Endothelin DNA and use thereof

Endothelin-DNA und Verwendung davon

ADN de l’endothéline et son utilisation

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- PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 85, September 1988, WASHINGTON US pages 6964 - 6967 MASASHI YANAGISAWA ET AL 'Primary structure, synthesis and biological activity of rat endothelin, an endothelium-derived vasoconstrictor peptide'
- PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 86, April 1989, WASHINGTON US pages 2863 - 2867 AKIHIROINOUE ET AL 'The human endothelin family:Three structurally and pharmacologically distinct isopeptides predicted by three separate genes'

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BACKGROUND OF THE INVENTION

[0001] The present invention relates to a DNA sequence coding for a human vasoconstrictive peptide, namely endothelin-3, a transformant containing a vector comprising said DNA, a precursor protein (or a precursor polypeptide) of endothelin-3, and a method for preparing the mature protein (endothelin-3).

[0002] In this specification, the term "precursor protein" is preferably used to describe a protein which includes an amino acid sequence of a mature peptide and has a portion or all of an amino acid sequence coded with a DNA segment of the peptide at the N-terminus, the C-terminus or both termini thereof.

[0003] There have been reports of endothelium-dependent vasoconstrictor reactions to various mechanical and chemical stimuli as well as endothelium-dependent vasodilative reactions. For example, it is known that vasoconstriction can be induced by mechanical loads such as vascular stretch and increased vascular inner pressure, or can be chemically induced by such agents as thrombin. Further, vasoconstriction can be induced by conditions of anoxia. Noradrenaline-induced vasoconstriction can be enhanced by use of neuropeptide Y [K. Takemoto, Proc. Natl. Acad. Sci. U.S.A. 79, 5485 (1982); C. Minth et al., ibid. 81, 4577 (1984)]. Endothelial cell-derived coronary vascular constrictor factors (each having molecular weights of 8,500 and 3,000) are described in K. A. Hickey et al., Am. J. Physiol. 248, C550 (1985); and in R. F. O'Brien, J. Cell Physiol. 132, 263 (1987). However, their structures are unknown. An endothelial cell-derived peptide-like substance is also described in M. N. Gillespie et al., J. Pharmac. Exp. Ther. 236, 339 (1985). However, the structure of that substance is also unknown.

[0004] Vasopressin is known as a peptide having a vasoconstrictor activity, and the amino acid sequence thereof was determined. There have been no reports, however, that vasopressin was obtained from mammalian or bird vascular endothelial cells. Although there is a report that an angiotensin having a vasoconstrictor activity was obtained from the endothelial cells of bovine aortas [I. Kifor and V. J. Dzav, Circ. Res. 60, 422 (1987)], the angiotensin is a peptide having a molecular weight of only about 1,000.


[0006] Further, the present inventors have filed patent applications with respect to the isolation of rat endothelin and the cloning of its cDNA (Japanese Patent Application Nos. 174935/1988 and 188083/1988), and this rat endothelin is referred to as endothelin-3. The amino acid sequence of rat mature endothelin-3 was further known from Proc. Natl. Acad. Sci. USA, vol 85, pp. 6964-6967, September 1988.

[0007] Furthermore, the present inventors have also filed a patent application with respect to the isolation of mouse endothelin and the cloning of its cDNA (Japanese Patent Application No. 223389/1988), and this mouse endothelin is referred to as endothelin B.

[0008] The amino acid sequences of the endothelin-1, endothelin B and endothelin-3 are shown in Fig. 3 in comparison to one another.

[0009] Endothelin is a general term for peptides having a molecular weight of 2500 ± 300 and having 21 amino acid residues, including four cysteine groups located at the 1st, 3rd, 11th and 15th residues from the N-terminus of the amino acid sequence, which form two sets of disulfide bonds. One of the combinations of the disulfide bonds may be 1-15 and 3-11 cysteine groups, and the other may be 1-11 and 3-15. The former is higher in ratio of formation and in activity than the latter.

