ligated. As controls, with the use of commercial pTracer (registered trademark)-EF/V5-His-A (Invitrogen) and pEF6/Myc-His-A expression plasmids (Invitrogen), human erythropoietin-coding DNA was inserted into the *Eco*R 1-*Xba* 1 restriction enzyme site of each plasmid.

Western blotting was carried out using an anti-6His antibody (MBL). At 24 hours after transfection, 1 mL of a culture solution was subjected to trapping with 6His amino acid residues. The total amount of the thus collected protein was used for Western blotting.

Fig. 46 shows the results. Lane 1 shows the results of expression using pTracer (registered trademark)-EF/V5-His-A, lane 2 shows the results of expression using pEF6/Myc-His-A, and lane 3 shows the results of expression using expression plasmid construct No. 14. As shown in Fig. 46, the strongest

expression was observed with the use of expression plasmid construct No. 14. This result demonstrates that this system (the backbone of construct No.14) is useful for production of proteins to be used as pharmaceutical products such as erythropoietin, diagnostic agents, or reagents, in view of mass production based on gene enhancement.

Example 4 Expression of human IgG

The expression cassette of the present invention containing a human IgG light chain and a human IgG heavy chain as target genes (insertion genes) was prepared. HEK293 cells were transfected with the expression cassette, and then the thus expressed protein was analyzed by Western blot. Transfection was carried out using FuGENE (trademark)-HD. After 24 hours, the human IgG light chain and the human IgG heavy chain in a cell extract and a cell culture solution were detected by Western blotting.

The construct of the expression cassette used herein was construct No. 14 (Fig. 20) used in Example 1, to which a human IgG light chain- or a human IgG heavy chain-coding DNA was inserted as a target gene (insertion gene). Fig. 47 shows the nucleotide sequence (SEQ ID NO: 26 or SEQ ID NO: 27) of DNA encoding a human IgG light chain (Fig. 47A) or a human IgG heavy chain (Fig. 47B), to which a restriction enzyme site was ligated. As a control, with the use of commercial pTracer (registered trademark)-EF/V5-His-A (Invitrogen) and pEF6/Myc-His-A (Invitrogen) expression plasmids, the human IgG light chain- or the human IgG heavy chain-coding DNA was inserted into the *Eco*R 1-*Xba* 1 restriction enzyme site of each plasmid.

Western blotting was carried out using an anti-6His antibody (MBL). At 24 hours after transfection, a cell extract (total protein amount: 10 μg) and 1 mL of a culture solution were subjected to trapping with 6His amino acids. The

total amount of the thus collected protein was used.

Fig. 48 shows the results. Fig. 48A shows the result for the human IgG light chain and Fig. 48B shows the result for the human IgG heavy chain. In both Fig. 48A and Fig. 48B, lane 1 indicates the result of expression using pTracer (registered trademark)-EF/V5-His-A, lane 2 shows the result of expression using pEF6/Myc-His-A, and lane 3 shows the result of expression using construct No. 14. Also, Fig. 48 shows the results of intracellular expression using the cell extract and the results thereof using the culture solution. As shown in Fig. 48, the strongest expression was observed in the case of using construct No. 14. The results demonstrate that this system (the backbone of construct No. 14) is useful for production of proteins to be used for pharmaceutical products such as antibodies, diagnostic agents, or reagents in view of mass production based on gene enhancement.

Example 5 Expression of REIC protein using expression vector constructed by modification of commercial plasmid vector

The expression cassette of the present invention containing a human REIC gene as a foreign gene (insertion gene) was prepared. HEK293 cells were transfected with the expression cassette and then the thus expressed protein was analyzed by Western blot. Transfection was carried out using FuGENE (trademark) HD. After 24 hours, the REIC protein in a cell extract was detected by Western blotting.

