SPECIFICATION

HIGH-EXPRESSION VECTOR FOR ANIMAL CELLS

TECHNICAL FIELD

The present invention relates to a vector DNA for animal cells, which realizes high-level production of recombinant proteins using an animal cell as a host. The vector of the present invention is particularly useful for expressing a protein which cannot be produced as an active protein when a bacterium, eukaryotic microorganism or insect cell is used as a host.

BACKGROUND ART

A large number of vectors used for production of recombinant proteins have been developed, and such vectors enable the production of such recombinant proteins at high yield in an expression system in which a bacterium, eukaryotic microorganism or insect cell is used as a host. However, in the expression system in which these hosts are used, there may be problems regarding modification of proteins, or the characteristics of mammalian proteins may be lost. Thus, for example, in the case of a protein having a biological activity which depends on sugar chains, it is necessary to produce the protein using a mammalian cell as a host. Although there is such a requirement, the productivity of recombinant proteins is generally found to be low in an expression system in which a mammalian cell is used as a host. Also, there is a problem regarding the stability of an introduced gene in many cases.

Examples of a case of producing a recombinant protein using a mammalian cell as a host include erythropoietin (Japanese Patent Laid-Open No. 2002-529100) and IFN-β (Japanese Patent Laid-Open No. 7-265084). In addition, there have been many reports regarding production of a genetically recombinant monoclonal antibody (Japanese Patent Laid-Open Nos. 7-67648, 6-30788 and 6-217786).

DISCLOSURE OF THE INVENTION

Improvement of productivity of a recombinant protein using a mammalian cell as a host is not only very important for the production of therapeutic agents or diagnostic agents, but it largely contributes to research for development of these agents. Thus, the development of a vector that enables high-level productive strains to be easily obtained, using, as a host, a mammalian cell, and especially, a Chinese hamster ovary cell (CHO cell), is considered to be an urgent necessity.

In order to easily obtain high-level productive strains, it is adequate to allow high-level productive strains producing a large number of substances of interest to exist in a small number of transformed cells. Thus, it becomes easy to select high-level productive clones.

It has been found that the expression level of a gene differs significantly depending on positions on a chromosome (Annu. Rev. Cell Biol., 6, p. 679, 1990). In order to obtain high-level productive strains, it seems to be important that a vector DNA used in transformation is targeted to a site on a chromosome which allows high gene expression level, considering the properties or performances of the vector DNA.

As stated above, an object of the present invention is to provide an expression vector which enables easy obtainment of high-level productive strains, using a mammalian cell, and especially, a Chinese hamster ovary cell (CHO cell) as a host.

The present inventors have developed a vector having a mechanism in which strains wherein a gene cassette of interest is incorporated into a position which allows a high gene expression level on the chromosome of a host cell eventually survive as neomycine-resistant strains. In order to enable targeting to a position which allows a high gene expression level on a chromosome, it is important to set the expression level of a neomycine phosphotransferase (hereinafter referred to as NPT) gene to be infinitesimal. Moreover, when the above vector has a dihydrofolate reductase (hereinafter referred to as DHFR) gene and is designed in such a way that the expression level of the gene is controlled, it enables efficient gene amplification by stimulation of methotrexate. In the present invention, a vector meeting the two above requirements has been developed. As a result, a vector that enables high-level and stable production of proteins can be produced, thereby completing the present invention.

Thus, the present invention provides the followings:

(1) An expression vector inducing high productivity of genetically recombinant proteins in animal host cells, which comprises a highly expression-inducible promoter, a multicloning site for insertion of gene and a polyadenylation signal sequence in this order from upstream, and further comprises downstream thereof a drug resistant gene that does not have a promoter capable of acting in animal cells.

(2) The expression vector according to (1) above, wherein the highly expression-inducible promoter is a human cytomegalovirus Major Immediately-Early antigen promoter, CMV5 promoter (a synthetic chimeric promoter), a β-actin promoter, or an SV40 early promoter.

(3) The expression vector according to (1) or (2) above, wherein the drug resistant gene

is a neomycine resistant gene, codon-disoptimized neomycine resistant gene, a hygromycin resistant gene, a zeocin resistant gene, or a blasticidin resistant gene.

(4) The expression vector according to any one of (1) to (3) above, wherein the drug resistant gene is a neomycine phosphotransferase gene.

(5) The expression vector according to any one of (1) to (4) above, wherein the drug resistant gene is a neomycine phosphotransferase gene derived from *Escherichia coli* transposon Tn5.

(6) The expression vector according to any one of (1) to (5) above, wherein a trace amount of the messenger RNA or protein of a drug resistant gene is generated by a read-through effect which is achieved by the fact that the drug resistant gene exists downstream of the highly expression-inducible promoter blocked by the polyadenylation signal sequence.

(7) The expression vector according to any one of (1) to (6) above, which further comprises, downstream of the drug resistant gene, a dihydrofolate reductase gene or codon-disoptimized dihydrofolate reductase gene that does not have a promoter capable of acting in animal cells.

(8) The expression vector according to any one of (1) to (7) above, which further comprises, downstream of the drug resistant gene, a highly expression-inducible promoter, a multicloning site for insertion of gene and a polyadenylation signal sequence in this order from upstream, and further comprises downstream thereof a dihydrofolate reductase gene or codon-disoptimized dihydrofolate reductase gene that does not have a promoter capable of acting in animal cells.

(9) The expression vector according to any one of (1) to (6) above, which comprises a dihydrofolate reductase gene or codon-disoptimized dihydrofolate reductase gene ligated downstream of a promoter with low expression inductivity.

(10) The expression vector according to any one of (7) to (9) above, wherein the promoter with low expression inductivity is a promoter derived from a gene of a protein that is generally expressed in trace amounts in mammalian cells, a promoter derived from a viral antigen gene that hardly ever infects mammals, and a promoter obtained by removing an enhancer sequence from the above promoters.

(11) A recombinant expression vector which is obtained by incorporating a gene of interest into the multicloning site for insertion of gene of the expression vector according to any one of (1) to (6) above.

(12) A recombinant expression vector which is obtained by incorporating a gene of interest into the multicloning site for insertion of gene of the expression vector according

to any one of (7) to (10) above.

(13) A transformant comprising the expression vector according to any one of (1) to (10) above or the recombinant expression vector according to (11) or (12) above.

(14) A method for producing a transformant that highly expresses a gene of interest, which comprises introducing the recombinant expression vector according to (11) or (12) above into animal host cells.

(15) A method for obtaining a transformant capable of stably producing proteins in a serum free medium, which comprises introducing the recombinant expression vector according to (11) or (12) above into animal host cells so as to obtain a transformant, and acclimating the obtained transformant to the serum free medium.

(16) A method for amplifying a gene of interest, which comprises introducing the recombinant expression vector according to (12) above into a dihydrofolate reductase-deficient CHO cell, and culturing the cell in the presence of methotrexate.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a flow chart for construction of a neomycine resistant vector pSV/GKT-Neo.

Figure 2 is a flow chart for construction of a neomycine resistant/methotrexate resistant vector pND.

Figure 3 is a flow chart for construction of a cassette plasmid pCB used for expression of a desired gene.

Figure 4 is a flow chart for construction of a foreign gene expression vector pNOS-GKT2 having an NPT gene cistron that does not have a transcription promoter acting in mammalian cells.

Figure 5 is a flow chart for construction of a foreign gene expression vector pNOS-GKT2B, in which bGH-poly A for termination of transcription is located upstream of the NPT gene cistron having no promoters.

Figure 6 is a flow chart for construction of a foreign gene expression vector pNOS-GKT2B comprising a rabbit β globin gene intron for improvement of translation efficiency.

Figure 7 is a map of the expression vector pNOS-GKT2B of the present invention.

Figure 8 is a flow chart for construction of an hG-CSF expression vector pNOS-GKT2B-R/hG-CSF.

Figure 9 is an example of detection of hG-CSF by the dot blot method, which

was produced by clones transfected with the expression vector pNOS-GKT2B of the present invention.

Figure 10 is a distribution of the expression level of hG-CSF of each clone transfected with the expression vector pNOS-GKT2B of the present invention.

Figure 11 is a map of the expression vector pNOS of the present invention used in Example 4.

Figure 12 is an example of detection of hG-CSF by the dot blot method, which was produced by clones transfected with the expression vector pNOS/human G-CSF of the present invention.

Figure 13 is another example of detection of hG-CSF by the dot blot method, which was produced by clones tranfected with the expression vector pNOS/human G-CSF of the present invention.

Figure 14 is a distribution of the expression level of hG-CSF of each clone transfected with the expression vector pNOS/human G-CSF of the present invention.

Figure 15 is a flow chart for preparation of CMV5 promoter.

Figure 16 is a flow chart for preparation of CMV5 promoter.

Figure 17 is a flow chart for preparation of pCMV5-luciferase.

Figure 18 is the comparison of the activity of the CMV5 promoter with CMV promoter by luciferase assay.

BEST MODE FOR CARRYING OUT THE INVENTION

The embodiments of the present invention will be described in detail below.

If an introduced drug resistant gene (e.g., an NPT gene) is not expressed or its expression level is too low, a host cell into which vector DNA has been introduced cannot obtain resistance to the corresponding drug (e.g., neomycine (hereinafter referred to as NEO)). However, in studies that the present inventors have been made to date, in all cases of using vector DNA comprising a common NPT gene, including commercially available vectors, numerous NEO resistant strains have been obtained. From these results, it has been assumed that the expression of an NPT gene provided from vector DNA contributes as a selective marker for determining whether or not a cell obtained as a result of transfection is a transformant, but that it has no effects other than that.

The present inventors have limited the induction of NPT expression to an extreme extent, so that they have designed NPT such that NPT is not expressed at a level necessary for obtaining resistance to NEO, as long as an NPT gene cistron is not incorporated into a site which allows a high gene expression level on the chromosome of

a host cell. Thus, they have designed vector DNA such that a transformed host cell does not acquire resistance to NEO and cannot survive in a medium containing a certain amount of NEO, as long as the vector DNA is introduced into a site which allows an extremely high level of gene expression on the chromosome. There have been several reports on the use of a weak promoter for NPT gene cistrons of vector DNA (Mol. Cell Biol., 6, p. 2593, 1986; Mol. Cell Biol., 7, p. 1296, 1987; DNA, 7, p. 651, 1988). However, it is difficult to think that highly productive transformants are effectively and sufficiently selected in these cases.