[0010] These above-described endothelins have been called variously, and in the present invention, newly unified names for the endothelins are employed in the present invention. They are shown as compared with the previous names as follows:

<table>
<thead>
<tr>
<th>Newly unified names</th>
<th>Previous names</th>
</tr>
</thead>
<tbody>
<tr>
<td>endothelin-1</td>
<td>endothelin A</td>
</tr>
<tr>
<td>human endothelin</td>
<td></td>
</tr>
<tr>
<td>porcine endothelin</td>
<td></td>
</tr>
<tr>
<td>endothelin α</td>
<td></td>
</tr>
</tbody>
</table>
As described above, homologous endothelin peptides have been discovered from various animals. However, no novel homologous genes have been discovered from the same animal species. It is therefore a current subject that novel homologous endothelin is further screened, and the structure and activity of the endothelin is studied, thereby examining its usefulness, and that the novel peptide is cloned by gene recombination to pioneer mass production thereof.

SUMMARY OF THE INVENTION

Namely, the present inventors have succeeded in cloning a new human endothelin DNA which is the same as the above endothelin-3 [rat endothelin (endothelin C)] in amino acid sequence of the mature protein, but different therefrom in nucleotide sequence coding for the mature protein and in precursor amino acid sequence. The present inventors have named this DNA "human endothelin-3 DNA", and the precursor "human endothelin-3 precursor protein".

In accordance with the present invention, there are provided (1) a DNA sequence coding for a precursor protein of human endothelin-3, (2) a human endothelin-3 precursor protein, (3) a transformant containing a vector comprising a DNA sequence described in (1) and (4) a method for preparing mature endothelin-3 which comprises culturing the transformant described in (3), producing and accumulating a protein in a culture medium, and collecting the protein thus obtained.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows simplified restriction enzyme maps of a DNA sequence containing an endothelin-2 precursor or mature peptide DNA segment and of a DNA sequence containing a human endothelin-3 DNA segment;

Fig. 2 shows a nucleotide sequence of the endothelin-2 precursor or mature peptide DNA segment, a nucleotide sequence of the human endothelin-3 DNA segment, nucleotide sequences of endothelin-1 and rat endothelin-3 as comparative examples, and their amino acid sequences presumed therefrom;

Fig. 3 shows an amino acid sequence of the human endothelin-2 mature peptide presumed from the nucleotide sequence shown in Fig. 2, and amino acid sequences of endothelin-1, endothelin B and endothelin-3.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The DNA of the present invention coding for a precursor of human endothelin-3 is represented by the following formula (3) or a portion thereof:
It is shown in Fig. 2 that this DNA sequence is different from the known sequences. [0017] The DNA sequence corresponding to mature proteins is represented by Nos. 34 to 96 in formula (3).
[0018] Human endothelin-3 precursor comprises the following amino acid sequence represented by the formula (4), which differs from the amino acid sequence of rat endothelin-3 precursor as shown in Fig. 2,

\[
\begin{aligned}
1 & \quad 2 & \quad 3 & \quad 4 & \quad 5 & \quad 6 & \quad 7 & \quad 8 & \quad 9 & \quad 10 \\
\text{Glu} & \text{Gly} & \text{Ala} & \text{Pro} & \text{Glu} & \text{His} & \text{His} & \text{Arg} & \text{Ser} & \text{Arg} \\
11 & 12 & 13 & 14 & 15 & 16 & 17 & 18 & 19 & 20 \\
\text{Arg} & \text{Cys} & \text{Thr} & \text{Cys} & \text{Phe} & \text{Thr} & \text{Tyr} & \text{Lys} & \text{Asp} & \text{Lys} \\
21 & 22 & 23 & 24 & 25 & 26 & 27 & 28 & 29 & 30 \\
\text{Glu} & \text{Cys} & \text{Val} & \text{Tyr} & \text{Tyr} & \text{Cys} & \text{His} & \text{Leu} & \text{Asp} & \text{Ile} \\
31 & 32 & 33 & 34 & 35 & 36 & 37 \\
\text{Ile} & \text{Trp} & \text{Ile} & \text{Asn} & \text{Thr} & \text{Pro} & \text{Glu} \\
\end{aligned}
\]