The construct of the expression cassette used herein was construct No. 17 (Fig. 49). Construct No. 17 was constructed by inserting REIC-coding DNA into the *Xba* 1-*Kpn* 1 insertion site of a commercial pShuttle plasmid vector (Clontech), and then inserting 3 enhancers (an hTERT enhancer, an SV40 enhancer, and a CMV enhancer) located downstream of an expression gene of

expression plasmid construct No. 14 (Fig. 20) used in Example 1 into the *Kpn* 1-*Eco*R 1 insertion site downstream of the REIC-coding DNA in the form of BGH poly A + 3 enhancers. As a control, a vector constructed by inserting REIC-coding DNA into the *Xba* 1-*Kpn* 1 insertion site of a commercial pShuttle plasmid vector (Clontech) was used. Fig. 50 shows the full nucleotide sequence (SEQ ID NO: 28) of expression vector construct No. 17 into which REIC-coding DNA was inserted. Portions surrounded by frames (1) and (2) in the nucleotide sequence in Fig. 50 indicate REIC-coding DNA and 3 enhancers (3xenh), respectively.

Western blotting was carried out using an anti-REIC antibody and a cell extract (total protein amount: 10 μg) obtained at 24 hours after transfection.

Fig. 51 shows the results. Lane 1 shows the result of a cell extract of cells not caused to express any foreign protein, lane 2 shows the result of a cell extract of cells transfected with a commercial pShuttle vector to which REIC-coding DNA had been inserted, lane 3 shows the result of a cell extract of cells transfected with an expression vector that had been constructed by inserting REIC-coding DNA into a commercial pShuttle vector, and then inserting 3 enhancers into a site downstream of the REIC-coding DNA (downstream of BGH poly A). As shown in Fig. 51, the strongest expression was observed in the case of using construct No. 17. The results demonstrate that incorporation of the portion of 3 enhancers of this system (the backbone of construct No. 14) into a site downstream of the expression gene cassette of a commercial plasmid such as a pShuttle vector enables to enhance the gene expression. Specifically, the results demonstrate that target gene expression can be enhanced by insertion of the region of 3 enhancers into various gene constructs.

Example 6 Expression of c-myc gene with the use of expression vector

constructed by modification of commercial plasmid vector

The expression cassette of the present invention containing a human c-mycC gene as a target gene (insertion gene) was prepared. HEK293 cells were transfected with the expression cassette. The thus expressed protein was analyzed by Western blot. Transfection was carried out using FuGENE (trademark)-HD. After 24 hours, the REIC protein in a cell extract was detected by Western blotting.

The construct of the expression cassette used herein was constructed by inserting REIC-coding DNA into the *Xba* 1-*Kpn* 1 insertion site of a commercial pShuttle plasmid vector (Clontech), and then inserting 3 enhancers (hTERT enhancer, SV40 enhancer, and CMV enhancer) located downstream of the expression gene of expression plasmid construct No. 14 (Fig. 20) used in Example 1 into the *Kpn* 1-*Eco*R 1 insertion site downstream of the REIC-coding DNA in the form of BGH poly A + 3 enhancers. As a control, a vector constructed by inserting human c-myc-coding DNA into the *Xba* 1-*Kpn* 1 insertion site of a commercial pShuttle plasmid vector (Clontech) was used. Fig. 52 shows the nucleotide sequence (SEQ ID NO: 29) of the inserted c-myc gene. Fig. 53 shows the nucleotide sequence (SEQ ID NO: 30) of a region containing BGH poly A and 3 enhancers contained in construct No. 14.

Western blotting was carried out using an anti-c-mycC antibody (Santa Cruz Biotechnology, Inc., Cat No.: sc-70469) and a cell extract (total protein amount: 10 μg) obtained at 24 hours after transfection.