The present invention is based on the principle that a DNA fragment comprising an NPT gene cistron derived from *Escherichia coli* transposon Tn5, while it has no promoters having transcriptional activity in mammalian cells, is allowed to locate downstream of a promoter having transcriptional activity in mammalian cells and the following optimal polyadenylation signal haing strong transcription termination activity, so that the NPT expression mechanism in the transformed host cells is significantly damaged and attenuated. Since the NPT gene cistron of the present invention has no promoters, it is defined as a "promoterless NPT gene cistron."

Thus, the present invention provides an expression vector, which comprises a highly expression-inducible promoter, a multicloning site for insertion of gene and a polyadenylation signal sequence in this order from upstream, and further comprises downstream thereof a drug resistant gene that does not have a promoter capable of acting in animal cells. The expression vector of the present invention can induce high productivity of genetically recombinant proteins in animal host cells.

In the present invention, examples of a drug resistant gene may include a neomycine resistant gene, a hygromycin resistant gene, a zeocin resistant gene, and a blasticidin resistant gene. Particularly preferably, the drug resistant gene is a neomycine phosphotransferase gene. Most preferably, it is a neomycine phosphotransferase gene derived from *Escherichia coli* transposon Tn5. In the present invention, a neomycine phosphotransferase gene of SEQ ID NO.50 having a weak expression level, which is prepared by substituting a part or all of the codons of neomycine phosphotransferase gene with codons which are used at a low frequency in CHO cells, defined as codon-disoptimized neomycine resistant gene, can be used as a drug resistant marker to construct a foreign gene expression vector.

In the present invention, a trace amount of the messenger RNA or protein of drug resistant gene is generated by a read-through effect obtained by the fact that the drug resistant gene exists downstream of the highly expression-inducible promoter

blocked by the polyadenylation signal sequence.

Based on properties of a drug resistant gene cistron, the incorporation of the expression vector of the present invention into a high expression site on a host cell chromosome is achieved by selection of survival cells in a medium containing the corresponding drug. In this case, it is necessary to strongly induce the expression of a protein of interest at the above site on the chromosome. Accordingly, a promoter comprised in a multicloning site (hereinafter referred to as MCS) for insertion of a protein gene and a polyadenylation signal (hereinafter referred to as polyA) are selected from those having the strongest expression inductivity. Examples of such promoters may include a human cytomegalovirus Major Immediately-Early antigen promoter (hCMV MIE: Cell, 41, p. 521, 1985), a β-actin promoter (Proc. Natl. Acad. Sci. USA, 84, p. 4831, 1987), and an SV40 early promoter. In the present invention, CMV 5 promoter having a nucleotide sequence of SEQ ID NO.49 which is prepated by the method according to Massie B et al. (Journal of Virology, 1998 Mar;72(3):2289-96. Inducible overexpression of a toxic protein by an adenovirus vector with a tetracycline-regulatable expression cassette. Massie B, Couture F, Lamoureux L, Mosser DD, Guilbault C, Jolicoeur P, Belanger F, Langelier Y.) can be used instead of the aforementioned CMV promoter to construct a foreign gene expression vector.

An example of such poly A signals may include a poly A sequence derived from human growth hormone. In the present specification, a cistron having MCS into which the above gene of protein of interest is incorporated is referred to as an "expression cassette".

The present invention also relates to a transformant obtained by introducing into a host the expression vector of the present invention into which a gene of interest is incorporated, and a method for producing the transformant.

Moreover, the expression vector of the present invention preferably has a DHFR gene cistron. For this expression vector, a dihydrofolate reductase-deficient CHO cell can be used as a host cell. A gene in the vector DNA gene is introduced into the above host cell, and thereafter, the cell is cultured in the presence of methotrexate, so that the gene contained in the above vector DNA can be efficiently amplified. In the present invention, a dihydrofolate reductase gene of SEQ ID NO.51 having a weak expression level, which is prepared by substituting a part or all of the codons of dihydrofolate reductase gene with codons which are used at a low frequency in CHO cells, defined as codon-disoptimized dihydrofolate reductase gene, can be used as a selective marker to construct a foreign gene expression vector. The dihydrofolate reductase gene of SEQ

ID NO.51 having a weak expression level can be used in combination with the neomycine phosphotransferase gene of SEQ ID NO.50 having a weak expression level.

With regard to a DHFR gene cistron, as in the case of the NPT gene cistron, the DHFR gene cistron, while it has no promoters capable of expressing in mammalian cells, is allowed to locate downstream of multicloning site for insertion of genes having a highly expression-inducible promoter and the following optimal polyadenylation signal sequence, and it is used. Alternatively, a promoter whose expression inductivity is reduced, for example, a promoter obtained by removing an enhancer region from an SV40 viral antigen promoter generally noninfectious to CHO cells, is ligated to the DHFR gene cistron, and it is then used. The present inventors have considered that, in order to promote gene amplification, it is more effective to reduce the expression inductivity of a DHFR gene or to repress such inductivity, so as to make MTX contained in a medium promote the amplification of vector DNA incorporated into a host cell chromosome.

Moreover, the present invention provides a method for obtaining a high-level productive cell clone capable of producing a high level of proteins, which comprises transforming a host cell by gene transfer using the above vector DNA having the DHFR gene cistron into which a protein gene is incorporated, and amplifying the vector DNA incorporated into the host cell chromosome.

Furthermore, the present invention provides a method for obtaining a high-level productive cell clone capable of stably producing a high level of proteins in a serum free medium, which comprises transforming a host cell by gene transfer using the above vector DNA into which a protein gene is incorporated, and acclimating the obtained transformant to a serum free medium.

Still further, the present invention provides vector DNA, which has the above-described DHFR gene cistron and a multicloning site for insertion of gene located between a highly expression-inducible promoter and an optimal polyadenylation signal. This vector DNA can be used to construct vector DNA comprising the above-described promoterless NPT gene cistron.

In the present invention, the term "cistron" is used to mean a unit, which expresses proteins by transcription and translation, using a promoter, a structural gene and a polyadenylation signal (poly A), as a basic structure. The cistron may also comprise, as an insertion sequence, a DNA sequence associated with any of them or any given DNA sequence. The term "highly expression-inducible promoter" is used to mean a promoter commonly used for protein expression in ordinary vector DNA, and it

preferably means a promoter having particularly high expression inductivity, such as a human cytomegalovirus Major Immediately-Early antigen promoter, β-actin promoter, or SV40 early promoter.

The vector DNA of the present invention can be obtained by incorporating an NPT gene cistron and an expression cassette into a backbone vector, and by further incorporating a DHFR gene cistron, if desired. The backbone vector is not particularly limited. For example, vector DNA (pUC series, Gene, 33: p. 103, 1985, etc.) having an *Escherichia coli* replication origin (ColE1 ori) can be used as such a backbone vector.

An example of a method of using the vector of the present invention may include a method comprising incorporating a gene of protein into the MCS of the vector of the present invention, introducing the gene into a host cell by the cationic liposome method, selecting strains surviving in a medium containing NEO, so as to obtain cell clones capable of producing a high level of proteins. A majority of cells obtained by selecting strains surviving in a medium containing NEO has already achieved a relatively high expression level. However, in order to select cells having further high productivity from among them, the expression level of proteins may also be measured. The obtained cells having high productivity can be repeatedly subjected to limiting dilution, followed by subculture and stabilization. When further higher productive strains are required to be obtained, a vector further comprising a DHFR gene cistron is used as the vector of the present invention. MTX is added to a medium, and the DHFR gene incorporated into a host cell is stimulated by MTX, so as to amplify the gene.

Moreover, a more specific method of using the expression vector (pNOS-GKT2B or pNOS-GKT2B-R) of the present invention that will be described later in examples comprises the following steps:

(1) A gene of a substance (protein) of interest is cloned, and then (2) it is incorporated into the multicloning site (MCS) of the expression vector (pNOS-GKT2B or pNOS-GKT2B-R) of the present invention to make expression construct. (3) The expression construct is amplified in *Escherichia coli*, (4) the gene is introduced in a CHO cell (DHRF negative) by the lipofectin method or electroporation, and (5) G418 is added to a medium followed by culture, so as to select G418 resistant cells. (6) The obtained cells are repeatedly cloned while culturing them, (7) clones producing a high amount of substances of interest are selected, and (8) the culture is continued until the growth rate becomes faster and is stabilized. (9) FCS is gradually removed from the FCS-added medium containing stabilized clones, so as to acclimate the clones to a serum free medium. (10) The DNA of introduced construct is amplified by stepwise addition of

MTX (15 nM-1 μM) to the medium containing the highly productive clones, and then (11) highly productive clones are selected again, and (12) the culture is continued until the growth rate becomes faster and is stabilized. (13) FCS is gradually removed from the FCS-added medium containing stabilized clones, so as to acclimate the clones to a serum free medium. It is to be noted that the conversion of the medium into a serum free medium may be carried out either in the step (9) or (13).

The method for producing the expression vector of the present invention and the method of using the same will be further described in the following examples. A person skilled in the art can readily substitute the constituent elements used in the present examples, such as a starting plasmid or promoter, with items equivalent thereto. Accordingly, the examples are not intended to limit the scope of the present invention.

EXAMPLES

Example 1: Construction of the expression vector of the present invention

(1) Production of neomycine resistant vector [pSV/GKT-Neo]

An NEO vector [pSV/GKT-Neo] comprises an *Escherichia coli* replication origin (ColE1 ori) derived from a plasmid pUC18 (Gene, 33, p. 103, 1985), and a neomycine phosphotransferase (NPT) gene cistron. The *Escherichia coli* replication origin was amplified from the plasmid pUC18 by the PCR method, using primers having nucleotide sequences shown in SEQ ID NOS: 1 and 2. The PCR was carried out under the following conditions. A kit commercially available from Takara Shuzo Co., Ltd. was used as a PCR kit, and as a 10 x reaction buffer and a dNTP solution, items attached to the kit were used. First, the 10 x reaction buffer, the dNTP solution, 1 μl each of 100 μM primers shown in SEQ ID NOS: 1 and 2, 1 μl of template DNA and 0.5 μl of Pyrobest DNA polymerase (5 U/μl) were added to 36.5 μl of sterile distilled water, and the obtained mixture was completely blended. Thereafter, a PCR reaction was carried out using Takara Thermal Cycler PERSONAL. This is to say, after heating at 94°C for 5 minutes, a cycle consisting of the following 3 steps, denaturation (94°C, 30 seconds), primer annealing (55°C for 30 seconds), and elongation (72°C, 1 minute), was repeated 35 times, and finally the reaction product was heated at 72°C for 5 minutes, so as to terminate the reaction. It is to be noted that the PCR reactions described hereinafter were carried out using the same reaction cycle as described above. After completion of the reaction, the obtained PCR product consisting of approximately 600 base pairs was cleaved with restriction enzymes (all the restriction enzymes described below were manufactured by NEB), that is, 1 μl of *Sac*II (20000 U/μl) and 1 μl of *Cla*I (5000 U/μl),

at 37°C for 2.5 hours. A total amount of the reaction solution obtained by the treatment with the restriction enzymes was added to 1% agarose gel, followed by electrophoresis at 60 mA for 25 minutes. A DNA band consisting of approximately 600 base pairs was recovered together with the gel, and it was frozen at -135°C for 10 minutes. Thereafter, it was subjected to centrifugal separation, and a DNA fragment was recovered from the supernatant (ColE1 ori cassette; 36 ng/μl). The following restriction enzyme treatment and recovery of DNA fragments were carried out in the same manner as described above.