[0019] The mRNA coding for endothelin-3 can be obtained from various endothelin-producing cells such as endothelial cells of human aortas and human placentas.
[0020] Methods for preparing the mRNA from the endothelin-3-producing cells include the guanidine thiocyanate method [J. M. Chirgwin et al., Biochemistry 18, 5294 (1979)] and the like.
[0021] Using the mRNA thus obtained as a template, cDNA is synthesized by use of reverse transcriptase, for example, in accordance with the method of H. Okayama et al. [Molecular and Cellular Biology 2, 161 (1979); ibid. 3, 280 (1983)]. The cDNA thus obtained is introduced into the plasmid.
[0022] The plasmids into which the cDNA may be introduced include, for example, pBR322 [Gene 2, 95 (1977)], pBR325 [Gene 4, 121 (1978)], pUC12 [Gene 19, 259 (1982)] and pUC13 [Gene 19, 259 (1982)], each derived from
Escherichia coli, and pUB110 derived from Bacillus subtilis [Biochemical and Biophysical Research Communication 112, 678 (1983)]. However, any other plasmid can be used as long as it is replicable and growable in the host cell. Examples of the phage vectors into which the cDNA may be introduced include λgt11 [R. Young and R. Davis, Proc. Natl. Acad. Sci. U.S.A., 80, 1194 (1983)]. However, any other phage vector can be used as long as it is growable in the host cell.

[0023] Methods for introducing the cDNA into the plasmid include, for example, the method described in T. Maniatis et al., Molecular Cloning, Cold Spring Harbor Laboratory, p.239 (1982). Methods for introducing the cDNA in the phage vector include, for example, the method of T. V. Hyun et al. [DNA Cloning, A Practical Approach 1, 49 (1985)].

[0024] The plasmid thus obtained is introduced into an appropriate host cell such as Escherichia and Bacillus.


[0027] Methods for transforming the host cell with the plasmid include, for example, the calcium chloride method or the calcium chloride/ribidium chloride method described in T. Maniatis et al., Molecular Cloning, Cold Spring Harbor Laboratory, p.249 (1982).

[0028] When a phage vector is used, for example, the phage vector can be transduced into multiplied Escherichia coli, using the in vitro packaging method.

[0029] Human cDNA libraries containing endothelin-3 cDNA can be obtained by numerous techniques well known in the art including purchasing them from the market, though obtainable by the methods described above. For example, a cDNA library from human placenta is available from Clontech Laboratories, Inc., U.S.A.

[0030] Methods for cloning an endothelin-3 DNA from the human DNA library include, for example, the plaque hybridization method using oligonucleotides chemically synthesized on the basis of phage vector 4A and the amino acid sequence of endothelin-2 as a probe [T. Maniatis et al., Molecular Cloning, Cold Spring Harbor Laboratory, (1982)]. The endothelin-2 DNA thus cloned is subcloned in, for example, pBR322, pUC12, pUC13, pUC19, pUC118 and pUC119 to obtain the endothelin-2 DNA, if necessary.

[0031] The nucleotide sequence of the DNA sequence thus obtained is determined by, for example, the Maxam-Gilbert method [A. M. Maxam and W. Gilbert, Proc. Natl. Acad. Sci. U.S.A., 74, 560 (1977)] or the dideoxy method [J. Messing et al., Nucleic Acids Research 9, 309 (1981)] and the existence of the endothelin-2 DNA is confirmed in comparison with the known amino acid sequence.

[0032] As described above, the DNA sequence [endothelin-3 DNA] represented by formula (3) coding for endothelin-3 is obtained.

[0033] Fig. 1 shows the restriction enzyme fragment maps of the DNA sequence containing the DNA segment coding for endothelin-3 obtained in Example 2 described below and of the DNA sequence containing the human endothelin-3 DNA obtained in Example 3 described below. Fig. 2 shows the nucleotide sequences of the DNA sequences as determined by the dideoxy method, and Fig. 3 shows the amino acid sequences ascertained from the nucleotide sequences.

[0034] The DNA sequence encoding for endothelin-3 cloned as described above can be used as it is, or after digestion with a restriction enzyme if desired, depending on the intended use.

[0035] A region intended to be expressed is cut out from the cloned DNA and ligated downstream from the promoter in a vehicle (vector) suitable for expression, whereby the expression vector can be obtained.

[0036] The DNA sequence has ATG as a translation initiating codon at the 5'-terminus thereof and may have TAA, TGA or TAG as a translation terminating codon at the 3'-terminus. The translation initiating codon and translation terminating codon may be added by use of an appropriate synthetic DNA adaptor. A promoter is further ligated in the upstream thereof for the purpose of expressing the DNA sequence.