Fig. 54 shows the results. Lane 1 shows the result of a cell extract of cells transfected with a commercial pShuttle vector to which an REIC-coding DNA had been inserted, lane 2 shows the result of a cell extract of cells transfected with an expression vector constructed by inserting c-myc-coding DNA into a commercial pShuttle vector and then inserting 3 enhancers to a site

downstream (downstream of BGH poly A) of the c-myc-coding DNA. As shown in Fig. 54, the strongest expression was observed when the expression vector constructed by inserting c-myc-coding DNA into a commercial pShuttle vector and then inserting 3 enhancers into a site downstream of the c-myc-coding DNA (downstream of BGH poly A) had been used. This result demonstrates that the gene expression can be enhanced by incorporation of the portion of 3 enhancers of this system (the backbone of construct No. 14) into a site downstream of the expression gene cassette of a commercial plasmid such as a pShuttle vector. Specifically, the result demonstrates that insertion of the region of 3 enhancers to various gene constructs can enhance target gene expression. The result of this example demonstrates that c-myc gene expression is enhanced by incorporation of the portion of 3 enhancers of this system (the backbone of construct No. 14) into a site downstream of the expression gene cassette of a plasmid for expression of a c-myc gene to be used for preparation of iPS cells. iPS cells are thought to be useful for various types of regeneration medicine. Specifically, the result demonstrates that the enhancer portion is useful for regeneration medicine and the like as a means for enabling stronger expression of namely, reprogramming genes, such as c-myc, or a means for using the thus expressed protein itself as a regeneration factor *in vivo* or *ex vivo*.

Example 7 Enhanced expression upon the use of hTERT as promoter

The expression cassettes of the present invention containing GFP (Green fluorescent protein) as a foreign gene (insertion gene) were prepared. HEK293 cells were transfected with the expression cassettes, and the thus expressed proteins were analyzed by Western blot. Transfection was carried out using FuGENE (trademark)-HD. After 24 hours, GFP in cell culture

solutions was detected by Western blotting.

The constructs of the expression cassettes used herein were: construct No. 2 (Fig. 8) used in Example 1 and constructs No. 18, 19, 20, and 21. Constructs No. 18, 19, 20, and 21 are shown in Fig. 55, Fig. 56, Fig. 57, and Fig. 58, respectively. Construct No. 18 is a general gene expression vector containing an hTERT promoter. Such a gene expression vector is advantageous in strong gene expression in an environment where hTERT is expressed at a high level; that is, in cancer cells, and is used when cancer cell-specific gene expression is expected. However, the use of this vector (construct No. 18) results in extremely weak gene expression and this hinders the vector from being clinically applied to gene therapy, protein (drug) production in specific cells, and the like. Construct No. 19 is a plasmid constructed by inserting an RU5 sequence into a site upstream of an expression gene of a general gene expression vector (construct No. 18) containing an hTERT promoter. Construct No. 20 is a plasmid constructed by inserting an hTERT enhancer sequence into a site downstream of a BGH poly A sequence of a general gene expression vector (construct No. 18) containing an hTERT promoter. Construct No. 21 is a plasmid constructed by inserting an RU5' sequence into a site upstream of an expression gene of a general gene expression vector (construct No. 18) containing an hTERT promoter and further inserting an hTERT enhancer sequence into a site downstream of the BGH poly A sequence. Fig. 59-1 and Fig. 59-2 (a continuation from Fig. 59-1) show the full nucleotide sequence (SEQ ID NO: 31) of expression vector construct No. 21. Portions surrounded by frames (1), (2), (3), (4), and (5) in the nucleotide sequence shown in Fig. 59-1 and Fig. 59-2 indicate an hTERT core promoter, a minimal CMV promoter, RU5', a GFP gene, BGH poly A, and an hTERT core promoter, respectively.

Fig. 60 shows the results of expression. Lane 1 shows the results for a

cell extract of cells not caused to express any foreign protein, and lanes 2 to 6 show the results of expression using constructs No. 2, No. 18, No. 19, No. 20, and No. 21, separately. As shown in Fig. 60, expression was observed descending order of strength in constructs No. 18, No. 19, No. 20, No. 21 and No. 2. The results for this example demonstrate that this system (the form of the constructs wherein a target expression gene is flanked by a promoter and an enhancer) is useful for significantly enhancing the gene expression of an hTERT promoter that enables cancer-specific gene expression but has extremely weak gene expression capacity.