Next, 3 μg of a plasmid pSV(D)S/Neo (Japanese Patent Laid-Open No. 10-179169) was cleaved with 1 μl each of restriction enzymes *Sac*II (20000 U/μl) and *Cla*I (5000 U/μl), and a DNA fragment (NEO cassette 1; 28 ng/μl) consisting of approximately 2200 base pairs was prepared, which had an NPT gene cistron downstream of a monkey virus 40 (SV40) early promoter from which an enhancer portion had been removed. The two obtained fragments were ligated to each other using a Takara DNA ligation kit ver. 2 (manufactured by Takara Shuzo Co., Ltd.), so as to prepare pSVNEO/Ori'. Practically, 1 μl of a ColE1 ori cassette was mixed with 1 μl of an NEO cassette 1, and a Takara DNA ligation kit ver. 2-solution I was then added to the mixture in an amount equal to the mixed solution. The obtained mixture was reacted at 16°C for 30 minutes, so as to ligate these DNA. Subsequently, the DNA was transformed in *Escherichia coli* (*E. Coli*) XL-1BlueTM competent cells according to the rubidium-chloride method. The obtained *Escherichia coli* colonies on the LB-agar (Difco) containing 50 μg/ml kanamycin sulfate (Sigma) were inoculated into a Terrific broth (containing per liter: 12 g of bacto-tryptone (manufactured by Difco), 24 g of bacto yeast extract (manufactured by Difco), 4 ml of glycerol (manufactured by Wako Pure Chemical Industries, Ltd.), 2.31 g of KH_2PO_4 (manufactured by Wako Pure Chemical Industries, Ltd.), and 12.5 g of K_2HPO_4 (manufactured by Wako Pure Chemical Industries, Ltd.)), which contained kanamycin sulfate (50 μg/ml; Sigma). After culture for about 20 hours, cells were collected, and plasmids were isolated by the alkali-SDS method (Current Protocols in Molecular Biology, p.1-6-3, 1986), so as to prepare pSVNEO/Ori'. It is to be noted that all of the ligation, transformation and the isolation of plasmids described hereinafter were carried out by the same methods as described above.

Next, pSVNEO/Ori' was cleaved with 1 μl each of restriction enzymes *Apa*I (1000 U/μl) and *Cla*I (5000 U/μl), and then purified. To the obtained DNA fragment (38 ng/μl) consisting of approximately 2800 base pairs, a linker (pSVNEO linker) having multiple restriction enzyme cleavage sites obtained by annealing newly synthesized

oligonucleotides having nucleotide sequences shown in SEQ ID NOS: 3 and 4 was ligated. It was then transformed, and plasmids were isolated, so as to prepare pSVNEO/Ori. The linker was produced by placing 50 μl each of oligonucleotides (100 μM) shown in SEQ ID NOS: 3 and 4 into a microtube (1.5 ml; manufactured by Tref), and heating it with heat block at 70°C for 5 minutes, followed by cooling it to 25°C for about 1 hour. Hereinafter, a linker was produced in the same manner as described above.

At the same time, a DNA fragment comprising a promoter sequence controlling the expression inductivity of the NPT gene cistron in *Escherichia coli* was amplified from a plasmid pSV2NEO (manufactured by Stratagene) by PCR using primers having nucleotide sequences shown in SEQ ID NOS: 5 and 6. A kit commercially available from Takara Shuzo Co., Ltd. was used as a PCR kit, and the attached items were used as 10 x reaction buffer, a dNTP solution and 10 x magnesium chloride solution. First, 5 μl of 10 x reaction buffer, 5 μl of the dNTP solution, 5 μl of 10 x magnesium chloride solution, 1 μl each of 100 μM primers shown in SEQ ID NOS: 5 and 6, 1 μl of template DNA and 0.5 μl of LA Taq DNA polymerase (5 U/μl) were added to 31.5 μl of sterile distilled water, and the obtained mixture was completely blended. Thereafter, a PCR reaction was carried out using Takara Thermal Cycler PERSONAL. It is to be noted that PCR reactions using LA Taq DNA polymerase described hereinafter were carried out applying the same mixing ratio. The obtained PCR product consisting of approximately 400 base pairs was run on agarose gel, and then separated and purified (GKT fragment; 58 ng/μl). It was then ligated with a plasmid for cloning PCR products pT7Blue(R) (50 ng/μl; Novagen) by ligation, followed by transformation and the isolation of plasmids, so as to prepare pT7B-GKT.

Subsequently, 6 μg of pSVNEO/Ori was cleaved with 1 μl of restriction enzyme *Pst*I (20000 U/μl). The obtained fragment (38 ng/μl) consisting of approximately 2800 base pairs and comprising an *Escherichia coli* replication origin, an SV40 early promoter from which an enhancer portion had been removed, and a partial sequence of the NPT gene cistron, was ligated to a fragment (GKT cassette; 9 ng/μl) consisting of approximately 400 base pairs and comprising a partial sequence of the NPT gene cistron, which was obtained by cleaving 6 μg of pT7B-GKT with *Pst*I. Thereafter, the obtained product was transformed, and plasmids were isolated, so that pSV/GKT-Neo was produced. A flow chart for construction of [pSV/GKT-Neo] is shown in Figure 1.

(2) Production of neomycine resistant/methotrexate resistant vector [pND]

5 μg of the above pSV/GKT-Neo was cleaved with 1 μl each of restriction enzymes *Eco*RI (20000 U/μl) and *Cla*I (5000 U/μl). The obtained fragment was run on agarose gel, and it was then separated and purified, so as to obtain a fragment pSV/GKT-Neo cassette (44 ng/μl) consisting of approximately 3000 base pairs. Moreover, pSVP(D)S/DHFR (Japanese Patent Laid-Open No. 10-179169) was cleaved with 1 μl each of restriction enzymes *Eco*RI (20000 U/μl) and *Cla*I (5000 U/μl), so as to obtain a fragment (DHFR cassette; 25 ng/μl) consisting of approximately 1800 base pairs, and comprising an SV40 early promoter from which an enhancer portion had been removed and a dihydrofolate reductase (DHFR) gene cistron. The pSV/GKT-Neo cassette was ligated with the DHFR cassette by ligation, followed by transformation and the isolation of plasmids, so as to obtain pND. A flow chart for construction of [pND] is shown in Figure 2.

(3) Production of cassette plasmid [pCB] for desired gene expression

This vector was comprised of a promoter, a multicloning site (MCS) for insertion of gene of protein of interest, and a polyadenylation signal. A human cytomegalovirus (hCMV) MIE antigen promoter (PCMV) was used herein as a promoter, which is considered as a promoter having the highest expression inductivity. In addition, a bovine growth hormone poly A signal (bGH-poly A) was used as a poly A signal, which has been reported to be highly productive and excellent in reproducibility.

First, DNA was amplified from a plasmid pRc/CMV (manufactured by Invitrogen) by PCR with LA Taq DNA polymerase, using primers having nucleotide sequences shown in SEQ ID NOS: 7 and 8. The thus amplified DNA was run on agarose gel, and it was then separated and purified, so as to obtain a PCMV fragment consisting of approximately 650 base pairs. The PCMV fragment was ligated to a plasmid pT7Blue(R), followed by transformation and the isolation of plasmids, so as to produce pT7B-PCMV. At the same time, DNA was amplified from the plasmid pRc/CMV by PCR with LA Taq DNA polymerase, using primers having nucleotide sequences shown in SEQ ID NOS: 9 and 10, so as to obtain a bGH-poly A fragment consisting of approximately 650 base pairs. The thus obtained fragment was run on agarose gel, and it was then separated and purified. Thereafter, the fragment was ligated to a plasmid pT7Blue(R), followed by transformation and the isolation of plasmids, so as to produce pT7B-bGH-poly A. At the same time, 5 μg of a plasmid pUC18 was cleaved with 1 μl each of restriction enzymes *Eco*RI (20000 U/μl) and *Hind*III (20000 U/μl). To the obtained DNA fragment, a linker (CB linker) having

multiple restriction enzyme-cleavage sites obtained by annealing newly synthesized oligonucleotides having nucleotide sequences shown in SEQ ID NOS: 11 and 12 was ligated, so as to prepare pUC18-CB-linker.

Thereafter, 4.5 μg of the pUC18-CB-linker was cleaved with 1 μl each of restriction enzymes *Bam*HI (20000 U/μl) and *Hind*III (20000 U/μl) to obtain a DNA fragment consisting of approximately 2700 base pairs. The DNA fragment was run on agarose gel, and it was then separated and purified, so as to obtain a pUC18-CB-linker cassette (26 ng/μl). Moreover, 4.7 μg of the pT7B-PCMV was cleaved with 1 μl each of restriction enzymes *Not*I (10000 U/μl) and *Hind*III (20000 U/μl), and the obtained fragment was run on agarose gel, and it was then separated and purified, so as to obtain a DNA fragment (PCMV cassette; 12 ng/μl) consisting of approximately 650 base pairs and comprising PCMV. Furthermore, 1.3 μg of the pT7B-bGH-poly A was cleaved with 1 μl each of restriction enzymes *Not*I (10000 U/μl) and *Bam*HI (20000 U/μl), and the obtained fragment was run on agarose gel, and it was then separated and purified, so as to obtain a DNA fragment (bGH-poly A cassette; 2 ng/μl) consisting of approximately 650 base pairs and comprising bGH-poly A. The pUC18-CB-linker cassette, the PCMV cassette and the bGH-poly A cassette were ligated with one another by ligation, followed by transformation and the isolation of plasmids, so as to achieve [pCB]. A flow chart for construction of a cassette plasmid [pCB] for the expression of a desired gene is shown in Figure 3.

(4) Production of foreign gene expression vector [pNOS-GKT2] having NPT gene cistron having no transcription promoters acting in mammalian cells

5 μg of the pCB was cleaved with 1 μl each of restriction enzymes *Bam*HI (20000 U/μl) and *Hind*III (20000 U/μl), and the obtained fragment was run on agarose gel, and it was then separated and purified, so as to obtain a DNA fragment pCB cassette (31 ng/μl) consisting of approximately 2700 base pairs. Thereafter, a PCR product comprising the NPT gene cistron, which had been obtained by amplifying a DNA fragment by PCR with LA Taq DNA polymerase using primers having nucleotide sequences shown in SEQ ID NOS: 13 and 14, was cleaved with 1 μl each of restriction enzymes *Not*I (10000 U/μl) and *Nhe*I (5000 U/μl). Thereafter, the obtained fragment was run on agarose gel, and it was then separated and purified, so as to obtain a DNA fragment NEO cassette 2 (39 ng/μl) consisting of approximately 2700 base pairs. The pCB cassette was ligated to the NEO cassette 2, followed by transformation and the isolation of plasmids, so as to obtain pCB/GKT-Neo. Moreover, 3 μg of the

pCB/GKT-Neo was cleaved with 1 μl each of restriction enzymes *Hind*III (20000 U/μl) and *Eco*RI (20000 U/μl), and the obtained fragment was run on agarose gel, and it was then separated and purified, so as to obtain a DNA fragment (CNB cassette; 11 ng/μl) consisting of approximately 1000 base pairs and comprising PCMV, an NPT gene and bGH-poly A.

Furthermore, 5 μg of the pND was cleaved with 1 μl each of restriction enzymes *Sac*II (20000 U/μl) and *Eco*RI (20000 U/μl) and 1.5 μl of a restriction enzyme *Nae*I (10000 U/μl). The obtained fragment was run on agarose gel, and it was then separated and purified, so as to obtain a DNA fragment (DHFR cassette 2; 33 ng/μl) consisting of approximately 2300 base pairs and comprising ColE1-Ori and a DHFR gene cistron having an SV40 early promoter from which an enhancer portion had been removed.

Still further, a linker (HS linker) having restriction enzyme-cleavage sites was obtained by annealing newly synthesized oligonucleotides having nucleotide sequences shown in SEQ ID NOS: 15 and 16. 1 μl of the CNB cassette, 1 μl of the DHFR cassette 2, and 1 μl of the HS linker were connected with one another by ligation, followed by transformation and the isolation of plasmids, so as to produce [pNOS-GKT2]. A flow chart for construction of a foreign gene expression vector [pNOS-GKT2] having an NPT gene cistron having no transcription promoters acting in mammalian cells is shown in Figure 4.

(5) Production of foreign gene expression vector pNOS-GKT2B comprising bGH-poly A for termination of transcription located upstream of NPT gene cistron having no promoters

DNA was amplified from pNOW3 (Japanese Patent Laid-Open No. 10-179169) by PCR with LA Taq DNA polymerase, using primers having nucleotide sequences shown in SEQ ID NOS: 17 and 18. The thus amplified DNA was run on agarose gel, and it was then separated and purified, so as to obtain a DNA fragment (bGH-poly A; 42 ng/μl) consisting of approximately 200 base pairs. The DNA fragment was ligated to a plasmid pT7Blue(R), followed by transformation and the isolation of plasmids, so as to produce pT7Blue/BGHpA. 2.2 μg of the pT7Blue/BGHpA was cleaved with 1 μl each of restriction enzymes *Not*I (10000 U/μl) and *Nhe*I (5000 U/μl). The obtained fragment was run on agarose gel, and it was then separated and purified, so as to obtain a DNA fragment bGH-poly A cassette 2 (3 ng/μl) consisting of approximately 200 base pairs and comprising bGH-poly A. At the same time, 5 μg of the pNOS-GKT2 was cleaved with 1 μl each of restriction enzymes *Not*I (10000 U/μl) and *Xba*I (20000 U/μl). The

obtained fragment was run on agarose gel, and it was then separated and purified, so as to obtain a DNA fragment pNOS-GKT2 cassette (13 ng/μl) consisting of approximately 4200 base pairs. 1 μl of the bGH-poly A cassette 2 was ligated to 1 μl of the pNOS-GKT2 cassette, followed by transformation and the isolation of plasmids, so as to produce pNOS-GKT2B. A flow chart for construction of a foreign gene expression vector pNOS-GKT2B comprising bGH-poly A for termination of transcription located upstream of an NPT gene cistron having no promoters is shown in Figure 5.

(6) Production of foreign gene expression vector [pNOS-GKT2B-R] comprising rabbit β-globin gene intron for improvement of translation efficiency

DNA was amplified from rabbit genomic DNA (manufactured by CH Laboratories) by PCR with LA Taq DNA polymerase, using primers having nucleotide sequences shown in SEQ ID NOS: 19 and 20. The obtained PCR product (fragment length: approximately 600 base pairs), which corresponded to a portion (gloSA) encoding an intron comprising a rabbit β-globin gene splice donor and a splice acceptor sequence, was run on agarose gel, and it was then separated and purified. Thereafter, it was ligated to pT7Blue(R), followed by transformation and the isolation of plasmids, so as to prepare pT7B-gloSA.

5 μg of the pT7B-gloSA was cleaved with 1 μl each of restriction enzymes *Not*I (10000 U/μl) and *Xho*I (20000 U/μl). The obtained fragment was run on agarose gel, and it was then separated and purified, so as to obtain a DNA fragment (gloSA cassette; 22 ng/μl) consisting of approximately 600 base pairs and comprising gloSA. At the same time, 5 μg of the pNOS-GKT2B was cleaved with each of restriction enzymes *Not*I (10000 U/μl) and *Sal*I (20000 U/μl). The obtained fragment was run on agarose gel, and it was then separated and purified, so as to obtain a DNA fragment (pNOS-GKT2B cassette; 13 ng/μl) consisting of approximately 4400 base pairs. 1 μl of the gloSA cassette was ligated to 1 μl of the pNOS-GKT2B cassette, followed by transformation and the isolation of plasmids, so as to prepare pNOS-GKT2B-R.

A flow chart for construction of a foreign gene expression vector [pNOS-GKT2B-R] comprising a rabbit β-globin gene intron for improvement of translation efficiency is shown in Figure 6. In addition, the structure of [pNOS-GKT2B-R] is shown in Figure 7, and the entire nucleotide sequence is shown in SEQ ID NO: 21.

Example 2: Test of production of human granulocyte colony-stimulating factor using the

expression vector of the present invention

One type of colony-stimulating factors, human granulocyte colony-stimulating factor (hG-CSF), was selected as a substance of interest, which was used in the gene transfer of the hG-CSF gene using an expression vector pNOS-GKT2B-R, and in the establishment of G418 resistant early clones. This protein has a sugar chain, and a normal form of recombinant protein cannot be obtained in the production using *Escherichia coli* or yeast as a host.

(1) Production of hG-CSF expression vector [pNOS-GKT2B-R/hG-CSF]

DNA was amplified from human spleen cDNA by PCR with LA Taq DNA polymerase, using primers having nucleotide sequences shown in SEQ ID NOS: 22 and 23. The thus amplified DNA was run on agarose gel, and it was then separated and purified, so as to obtain a fragment (hG-CSF fragment; 13 ng/μl) consisting of approximately 600 base pairs and comprising hG-CSF cDNA. This fragment was ligated to a plasmid pT7Blue(R), followed by transformation and the isolation of plasmids, so as to prepare pT7Blue/hG-CSF. The pT7Blue/hG-CSF was cleaved with 1 μl each of restriction enzymes *Not*I (10000 U/μl) and *Xba*I (20000 U/μl). The obtained fragment was run on agarose gel, and it was then separated and purified, so as to obtain a DNA fragment (hG-CSF cassette; 6 ng/μl) consisting of approximately 600 base pairs and comprising hG-CSF cDNA. At the same time, the pNOS-GKT2B-R was cleaved with 1 μl each of restriction enzymes *Not*I (10000 U/μl) and *Xba*I (20000 U/μl). The obtained fragment was run on agarose gel, and it was then separated and purified, so as to obtain a DNA fragment (pNOS-GKT2B-R cassette; 19 ng/μl) consisting of approximately 5000 base pairs. 1 μl of the hG-CSF cassette was ligated to 1 μl of the pNOS-GKT2B-R cassette, followed by transformation and the isolation of plasmids, so as to prepare pNOS-GKT2B. A flow chart for construction of an hG-CSF expression vector [pNOS-GKT2B-R/hG-CSF] is shown in Figure 8, and the entire nucleotide sequence is shown in SEQ ID NO: 24.

(2) Introduction of hG-CSF expression vector [pNOS-GKT2B-R/hG-CSF] into mammalian cells, and selection of transformed strains using G418

1,500,000 DHFR-deficient CHO cells (DG44; obtained from Dr. Gail Urlaub; Somat. Cell Mol. Genet. 12, p. 555, 1986) placed in a 25cm^2 culture flask were transfected with 1 μg of pNOS-GKT2B-R/hG-CSF by the lipofectin method (using Effectene Transfection Reagent manufactured by Qiagen). The transfection was carried out according to an instruction book provided from the manufacturer. After a trypsin

treatment for 48 hours, the cells were peeled off, and the number of the cells was counted. Thereafter, 300,000 cells were diluted with 100ml of 10% dialyzed fetal bovine serum-added Iscove's Modified Dalbecco's Medium containing 0.8 mg/ml G418. Thereafter, the dilution was dispersed in ten 96-well microtiter plates (960 wells) and then cultured in the presence of 5% carbon dioxide at 37°C for about 3 weeks. As a result, it was found that surviving cells (G418 resistant strains) were observed only in 117 wells. 61 G418 resistant clones were randomly picked up from the surviving cells. The clones were transferred to a 24-well plate together with 1 ml of 10% dialyzed fetal bovine serum-added Iscove's Modified Dalbecco's Medium containing 0.8 mg/ml G418, and they were then cultured until they became confluent. After the culture supernatant was discarded, 1 ml of PBS (manufactured by Invitrogen) was added to each well, and it was then discarded. Thereafter, 0.5 ml of a serum free medium for CHO cells, CHO-S-SFM II (manufactured by Invitrogen), was added thereto, followed by culture in the presence of 5% carbon dioxide at 37°C for 72 hours. Thereafter, the production amount of hG-CSF in the culture supernatant was analyzed.