Example 8 Expression upon simultaneous transfection with a plurality of expression vectors having different foreign genes incorporated therein

Cells were transfected simultaneously with a plasmid constructed by incorporating DsRed (red fluorescent protein)-coding DNA into construct No. 2 (Fig. 20), a plasmid constructed by incorporating Yeast GST (Glutathione S transferase)-coding DNA into construct No. 21 (Fig. 58), and a plasmid constructed by incorporating GFP-coding DNA into construct No. 21 (Fig. 58). The thus expressed proteins were analyzed by Western blot. Transfection was carried out using FuGENE (trademark)-HD. As cells, cells of cancer cell lines, HEK293 cells, Hela cells, PC3 cells, HepG2 cells, HCT116 cells, and MCF7 cells were used. Furthermore, as cells of a normal cell line, OUMS-24 cells (human-derived fibroblasts) and NHK cells (human-derived keratinocyte cells) were used. At 48 hours after transfection, each protein in cell extracts was detected by Western blotting. For DsRed-coding DNA and Yeast GST-coding DNA, cDNAs were prepared by artificial synthesis based on known cDNA sequences.

Western blotting was carried out using an anti-GFP antibody, an anti-

6His antibody (MBL, used for detection of 6His-added Telomerase), an anti-tubulin antibody (Sigma), an anti-DsRed antibody (Clontech), and an anti-Yeast GST antibody and cell extracts obtained at 24 hours after transfection (total protein amount: 10 μg).

Fig. 61 shows the results. As shown in Fig. 61, a plasmid construct (construct No. 21) containing an hTERT promoter is useful for carrying out gene expression specific to only cancer cells in various cancer cells and normal cells. Specifically, in this case, genes were expressed strongly only in cancer cells, and gene expression in normal cells can be suppressed. In this regard, for example, gene expression is observed in both human cancer cells and normal cells in the case of a plasmid construct (construct No. 2) containing a CMV promoter, so that cancer-cell-specific gene expression cannot be achieved. As described above, a plasmid containing an hTERT promoter is useful in that cancer-cell-specific gene expression can be achieved.

Example 9 Examination of the production amount of human erythropoietin (Human EPO) by HEK293 cells (Human embryonic kidney cells) transfected with expression vector containing the gene expression cassette of the present invention

For secretory expression of human erythropoietin, the DNA fragment of EPO-His tag having the His-tagged C-terminus was incorporated into a vector (referred to as SGE (Super Gene Expression) vector) containing construct No. 14 and the same was incorporated into a pTracer (registered trademark)-EF vector (EF-1α promoter, Invitrogen) as a control.

As host cells for secretory expression of human erythropoietin, 30 mL of human kidney-derived cells (FreeStyle 293-F cells (Invitrogen)) at the logarithmic growth phase was seeded at a concentration of 5-6 x 10^5 cells/mL to

a 125 mL-flask. The cells were cultured with shake (125 rpm) overnight at 37°C in the presence of 8% CO_2 using Freestyle 293 Expression 1 Media (Invitrogen). On the next day, the cell concentration was adjusted to 1 x 10^6 cells/mL. To 125-mL flask to which 30 mL of 293-F cells had been seeded, 30 μg each of SGE-EPO-His-tag plasmid DNA and pTracer (registered trademark)-EF-EPO-His-tag plasmid DNA were mixed with a transfection reagent: 293 Fectin (Invitrogen) and then transfection was carried out. During 4 days after transfection, cells were cultured with shake at 37°C in the presence of 8% CO_2, and then a culture supernatant was collected. 18 μL of the culture supernatant was separated on SDS-PAGE, and then a glycosylation-type EPO protein with a molecular weight of about 35 kDa was detected by CBB staining (Fig. 62).