(3) Determination of the amount of hG-CSF produced by pNOS-GKT2B-R/hG-CSF transformed cell clones

The production amount was determined by the dot blot method. 1 μl of a culture supernatant obtained by culturing for 72 hours, 1 μl of a commercially available recombinant hG-CSF (manufactured by PeproTech) serially diluted with a serum free medium for CHO cells CHO-S-SFM II (8 ~ 0.125 ng/μl), and 1 μl of CHO-S-SFM II were dotted on a nylon membrane (Nytran, 0.45 μm, manufactured by Schleicher & Schuell). It was air-dried for 1 hour and then subjected to blocking with Block Ace. Thereafter, it was reacted with an anti-hG-CSF antibody (1 μg/μl, manufactured by PeproTech) and then with a horseradish peroxidase (HRP)-labeled anti-rabbit IgG antibody (manufactured by DAKO). Using the reacted antibodies, the production amount of hG-CSF was determined by using a method of detecting the activity of HRP with a Super Signal West Pico reagent (manufactured by Pierce), applying LuminoCapture (manufactured by Ato). The results obtained by the dot blot method are shown in Figure 9.

(4) Amount of hG-CSF produced by pNOS-GKT2B-R/hG-CSF transformed cell clones

Figure 10 shows a distribution of the expression level of hG-CSF of each clone transfected with the expression vector pNOS-GKT2B of the present invention.

Approximately the two third (39 strains) out of sixty-one G418 resistant strains produced a significantly high level of hG-CSF (0.25 μg/μl or more). Moreover, 33 strains (54.1%) out of the 61 strains produced hG-CSF at a level of 1 μg/μl or more. Further surprisingly, 12 strains (19.7%) out of the 61 strains produced hG-CSF at a level of 8 μg/μl or more. The highest production level was 178 μg/ml/3 days. When this value was compared with the data regarding early clones before gene amplification that had been obtained by representative expression vectors reported on publications or the like, it was found that this was the highest standard (DNA, 7, p. 651, 1988; Biotechnology, 10, p.1455, 1992; Biotechnology, 8, p. 662, 1990; Gene, 76, p. 19, 1989; Biotechnology, 9, p. 64, 1991).

Generally, it takes a period ranging from 6 months to 1 year to screen recombinant cells by gene amplification, and the results differ significantly depending on culture conditions or the concentration of a stimulant for amplification. Accordingly, it is considered to be adequate that primary performance comparison of expression vectors is carried out at the expression level of early clones before being amplified. It was found by such a comparison that the expression vector of the present invention has extremely high performance. As a result, it was confirmed that the "expression vector comprising a promoterless NPT gene cistron blocked by a strong polyadenylation signal" provides a very small number of G418 resistant strains, but enables the establishment of highly productive cell strains that produce proteins of interest with extremely high efficiency. From these results, it was proved that the expression vector of the present invention enables an extremely high level of protein expression.

Example 3: Gene amplification of G418 resistant cell strains

Out of G418 resistant cell strains, clones producing hG-CSF at a level of 77 μg/ml/3 days were subcultured and then stabilized. Thereafter, gene amplification was carried out using MTX. First, the above clone was amplified in a medium containing 15 nM MTX for 2 weeks, in a medium containing 60 nM MTX for 2 weeks, then in a medium containing 250 nM MTX for 2 weeks, and then in a medium containing 1 μM MTX for further 2 weeks. As a result, the production level of hG-CSF was found to be increased to 117 μg/ml/3 days per medium. The concentration of MTX used in gene amplification is generally multi-step ranging between 10 nM and 10 μM, and 10 μM is often applied as the final concentration. However, considering toxicity to cells, exposure to a high concentration of MTX is problematic to establish stable recombinant cell strains. Accordingly, achievement of the high productivity in a low concentration

of MTX is also an important evaluation standard. In the present invention, the concentration of MTX was set to 1 μM or lower. In addition, although the MTX-exposure period including selection is generally 6 to 12 months, it was set to about 8 weeks in the present experiment. In spite of these experimental conditions, an effective increase in the production amount of hG-CSF was observed, and it was confirmed that the vector of the present invention has high performance also in a gene amplification system.

Example 4: Expression of recombinant human G-CSF by using pNOS

(1) Production of pNOS/hG-CSF expression vector

An expression vector pNOS was constructed in the same manner as in Example 1 with exceptions that Tn5 derived from *Escherichia coli* was directly used as an NEO resistant gene and that HGHpA was used instead of BGHpA. Moreover, according to the method described in Example 2 (1), a human granulocyte colony-stimulating factor (hG-CSF) gene was inserted into a cloning site, so as to construct an expression vector pNOS/hG-CSF. The structure of pNOS/hG-CSF is shown in Figure 11, and the entire nucleotide sequence is shown in SEQ ID NO: 25.

(2) Measurement of expression level of recombinant human G-CSF obtained by using pNOS

When CHO cells that were being cultured in a 25 cm^2 flask were 40% to 80% confluent, a pNOS-human G-CSF expression vector was incorporated into the CHO cells using a transfection reagent commercially available from Qiagen. Two days later, the cells were treated with trypsin and then peeled off from the culture flask. Thereafter, the number of cells was counted using a blood cell counting chamber, and the cells were then dispersed in ten 96-well plates such that the concentration of the cells was set to 5 x 10^5 cell/100 ml. Two weeks later, appearing colonies were counted, and as a result, 146 colonies were confirmed. 72 single colonies appearing in the ten 96-well plates were transferred to a 24-well plate. When the clones transferred to the 24-well plate became confluent, they were washed with 1 x PBS once. Thereafter, a serum free medium was added thereto, and after 3 days, the culture supernatant was collected.

1 μl of the culture supernatant obtained after 3 days and 1 μl of a standard diluted line were spotted on a membrane. It was air-dried for 1 hour and then blocked. Thereafter, it was washed with TBS/T once. After completion of the washing, the primary antibody reaction was carried out using a 1 μg/ml rabbit-anti human G-CSF

antibody. The reaction product was then washed with TBS/T three times. Thereafter, the secondary antibody reaction was carried out using HRP-anti-rabbit IgG. The reaction product was then washed with TBS/T three times. Thereafter, a chemiluminescence reaction was carried out using a Super Signal West Pico reagent, so as to confirm the expression level of human G-CSF. Examples of detecting hG-CSF by the dot blot method, which was expressed by the expression vector of the present invention pNOS/human G-CSF, are shown in Figures 12 and 13. In addition, a distribution of the expression level of hG-CSF of each clone, which was expressed by pNOS/human G-CSF, is shown in Figure 14.

The expression level of human G-CSF obtained by using a pNOS expression vector was determined by the dot blot method (the number of clones subjected to concentration test was 46). As a result, it was found that 65% or more clones produced hG-CSF at an expression level of 1 μg/ml or more by using pNOS, and that further 33% clones thereof produced hG-CSF at an expression level of 8 μg/ml or more.

Example 5: Preparation of CMV5 promoter (Figures 15 and 16)

(I-1) The L1 region was amplified by PCR from 1 μl of adenovirus type 2 genome. The PCR kit which was commercially available from TAKARA SHUZO CO., LTD was used, and the buffers and dNTP attached to the kit were used. First, 5 μl of 2.5 mM dNTP and 5 μl of 10 × Pyrobest PCR buffer were added to 36.5 μl of sterile distilled water, to which 1 μl of the sense primer [SEQ ID No. 26] (100 μM) and 1 μl of the antisense primer [SEQ ID No. 27] (100 μM) for the L1 region, and 1 μl of the template DNA were added. Then, 0.5 μl of a DNA polymerase Pyrobest (5 U/μl) was added to the resulting mixture. The mixture was completely mixed by pipetting, and PCR was carried out under the following conditions. After treatment under heating at 94 °C for 5 minutes, a cycle of three steps of 94 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 30 seconds was repeated 35 times. Finally, the mixture was treated at 72 °C for 5 minutes to complete the reaction. After the completion of PCR, the amplification of the gene region of interest was confirmed by electrophoresis using 1 % agarose gel. A band around 40 bp could be confirmed.

(I-2) The L2 region was amplified by PCR from 1 μl of adenovirus type 2 genome. The PCR kit which was commercially available from TAKARA SHUZO CO., LTD was used, and the buffers and dNTP attached to the kit were used. First, 5 μl of 2.5 mM dNTP and 5 μl of 10 × Pyrobest PCR buffer were added to 36.5 μl of sterile distilled

water, to which 1 μl of the sense primer [SEQ ID No. 28] (100 μM) and 1 μl of the antisense primer [SEQ ID No. 29] (100 μM) for the L2 region, and 1 μl of the template DNA were added. Then, 0.5 μl of a DNA polymerase Pyrobest (5 U/μl) was added to the resulting mixture. The mixture was completely mixed by pipetting, and PCR was carried out under the following conditions. After treatment under heating at 94 °C for 5 minutes, a cycle of three steps of 94 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 30 seconds was repeated 35 times. Finally, the mixture was treated at 72 °C for 5 minutes to complete the reaction. After the completion of PCR, the amplification of the gene region of interest was confirmed by electrophoresis using 1 % agarose gel. A band around 70 bp could be confirmed.

(I-3) The L3 region was amplified by PCR from 1 μl of adenovirus type 2 genome. The PCR kit which was commercially available from TAKARA SHUZO CO., LTD was used, and the buffers and dNTP attached to the kit were used. First, 5 μl of 2.5 mM dNTP and 5 μl of 10 × Pyrobest PCR buffer were added to 36.5 μl of sterile distilled water, to which 1 μl of the sense primer [SEQ ID No. 30] (100 μM) and 1 μl of the antisense primer [SEQ ID No. 31] (100 μM) for the L3 region, and 1 μl of the template DNA were added. Then, 0.5 μl of a DNA polymerase Pyrobest (5 U/μl) was added to the resulting mixture. The mixture was completely mixed by pipetting, and PCR was carried out under the following conditions. After treatment under heating at 94 °C for 5 minutes, a cycle of three steps of 94 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 30 seconds was repeated 35 times. Finally, the mixture was treated at 72 °C for 5 minutes to complete the reaction. After the completion of PCR, the amplification of the gene region of interest was confirmed by electrophoresis using 1 % agarose gel. A band around 200 bp could be confirmed.

(I-4) The R1,2,3 region was amplified by PCR from 1 μl of adenovirus type 2 genome. The PCR kit which was commercially available from TAKARA SHUZO CO., LTD was used, and the buffers and dNTP attached to the kit were used. First, 5 μl of 2.5 mM dNTP and 5 μl of 10 × Pyrobest PCR buffer were added to 36.5 μl of sterile distilled water, to which 1 μl of the sense primer [SEQ ID No. 32] (100 μM) and 1 μl of the antisense primer [SEQ ID No. 33] (100 μM) for the R1,2,3 region, and 1 μl of the template DNA were added. Then, 0.5 μl of a DNA polymerase Pyrobest (5 U/μl) was added to the resulting mixture. The mixture was completely mixed by pipetting, and PCR was carried out under the following conditions. After treatment under heating at

98 °C for 1 minute, a cycle of three steps of 98 °C for 5 seconds, 55 °C for 20 seconds and 72 °C for 15 seconds was repeated 35 times. Finally, the mixture was treated at 72 °C for 2 minutes to complete the reaction. After the completion of PCR, the amplification of the gene region of interest was confirmed by electrophoresis using 1 % agarose gel. A band around 100 bp could be confirmed.

(I-5) The CMV promoter region was amplified by PCR using 1 μl of pCMVTnT (10 ng/μl) as template. The PCR kit which was commercially available from TAKARA SHUZO CO., LTD was used, and the buffer, dNTP and $MgCl_2$ attached to the kit were used. First, 5 μl of 2.5 mM dNTP, 5 μl of 25mM $MgCl_2$ and 5 μl of 10 × LA PCR buffer were added to 31.5 μl of sterile distilled water, to which 1 μl of the sense primer [SEQ ID No. 34] (100 μM) and 1 μl of the antisense primer [SEQ ID No. 35] (100 μM) for the CMV promoter, and 1 μl of the template DNA were added. Then, 0.5 μl of a DNA polymerase LA-Taq (5 U/μl) was added to the resulting mixture. The mixture was completely mixed by pipetting, and PCR was carried out under the following conditions. After treatment under heating at 94 °C for 5 minutes, a cycle of three steps of 94 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 30 seconds was repeated 35 times. Finally, the mixture was treated at 72 °C for 5 minutes to complete the reaction. After the completion of PCR, the amplification of the gene region of interest was confirmed by electrophoresis using 1 % agarose gel. A band around 650 bp could be confirmed (SEQ ID No.41).

(I-6) 5 μl of 3M NaoAc and 125 μl of 100 % ethanol were added to the PCR reaction solution in (I-1), and the mixture was centrifuged at 14,000 rpm for 10 minutes. After completion of the centrifugation, the pellet was confirmed, and the supernatant was gently discarded. 10 μl of 1 × DSB was added to the pellet to thoroughly dissolve the pellet. The total volume of the resulting solution was applied to 1 % agarose gel, and was subjected to electrophoresis at 50 mA for 25 minutes. By comparison with DNA markers, a band around 40 bp was confirmed and recovered, which was then frozen at -135 °C for 10 minutes and subsequently centrifuged at 14,000 rpm for 10 minutes. The supernatant from centrifugation was transferred to a fresh 1.5-ml Eppendorf tube. The resulting extract was defined as L1 (the extract concentration = 82 ng/μl).

(I-7) 5 μl of 3M NaoAc and 125 μl of 100 % ethanol were added to the PCR reaction solution in (I-2), and the mixture was centrifuged at 14,000 rpm for 10 minutes. After

completion of the centrifugation, the pellet was confirmed, and the supernatant was gently discarded. 10 μl of 1 × DSB was added to the pellet to thoroughly dissolve the pellet. The total volume of the resulting solution was applied to 1 % agarose gel, and was subjected to electrophoresis at 50 mA for 25 minutes. By comparison with DNA markers, a band around 70 bp was confirmed and recovered, which was then frozen at -135 °C for 10 minutes and subsequently centrifuged at 14,000 rpm for 10 minutes. The supernatant from centrifugation was transferred to a fresh 1.5-ml Eppendorf tube. The resulting extract was defined as L2 (the extract concentration = 104 ng/μl).

(I-8) 5 μl of 3M NaoAc and 125 μl of 100 % ethanol were added to the PCR reaction solution in (I-3), and the mixture was centrifuged at 14,000 rpm for 10 minutes. After completion of the centrifugation, the pellet was confirmed, and the supernatant was gently discarded. 10 μl of 1 × DSB was added to the pellet to thoroughly dissolve the pellet. The total volume of the resulting solution was applied to 1 % agarose gel, and was subjected to electrophoresis at 50 mA for 25 minutes. By comparison with DNA markers, a band around 200 bp was confirmed and recovered, which was then frozen at -135 °C for 10 minutes and subsequently centrifuged at 14,000 rpm for 10 minutes. The supernatant from centrifugation was transferred to a fresh 1.5-ml Eppendorf tube. The resulting extract was defined as L3 (the extract concentration = 88 ng/μl).

(I-9) 5 μl of 3M NaoAc and 125 μl of 100 % ethanol were added to the PCR reaction solution in (I-4), and the mixture was centrifuged at 14,000 rpm for 10 minutes. After completion of the centrifugation, the pellet was confirmed, and the supernatant was gently discarded. 10 μl of 1 × DSB was added to the pellet to thoroughly dissolve the pellet. The total volume of the resulting solution was applied to 1 % agarose gel, and was subjected to electrophoresis at 50 mA for 25 minutes. By comparison with DNA markers, a band around 100 bp was confirmed and recovered, which was then frozen at -135 °C for 10 minutes and subsequently centrifuged at 14,000 rpm for 10 minutes. The supernatant from centrifugation was transferred to a fresh 1.5-ml Eppendorf tube. The resulting extract was defined as R123.

(I-10) 5 μl of 3M NaoAc and 125 μl of 100 % ethanol were added to the PCR reaction solution in (I-5), and the mixture was centrifuged at 14,000 rpm for 10 minutes. After completion of the centrifugation, the pellet was confirmed, and the supernatant was gently discarded. 10 μl of 1 × DSB was added to the pellet to thoroughly dissolve the

pellet. The total volume of the resulting solution was applied to 1 % agarose gel, and was subjected to electrophoresis at 50 mA for 25 minutes. By comparison with DNA markers, a band around 650 bp was confirmed and recovered, which was then frozen at -135 °C for 10 minutes and subsequently centrifuged at 14,000 rpm for 10 minutes. The supernatant from centrifugation was transferred to a fresh 1.5-ml Eppendorf tube. The resulting extract was defined as PCMV (the extract concentration = 54 ng/μl).

(I-11) The L123 region was amplified by PCR using 1 μl of each of the extracts of I-6, I-7 and I-8. The PCR kit which was commercially available from TAKARA SHUZO CO., LTD was used, and the buffers, dNTP and $MgCl_2$ attached to the kit were used. First, 5 μl of 2.5 mM dNTP, 5 μl of 25mM $MgCl_2$ and 5 μl of 10 × LA PCR buffer were added to 29.5 μl of sterile distilled water, to which 1 μl of the sense primer [SEQ ID No. 26] (100 μM) for the L1 region and 1 μl of the antisense primer [SEQ ID No. 31] (100 μM) for the L3 region, and total 3 μl of the template DNAs were added. Then, 0.5 μl of a DNA polymerase LA-Taq (5 U/μl) was added to the resulting mixture. The mixture was completely mixed by pipetting, and PCR was carried out under the following conditions. After treatment under heating at 94 °C for 5 minutes, a cycle of three steps of 94 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 30 seconds was repeated 35 times. Finally, the mixture was treated at 72 °C for 5 minutes to complete the reaction. After the completion of PCR, the amplification of the gene region of interest was confirmed by electrophoresis using 1 % agarose gel. A band around 300 bp could be confirmed.

(I-12) 5 μl of 3M NaoAc and 125 μl of 100 % ethanol were added to the PCR reaction solution in (I-11), and the mixture was centrifuged at 14,000 rpm for 10 minutes. After completion of the centrifugation, the pellet was confirmed, and the supernatant was gently discarded. 10 μl of 1 × DSB was added to the pellet to thoroughly dissolve the pellet. The total volume of the resulting solution was applied to 1 % agarose gel, and was subjected to electrophoresis at 50 mA for 25 minutes. By comparison with DNA markers, a band around 300 bp was confirmed and recovered, which was then frozen at -135 °C for 10 minutes and subsequently centrifuged at 14,000 rpm for 10 minutes. The supernatant from centrifugation was transferred to a fresh 1.5-ml Eppendorf tube. The resulting extract was defined as L123 (the extract concentration = 80 ng/μl) (SEQ ID No. 42].

(I-13) The R123' region was amplified by PCR using the extract of I-9. The PCR kit which was commercially available from TAKARA SHUZO CO., LTD was used, and the buffers and dNTP attached to the kit were used. First, 5 μl of 2.5 mM dNTP and 5 μl of 10 × Pyrobest PCR buffer were added to 36.5 μl of sterile distilled water, to which 1 μl of the sense primer [SEQ ID No. 32] (100 μM) and 1 μl of the antisense primer [SEQ ID No. 36] (100 μM) for the R123' region, and 1 μl of the template DNA were added. Then, 0.5 μl of a DNA polymerase Pyrobest (5 U/μl) was added to the resulting mixture. The mixture was completely mixed by pipetting, and PCR was carried out under the following conditions. After treatment under heating at 98 °C for 1 minute, a cycle of three steps of 98 °C for 5 seconds, 55 °C for 20 seconds and 72 °C for 15 seconds was repeated 35 times. Finally, the mixture was treated at 72 °C for 2 minutes to complete the reaction. After the completion of PCR, the amplification of the gene region of interest was confirmed by electrophoresis using 1 % agarose gel. A band around 150 bp could be confirmed.

(I-14) 5 μl of 3M NaoAc and 125 μl of 100 % ethanol were added to the PCR reaction solution in (I-13), and the mixture was centrifuged at 14,000 rpm for 10 minutes. After completion of the centrifugation, the pellet was confirmed, and the supernatant was gently discarded. 10 μl of 1 × DSB was added to the pellet to thoroughly dissolve the pellet. The total volume of the resulting solution was applied to 1 % agarose gel, and was subjected to electrophoresis at 50 mA for 25 minutes. By comparison with DNA markers, a band around 150 bp was confirmed and recovered, which was then frozen at -135 °C for 10 minutes and subsequently centrifuged at 14,000 rpm for 10 minutes. The supernatant from centrifugation was transferred to a fresh 1.5-ml Eppendorf tube. The resulting extract was defined as R123-1 (the extract concentration = 107 ng/μl).