For estimation of the production amount of EPO, the human erythropoietin protein secreted in 25 mL of a culture supernatant collected 4 days later was purified using histidine affinity column chromatography (TALON-Affinity Resin (Clontech)). The eluate was separated on SDS-PAGE and then the purity of the EPO protein was confirmed by CBB staining (Fig. 63). The protein amount of the purified EPO protein was determined by Bradford assay, and then the protein amount obtained in the case of 1 L of the culture solution was calculated from the amount of the purified protein in the case of 25 mL of the culture solution (Fig. 64A and B). As a result, human erythropoietin could be produced with the use of the SGE vector at an expression level about 8 times higher than that in the case of using the pTracer (registered trademark)-EF vector. Therefore, an extremely highly efficient expression level; that is, about 150 mg (in terms of the level in the case of 1 L of the culture solution), was achieved.

Example 10 Examination of production amount of human REIC (Reduced

Expression in Immortalized Cells) protein by HEK293 cells (Human embryonic kidney cells) transfected with expression vector containing the gene expression cassette of the present invention

According to the method described in Example 1, 293-F cells were adjusted to a concentration of 1 x 10^6 cells/mL and then 180 mL of the solution was seeded in each of three 500-mL flasks. Each flask was subjected to transient transfection with an REIC expression SGE vector (the vector containing construct No. 14) (180 μg) using a transfection reagent: 293 Fectin (Invitrogen). During 4 days after transfection, cells were cultured with shake at 37°C in the presence of 8% CO_2, and thus culture supernatants were collected. Each culture supernatant (18 μL) was separated on SDS-PAGE, and then a glycosylated REIC protein with a molecular weight of about 55 kDa was detected by CBB staining (Fig. 65).

The collected culture supernatants were each concentrated by ultrafiltration from 520 mL to 35 mL. The solvent was substituted with 20 mM Hepes Buffer (pH7.2) using Sephadex (Trademark) G25M column chromatography (GE Healthcare), and then a REIC protein-containing fraction was collected. Thereafter, protein adsorption was carried out using anion exchange column chromatography (DEAE-Toyopearl (registered trademark) 650 M, TOSOH Corporation), and then elution was carried out in 20 mM Hepes Buffer (pH 7.2) with a linear concentration gradient (0 M to 0.7 M) of sodium chloride. Under the conditions of the sodium chloride concentration of about 0.35 M, the peak fractions of the REIC protein was confirmed. Each peak fraction was analyzed by SDS-PAGE, and thus fractions composed only of the REIC protein with high purity were collected (Fig. 66). The protein amount of the purified REIC protein was calculated using absorbance (280 nm) and the purified protein amount in the case of 520 mL of the culture solution, so that the

protein amount obtained in the case of 1 L of the culture solution was calculated (Fig. 67). As a result, about 50 mg of the purified protein with high purity could be obtained from the culture supernatant subjected to transfection with the REIC expression SGE vector. Specifically, it was demonstrated that the purified protein can be actually collected in an amount as large as about 100 mg in terms of 1 L of the culture solution.

Industrial Applicability

The gene expression cassette of the present invention comprises a DNA construct containing a gene to be expressed and a poly A addition sequence that are located downstream of a 1st promoter, wherein an enhancer or a 2nd promoter is ligated downstream of the DNA construct. The gene expression cassette realizes the mass production of a target protein through super-high expression, regardless of cell type, gene type, and transfection reagent type. The gene expression cassette can be applied as a reagent in the field of biotechnology and can also be applied extensively as a therapeutic protein remedy or for clinical treatment, examination, and diagnosis using genes. For example, the gene expression cassette can facilitate revolutionary evolution techniques in the field of biotechnology, such as: (1) the functional analysis of unknown genes in specific cells or tissues that have conventionally remained unanalyzed; (2) improvement of therapeutic effects via loading both viral and non-viral vectors with the gene expression cassette in gene therapy; (3) preparation of super cells capable of producing large amounts of a specific human functional protein with the use of human cells, as a method for producing biopharmaceutical products such as antibody drugs used in recent years; and (4) application for an efficient and inexpensive method for producing reagents or diagnostic agents to be used for various forms of assay or clinical diagnosis.