(I-15) The R123" region was amplified by PCR using the extract of I-14. The PCR kit which was commercially available from TAKARA SHUZO CO., LTD was used, and the buffers and dNTP attached to the kit were used. First, 5 μl of 2.5 mM dNTP and 5 μl of 10 × Pyrobest PCR buffer were added to 36.5 μl of sterile distilled water, to which 1 μl of the sense primer [SEQ ID No.32] (100 μM) and 1 μl of the antisense primer [SEQ ID No. 37] (100 μM) for the R123" region, and 1 μl of the template DNA were added. Then, 0.5 μl of a DNA polymerase Pyrobest (5 U/μl) was added to the resulting mixture. The mixture was completely mixed by pipetting, and PCR was carried out under the following conditions. After treatment under heating at 98 °C for 1 minute, a cycle of

three steps of 98 °C for 5 seconds, 55 °C for 20 seconds and 72 °C for 15 seconds was repeated 35 times. Finally, the mixture was treated at 72 °C for 2 minutes to complete the reaction. After the completion of PCR, the amplification of the gene region of interest was confirmed by electrophoresis using 1 % agarose gel. A band around 150 bp could be confirmed.

(I-16) 5 μl of 3M NaoAc and 125 μl of 100 % ethanol were added to the PCR reaction solution in (I-15), and the mixture was centrifuged at 14,000 rpm for 10 minutes. After completion of the centrifugation, the pellet was confirmed, and the supernatant was gently discarded. 10 μl of 1 × DSB was added to the pellet to thoroughly dissolve the pellet. The total volume of the resulting solution was applied to 1 % agarose gel, and was subjected to electrophoresis at 50 mA for 25 minutes. By comparison with DNA markers, a band around 150 bp was confirmed and recovered, which was then frozen at -135 °C for 10 minutes and subsequently centrifuged at 14,000 rpm for 10 minutes. The supernatant from centrifugation was transferred to a fresh 1.5-ml Eppendorf tube. The resulting extract was defined as R123-2 (the extract concentration = 95 ng/μl) (SEQ ID No. 43].

(I-17) 1 μl of the extract in I-12 as recovered by PCR (extract concentration expressed in ng/μl) and 1 μl of pT7Blue (at 50 ng/μl; Novagen) were mixed together. An equal volume of the ligation kit ver. 2 solution I (TAKARA SHUZO, Co., Ltd.) was added to the resulting mixture, and ligation reaction was carried out at 12 °C for 2 hours. Thereafter, transformants of Escherichia coli were obtained. After sequencing, then, a plasmid without any nucleotide substitution was selected. The plasmid thus recovered was defined as pT7B-L123.

(I-18) 1 μl of the extract in I-16 as recovered by PCR (extract concentration expressed in ng/μl) and 1 μl of pT7Blue (at 50 ng/μl; Novagen) were mixed together. An equal volume of the ligation kit ver. 2 solution I (TAKARA SHUZO, Co., Ltd.) was added to the resulting mixture, and ligation reaction was carried out at 12 °C for 2 hours. Thereafter, transformants of Escherichia coli were obtained. After sequencing, then, a plasmid without any nucleotide substitution was selected. The plasmid thus recovered was defined as pT7B-R123.

(I-19) 1 μl of the extract in I-10 as recovered by PCR (extract concentration expressed

in ng/μl) and 1 μl of pT7Blue (at 50 ng/μl; Novagen) were mixed together. An equal volume of the ligation kit ver. 2 solution I (TAKARA SHUZO, Co., Ltd.) was added to the resulting mixture, and ligation reaction was carried out at 12 °C for 2 hours. Thereafter, transformants of Escherichia coli were obtained. After sequencing, then, a plasmid without any nucleotide substitution was selected. The plasmid thus recovered was defined as pT7B-CMV.

(I-20) The L123' region was amplified by PCR using pT7B-L123 (10 ng/μl) of I-17 as templete. The PCR kit which was commercially available from TAKARA SHUZO CO., LTD was used, and the buffers and dNTP attached to the kit were used. First, 5 μl of 2.5 mM dNTP and 5 μl of 10 × Pyrobest PCR buffer were added to 36.5 μl of sterile distilled water, to which 1 μl of the sense primer [SEQ ID No. 26] (100 μM) and 1 μl of the antisense primer [SEQ ID No. 38] (100 μM) for the L123' region, and 1 μl of the template DNA were added. Then, 0.5 μl of a DNA polymerase Pyrobest (5 U/μl) was added to the resulting mixture. The mixture was completely mixed by pipetting, and PCR was carried out under the following conditions. After treatment under heating at 94 °C for 5 minutes, a cycle of three steps of 95 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 30 seconds was repeated 35 times. Finally, the mixture was treated at 72 °C for 5 minutes to complete the reaction. After the completion of PCR, the amplification of the gene region of interest was confirmed by electrophoresis using 1 % agarose gel. A band around 300 bp could be confirmed.

(I-21) The R123-IgG3' region was amplified by PCR using pT7B-R123 (10 ng/μl) of I-18 as templete. The PCR kit which was commercially available from TAKARA SHUZO CO., LTD was used, and the buffers and dNTP attached to the kit were used. First, 5 μl of 2.5 mM dNTP and 5 μl of 10 × Pyrobest PCR buffer were added to 36.5 μl of sterile distilled water, to which 1 μl of the sense primer [SEQ ID No. 39] (100 μM) and 1 μl of the antisense primer [SEQ ID No. 40] (100 μM) for the R123 region, and 1 μl of the template DNA were added. Then, 0.5 μl of a DNA polymerase Pyrobest (5 U/μl) was added to the resulting mixture. The mixture was completely mixed by pipetting, and PCR was carried out under the following conditions. After treatment under heating at 94 °C for 5 minutes, a cycle of three steps of 95 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 30 seconds was repeated 35 times. Finally, the mixture was treated at 72 °C for 5 minutes to complete the reaction. After the completion of PCR, the amplification of the gene region of interest was confirmed by electrophoresis using

1 % agarose gel. A band around 200 bp could be confirmed.

(I-22) 5 μl of 3M NaoAc and 125 μl of 100 % ethanol were added to the PCR reaction solution in (I-20), and the mixture was centrifuged at 14,000 rpm for 10 minutes. After completion of the centrifugation, the pellet was confirmed, and the supernatant was gently discarded. 10 μl of 1 × DSB was added to the pellet to thoroughly dissolve the pellet. The total volume of the resulting solution was applied to 1 % agarose gel, and was subjected to electrophoresis at 50 mA for 25 minutes. By comparison with DNA markers, a band around 300 bp was confirmed and recovered, which was then frozen at -135 °C for 10 minutes and subsequently centrifuged at 14,000 rpm for 10 minutes. The supernatant from centrifugation was transferred to a fresh 1.5-ml Eppendorf tube. The resulting extract was defined as L123-1 (the extract concentration = 87 ng/μl) (SEQ ID No. 44].

(I-23) 5 μl of 3M NaoAc and 125 μl of 100 % ethanol were added to the PCR reaction solution in (I-21), and the mixture was centrifuged at 14,000 rpm for 10 minutes. After completion of the centrifugation, the pellet was confirmed, and the supernatant was gently discarded. 10 μl of 1 × DSB was added to the pellet to thoroughly dissolve the pellet. The total volume of the resulting solution was applied to 1 % agarose gel, and was subjected to electrophoresis at 50 mA for 25 minutes. By comparison with DNA markers, a band around 300 bp was confirmed and recovered, which was then frozen at -135 °C for 10 minutes and subsequently centrifuged at 14,000 rpm for 10 minutes. The supernatant from centrifugation was transferred to a fresh 1.5-ml Eppendorf tube. The resulting extract was defined as R123-IgG3 (the extract concentration = 79 ng/μl) [SEQ ID No. 45].

(I-24) The L123-R123-IgG3' region was amplified by PCR using 1 μl of each of the extract of I-22 and 23 as templetes. The PCR kit which was commercially available from TAKARA SHUZO CO., LTD was used, and the buffer, dNTP and $MgCl_2$ attached to the kit were used. First, 5 μl of 2.5 mM dNTP, 5 μl of 25mM $MgCl_2$ and 5 μl of 10 × LA PCR buffer were added to 29.5 μl of sterile distilled water, to which 1 μl of the sense primer [SEQ ID No. 1] (100 μM) for the L1 region and 1 μl of the antisense primer [SEQ ID No. 15] (100 μM) for the R123-IgG3', and total 3 μl of the template DNA were added. Then, 0.5 μl of a DNA polymerase LA-Taq (5 U/μl) was added to the resulting mixture. The mixture was completely mixed by pipetting, and PCR was carried out under the

following conditions. After treatment under heating at 98 °C for 1 minute, a cycle of three steps of 98 °C for 5 seconds, 55 °C for 20 seconds and 72 °C for 15 seconds was repeated 35 times. Finally, the mixture was treated at 72 °C for 2 minutes to complete the reaction. After the completion of PCR, the amplification of the gene region of interest was confirmed by electrophoresis using 1 % agarose gel. A band around 500 bp could be confirmed (SEQ ID No. 46].

(I-25) 5 μl of 3M NaoAc and 125 μl of 100 % ethanol were added to the PCR reaction solution in (I-24), and the mixture was centrifuged at 14,000 rpm for 10 minutes. After completion of the centrifugation, the pellet was confirmed, and the supernatant was gently discarded. 10 μl of 1 × DSB was added to the pellet to thoroughly dissolve the pellet. The total volume of the resulting solution was applied to 1 % agarose gel, and was subjected to electrophoresis at 50 mA for 25 minutes. By comparison with DNA markers, a band around 500 bp was confirmed and recovered, which was then frozen at -135 °C for 10 minutes and subsequently centrifuged at 14,000 rpm for 10 minutes. The supernatant from centrifugation was transferred to a fresh 1.5-ml Eppendorf tube. The resulting extract was defined as LR123 (the extract concentration = 56 ng/μl).

(I-26) 1 μl of the extract in I-25 as recovered by PCR (extract concentration expressed in ng/μl) and 1 μl of pT7Blue (at 50 ng/μl; Novagen) were mixed together. An equal volume of the ligation kit ver. 2 solution I (TAKARA SHUZO, Co., Ltd.) was added to the resulting mixture, and ligation reaction was carried out at 12 °C for 2 hours. Thereafter, transformants of Escherichia coli were obtained. After sequencing, then, a plasmid without any nucleotide substitution was selected. The plasmid thus recovered was defined as pT7B-LR123.

(I-27) 2.5 μg of the plasmid recovered in I-25 was used and digested with 1 μl of each of restriction enzymes BamHI (20,000 U/ml) and EcoRI (20,000 U/ml) (both from NEB) at 25 °C for overnight so as to cut out LR123. After treatment with the restriction enzymes, 1 μl of the resulting reaction solution was applied to 1 % agarose gel, and was subjected to electrophoresis at 50 mA for 25 minutes. Consequently, bands around 2800 bp and 400 bp were confirmed by comparison with DNA markers. Again, the total volume of the reaction solution was applied to 1 % agarose gel, and was subjected to electrophoresis at 50 mA for 25 minutes. A band confirmed around 400 bp was recovered. The gel extract recovered was frozen at -135 °C for 10 minutes.

Subsequently, the product was centrifuged at 14,000 rpm for 10 minutes. The supernatant from centrifugation was transferred to a fresh 1.5-ml Eppendorf tube. The resulting extract was defined as LR123 digest (the extract concentration = 16 ng/μl).

(I-28) 2.5 μg of the plasmid recovered in I-19 was used and digested with 1 μl of each of restriction enzymes HindIII (20,000 U/ml) and BglII (10,000 U/ml) (both from NEB) at 25 °C for overnight so as to cut out the CMV promoter. After treatment with the restriction enzymes, 1 μl of the resulting reaction solution was applied to 1 % agarose gel, and was subjected to electrophoresis at 50 mA for 25 minutes. Consequently, bands around 2800 bp and 650 bp were confirmed by comparison with DNA markers. Again, the total volume of the reaction solution was applied to 1 % agarose gel, and was subjected to electrophoresis at 50 mA for 25 minutes. A band confirmed around 650 bp was recovered. The gel extract recovered was frozen at -135 °C for 10 minutes. Subsequently, the product was centrifuged at 14,000 rpm for 10 minutes. The supernatant from centrifugation was transferred to a fresh 1.5-ml Eppendorf tube. The resulting extract was defined as CMV promoter digest (the extract concentration = 14 ng/μl).

(I-29) 2.5 μg of pCB was used and digested with 1 μl of each of restriction enzymes HindIII (20,000 U/ml) and NotI (10,000 U/ml) (both from NEB) at 25 °C for overnight. After treatment with the restriction enzymes, total amount of the reaction solution was applied to 1 % agarose gel, and was subjected to electrophoresis at 50 mA for 25 minutes. Consequently, band around 3500 bp was confirmed by comparison with DNA markers, and was recovered. The gel extract recovered was frozen at -135 °C for 10 minutes. Subsequently, the product was centrifuged at 14,000 rpm for 10 minutes. The supernatant from centrifugation was transferred to a fresh 1.5-ml Eppendorf tube. The resulting extract was defined as pCB digest (the extract concentration = 30 ng/μl).

(I-30) 1 μl of the pCB digest (30 ng/μl) obtained in I-29, 1 μl of the LR123 digest (16 ng/μl) obtained in I-26 and 1 μl of the CMV promoter digest (14 ng/μl) obtained in I-27 were mixed together. An equal volume (3 μl) of the ligation kit ver. 2 solution I (TAKARA SHUZO, Co., Ltd.) was added to the resulting mixture, and ligation reaction was carried out at 16 °C for 30 minutes. Thereaftre, transformation of Escherichia coli was carried out to obtain a transformant. The plasmid recovered from the transformant was defined as pCMV5 [SEQ ID No. 47].

Example 6: Preparation of pCMV5-luciferase (Figure 17)

(II-1) 2.5 μg of pCMV5 was used and digested with 1 μl of each of restriction enzymes NotI (10,000 U/ml) and XbaI (20,000 U/ml) (both from NEB) at 25 °C for overnight. After treatment with the restriction enzymes, total amount of the reaction solution was applied to 1 % agarose gel, and was subjected to electrophoresis at 50 mA for 25 minutes. Consequently, band around 4000 bp was confirmed by comparison with DNA markers, and was recovered. The gel extract recovered was frozen at -135 °C for 10 minutes. Subsequently, the product was centrifuged at 14,000 rpm for 10 minutes. The supernatant from centrifugation was transferred to a fresh 1.5-ml Eppendorf tube. The resulting extract was defined as pCMV5 digest (the extract concentration = 19 ng/μl).

(II-2) 2.5 μg of pCB-luciferase was used and digested with 1 μl of each of restriction enzymes NotI (10,000 U/ml) and XbaI (20,000 U/ml) (both from NEB) at 25 °C for overnight. After treatment with the restriction enzymes, 1 μl of the resulting reaction solution was applied to 1 % agarose gel, and was subjected to electrophoresis at 50 mA for 25 minutes. Consequently, bands around 3500 bp and 1600 bp were confirmed by comparison with DNA markers. Again, the total volume of the reaction solution was applied to 1 % agarose gel, and was subjected to electrophoresis at 50 mA for 25 minutes. A band confirmed around 1600 bp was recovered. The gel extract recovered was frozen at -135 °C for 10 minutes. Subsequently, the product was centrifuged at 14,000 rpm for 10 minutes. The supernatant from centrifugation was transferred to a fresh 1.5-ml Eppendorf tube. The resulting extract was defined as luciferase gene digest (the extract concentration = 27 ng/μl) (SEQ ID NO.48).

(II-3) 1 μl of the pCMV5 digest (19 ng/μl) ontained in (II-1) and 1 μl of the luciferase gene digest (27 ng/μl) obtained in (II-2) were mixed together. An equal volume (2 μl) of the ligation kit ver. 2 solution I (TAKARA SHUZO, Co., Ltd.) was added to the resulting mixture, and ligation reaction was carried out at 16 °C for 30 minutes. Thereafter, transformation of Escherichia coli was carried out to obtain a transformant of Escherichia coli. The plasmid thus recovered from the transformant was defined as pCMV5-luciferase.

Example 7: Assay of the activity of the CMV5 promoter by luciferase assay

(III-1) In order to assay the activity of the CMV5 promoter with CHO DG44-S and HEK293RT, these cells were respectively cultured in 25-cm^2 flasks. The cells cultured in the 25-cm^2 flask were centrifuged in a 15-ml centrifuge tube on the day of transfection. After centrifugation, the culture supernatant was discarded. To one of the resulting centrifuge tubes was added 5 ml of a CHO-S-SFM II culture medium (GIBCO) containing 100 mM hypoxanthine and 10 mM thymidine. To the other centrifuge tube was added 5 ml of a Free Style medium (GIBCO). The cells were counted in both of the centrifuge tubes and then individually transferred to a 24-well plate to 2.5×10^5 cells/well.

(III-2) 0.35 ml of each of the culture media was added to 61 μl of a transfection reagent (QIAGEN) containing 166 ng of the DNA (pCMV5-luciferase) per well, and the mixture was added in the 24-well plates, and the culturing was carried out for 48 hours.

(III-3) 48 hours later, the cells were collected in a 1.5-ml tube, and the supernatants were discarded. 100 μl of PLD-30 (the reagent attached to the Picker Gene Dual sea pansy kit; manufactured by Toyo Ink Group Co., Ltd.) was added to the resulting cells. Then, the resulting mixture was gently agitated and left to stand for 15 minutes. 15 minutes later, the tube was centrifuged at 200 × g for 10 minutes. 5 μl of the supernatant recovered by centrifugation was added to 195 μl of the reagent PLD-30, and luciferase assay was carried out using a fluorescence assay apparatus (ATTO; Luminescencer PSN R). Thus, an activity which is stronger than that of the CMV promoter could be confirmed. The results are shown in Figure 18.

INDUSTRIAL APPLICABILITY

The present invention provides an expression vector capable of high-level production of genetically recombinant proteins using an animal cell as a host.

Claims

1. An expression vector inducing high productivity of genetically recombinant proteins in animal host cells, which comprises a highly expression-inducible promoter, a multicloning site for insertion of gene and a polyadenylation signal sequence in this order from upstream, and further comprises downstream thereof a drug resistant gene that does not have a promoter capable of acting in animal cells.

2. The expression vector according to claim 1, wherein the highly expression-inducible promoter is a human cytomegalovirus Major Immediately-Early antigen promoter, CMV5 promoter (a synthetic chimeric promoter), a β-actin promoter, or an SV40 early promoter.

3. The expression vector according to claim 1 or 2, wherein the drug resistant gene is a neomycine resistant gene, codon-disoptimized neomycine resistant gene, a hygromycin resistant gene, a zeocin resistant gene, or a blasticidin resistant gene.

4. The expression vector according to any one of claim 1 to 3, wherein the drug resistant gene is a neomycine phosphotransferase gene.

5. The expression vector according to any one of claims 1 to 4, wherein the drug resistant gene is a neomycine phosphotransferase gene derived from *Escherichia coli* transposon Tn5.

6. The expression vector according to any one of claims 1 to 5, wherein a trace amount of the messenger RNA or protein of a drug resistant gene is generated by a read-through effect which is achieved by the fact that the drug resistant gene exists downstream of the highly expression-inducible promoter blocked by the polyadenylation signal sequence.

7. The expression vector according to any one of claim 1 to 6, which further comprises, downstream of the drug resistant gene, a dihydrofolate reductase gene or codon-disoptimized dihydrofolate reductase gene that does not have a promoter capable of acting in animal cells.

8. The expression vector according to any one of claims 1 to 7, which further comprises, downstream of the drug resistant gene, a highly expression-inducible promoter, a multicloning site for insertion of gene and a polyadenylation signal sequence in this order from upstream, and further comprises downstream thereof a dihydrofolate reductase gene or codon-disoptimized dihydrofolate reductase gene that does not have a promoter capable of acting in animal cells.

9. The expression vector according to any one of claims 1 to 6, which comprises a dihydrofolate reductase gene, or codon-disoptimized dihydrofolate reductase gene ligated downstream of a promoter with low expression inductivity.

10. The expression vector according to any one of claims 7 to 9, wherein the promoter with low expression inductivity is a promoter derived from a gene of a protein that is generally expressed in trace amounts in mammalian cells, a promoter derived from a viral antigen gene that hardly ever infects mammals, or a promoter obtained by removing an enhancer sequence from the above promoters.

11. A recombinant expression vector which is obtained by incorporating a gene of interest into the multicloning site for insertion of gene of the expression vector according to any one of claims 1 to 6.

12. A recombinant vector which is obtained by incorporating a gene of interest into the multicloning site for insertion of gene of the expression vector according to any one of claims 7 to 10.

13. A transformant comprising the expression vector according to any one of claims 1 to 10 or the recombinant expression vector according to claim 11 or 12.

14. A method for producing a transformant that highly expresses a gene of interest, which comprises introducing the recombinant expression vector according to claims 11 or 12 into animal host cells.

15. A method for obtaining a transformant capable of stably producing proteins in a serum free medium, which comprises introducing the recombinant expression vector according to claim 11 or 12 into animal host cells so as to obtain a transformant, and acclimating the obtained transformant to the serum free medium.

16. A method for amplifying a gene of interest, which comprises introducing the recombinant expression vector according to claim 12 into a dihydrofolate reductase-deficient CHO cell, and culturing the cell in the presence of methotrexate.