The DNA expression cassette of the present invention can be applied as a reagent in the field of biotechnology and can also be extensively applied for clinical treatment, examination, and diagnosis using genes.

Sequence Listing Free Text

SEQ ID NOs: 1-16, 19-31 Synthesis

CLAIMS

1. A gene expression cassette, which comprises:
(i) a DNA construct in which a 1st promoter, a gene to be expressed, and a polyA addition sequence are linked in such order; and
(ii) an enhancer or an enhancer with UAS ligated to the upstream portion thereof, in such order,
wherein the enhancer or the enhancer with UAS ligated to the upstream portion thereof is ligated immediately downstream of the polyA addition sequence, and
can enhance the protein expression from the gene compared with a case in which an enhancer is inserted upstream of the 1st promoter.

2. The expression cassette according to claim 1, which does not have another mechanism for gene expression at a site downstream of the ligated enhancer, but has a structure in which a gene to be expressed is flanked by the 1st promoter and the enhancer.

3. The expression cassette according to claim 1, wherein the promoter is selected from the group consisting of a CMV i promoter, an SV40 promoter, an hTERT promoter, a β actin promoter, and a CAG promoter.

4. The expression cassette according to any one of claims 1 to 3, wherein the enhancer ligated downstream of the polyA addition sequence is at least one enhancer selected from the group consisting of a CMV enhancer, a SV40 enhancer, and an hTERT enhancer.

5. The expression cassette according to any one of claims 1 to 4, wherein the enhancers ligated downstream of the polyA addition sequence are an hTERT enhancer, an SV40 enhancer, and a CMV enhancer that are linked in such order.

6. The expression cassette according to any one of claims 1 to 5, wherein (i) 1 to 4 CMV enhancers are ligated upstream of the DNA construct in which a 1st promoter, a gene to be expressed, and a polyA addition sequence are linked in such order.

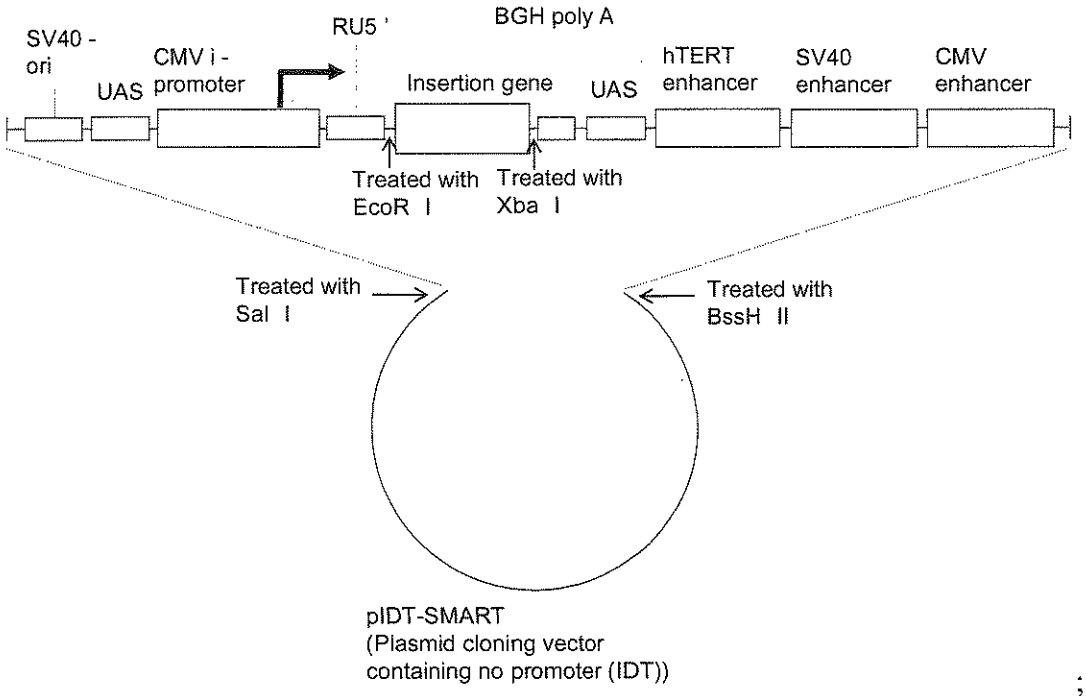
7. The expression cassette according to any one of claims 1 to 6, containing at least any one of the following elements (i) and (ii)
(i) RU5' ligated immediately upstream of a gene to be expressed; and
(ii) SV40-ori ligated to the most upstream portion of the expression cassette.

8. The expression cassette according to any one of claims 1 to 7, wherein DNA encoding the protein to be expressed is a therapeutic gene that can be used for treatment of disease, or DNA encoding a protein that can be used for a drug, a diagnostic agent, or a reagent.

9. The expression cassette according to claim 8, wherein the therapeutic gene is a REIC/Dkk-3 gene that is a cancer suppressor gene applicable for the treatment of tumors.

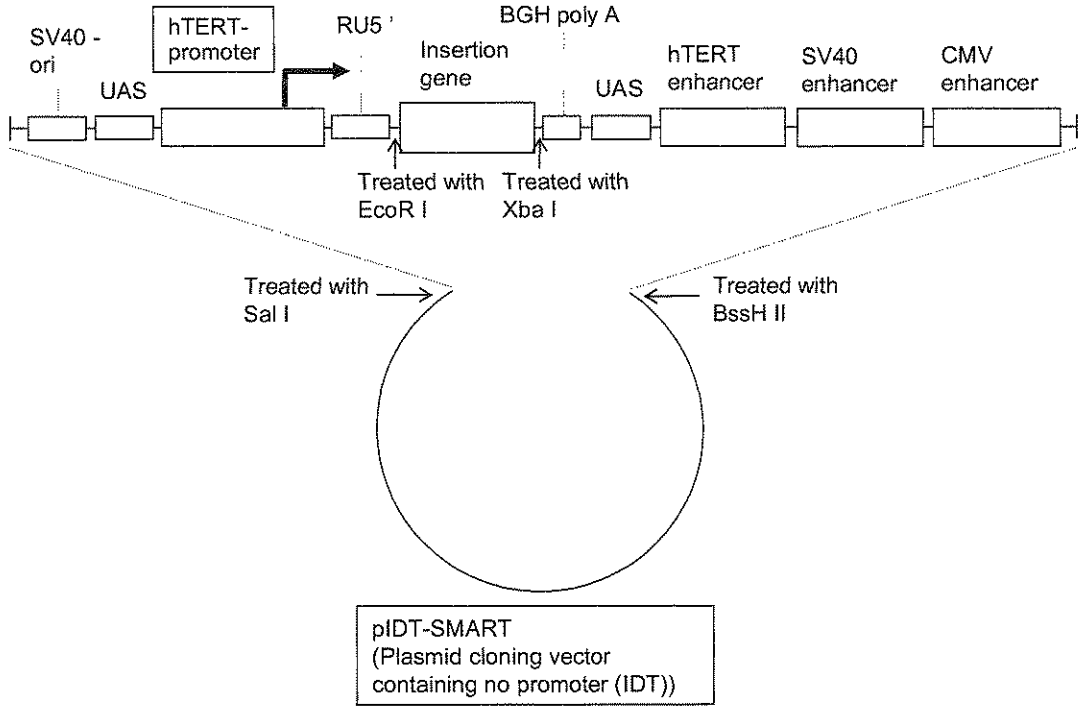
10. The expression cassette for a foreign gene according to claim 5, having the structure of a construct selected from the group consisting of construct No.14, construct No.16, and construct No.17 as the followings:

Construct No.14



;

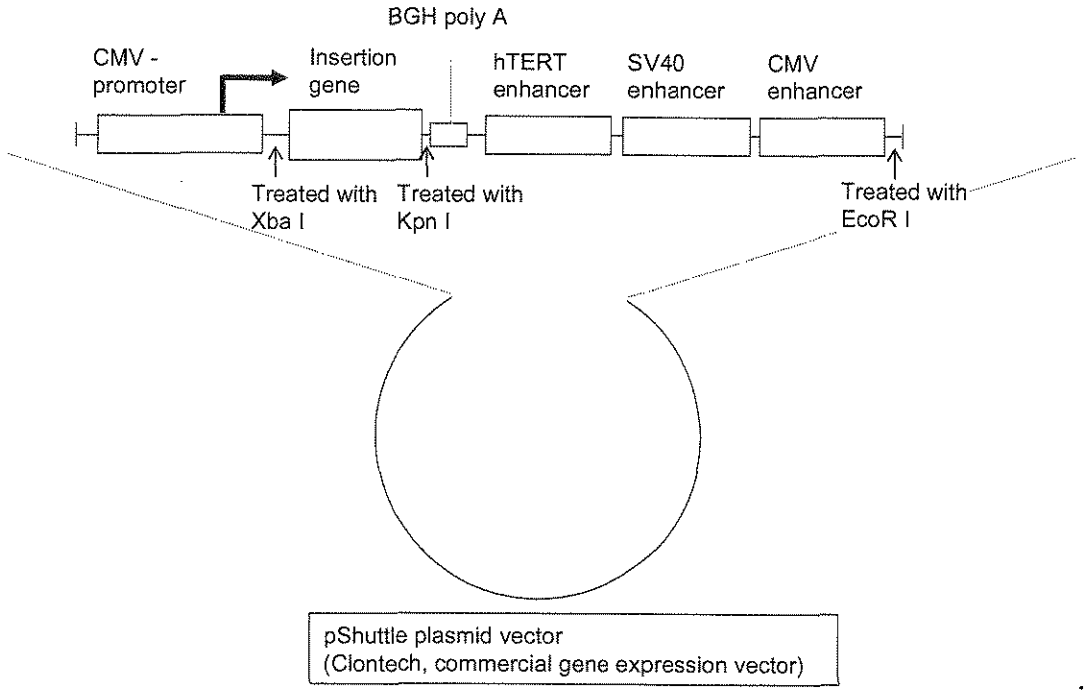
Construct No.16



;

and

Construct No.17



11. A vector, containing the expression cassette for a foreign gene according to any one of claims 1 to 10.

12. The vector according to claim 11, which is an adenovirus vector or an adeno-associated virus vector.

13. A host cell, containing the vector according to claim 11 or 12.

14. A preparation for disease detection or treatment, comprising the vector according to claim 11 or 12.

15. An in vitro method for expressing a gene to be expressed using the expression cassette according to any one of claims 1 to 10 or the vector according to claim 11 or 12.

16. An in vitro method for producing a protein encoded by a gene to be expressed, comprising introducing the expression cassette according to any one of claims 1 to 10 or the vector according to claim 11 or 12 into a cell, and then culturing the cell.

17. An in vitro method for enhancing protein expression from a gene compared with a case in which an enhancer is inserted upstream of a 1st promoter by: ligating an enhancer or an enhancer with UAS ligated to the upstream portion thereof to a site immediately downstream of a polyA addition sequence of a DNA construct in which a 1st promoter, a gene to be expressed, and the polyA addition sequence are linked in such order, so as to insert the enhancer to a site immediately downstream of the DNA construct comprising the gene to be expressed, in an expression cassette for gene expression comprising the 1st promoter, the gene to be expressed, the polyA addition sequence, and the enhancer.

18. The method according to claim 17, wherein the enhancer ligated downstream of the polyA addition sequence is at least one enhancer selected from the group consisting of a CMV enhancer, an SV40 enhancer, and an hTERT enhancer.

19. The method according to claim 17 or 18, wherein the enhancers ligated downstream of the polyA addition sequence are a TERT enhancer, an SV40 enhancer, and a CMV enhancer that are linked in such order.