[0037] Examples of the vectors include the above plasmids derived from Escherichia coli such as pBR322, pBR325, pUC12, and pUC13, the plasmids derived from Bacillus subtilis such as pUB110, pTP5 and pC194, plasmids derived from yeast such as pSH19 and pSH15, bacteriophage such as λphage, and animal viruses such as retroviruses and vaccinia viruses.

[0038] As the promoter used in the present invention, any promoter is appropriate as long as the promoter is suitable for expression in the host cell selected for the gene expression.

[0039] When the host cell used for transformation is Escherichia, it is preferable that a trp promoter, a lac promoter, a recA promoter, a λPL promoter or a λPP promoter is used. When the host cell is Bacillus, it is preferable that a PHO5 promoter, a PGK promoter, a GAP promoter or an ADH promoter is used. In particular, it is preferable that the host cell is Escherichia and the promoter is the trp promoter or the λPL promoter.

[0040] When the host cell is an animal cell, a SV-40 derived promoter, a retrovirus promoter, a metallothionein promoter and a heat shock promoter are each usable.
[0041] An enhancer, a certain DNA sequence important for promoter's activity in a cell, is also effectively used for expression.

[0042] Using a vector containing the DNA sequence coding for the endothelin-3 mature peptide (endothelin-3) thus constructed, transformants are prepared.

[0043] The host cells include, for example, Escherichia, Bacillus, yeast and animal cells.

[0044] As examples of the above Escherichia and Bacillus, strains similar to those described above can be mentioned.

[0045] Examples of the above yeast include Saccharomyces cerevisiae AH22, AH22R, NA87-11A and DKD-5D.

[0046] Examples of the animal cells include monkey cell COS-7, Vero, Chinese hamster cell (CHO), mouse L cell and human FL cell.

[0047] The transformation of the above Escherichia is carried out according to, for example, the method described in Proc. Natl. Acad. Sci. U.S.A. 69, 2110 (1972) or Gene 17, 107 (1982).

[0048] The transformation of the above Bacillus is conducted according to, for example, the method described in Molecular & General Genetics 168, 111 (1979).

[0049] The transformation of the yeast is carried out according to, for example, the method described in Proc. Natl. Acad. Sci. U.S.A. 75, 1929 (1978).

[0050] The transformation of the animal cells is carried out according to, for example, the method described in Virolology 52, 456 (1973).

[0051] Thus, there are obtained transformants transformed with an expression vector containing the DNA sequence coding for the endothelin-3 mature peptide (endothelin-3).

[0052] When bacterial transformants are cultured, a liquid medium is particularly suitable as a medium used for culture. Carbon sources, nitrogen sources, inorganic compounds and others necessary for growth of the transformant are contained therein. Examples of the carbon sources include glucose, dextrin, soluble starch and sucrose. Examples of the nitrogen sources include inorganic or organic materials such as ammonium salts, nitrates, corn steep liquor, peptone, casein, meat extracts, soybean meal and potato extract solution. The inorganic compounds include, for example, calcium chloride, sodium dihydrogenphosphate and magnesium chloride. Yeast extract, vitamins, growth promoting factors and so on may be further added thereto.

[0053] The pH of the medium is preferably 5 to 8.

[0054] As the medium used for cultivation of Escherichia, there is preferred, for example, M9 medium containing glucose and Casamino Acids (Miller, Journal of Experiments in Molecular Genetics 431-433, Cold Spring Harbor Laboratory, New York, 1972). In order to make the promoter act efficiently, a drug such as 3-indolylacrylic acid may be added thereto if necessary.

[0055] When the host cell is Escherichia, the cultivation is usually carried out at 15 to 43°C for 3 to 24 hours, with aeration or agitation if necessary.

[0056] When yeast transformants are cultured, there is used, for example, Burkholder minimum medium [K. L. Bostian et al., Proc. Natl. Acad. Sci. U.S.A. 77, 4505 (1980)] as the medium. The pH of the medium is preferably adjusted to 5 to 8. The cultivation is usually carried out at 20 to 35°C for 24 to 72 hours, with aeration or agitation if necessary.

[0057] When animal cell transformants are cultured, examples of the mediums include MEM medium containing 5 to 20% fetal calf serum [Science 122, 501 (1952)], DMEM medium [Virolology 8, 396 (1959)], RPMI1640 medium [Journal of the American Medical Association 199, 519 (1957)] and 199 medium [Proceeding of the Society for the Biological Medicine 73, 1 (1950)]. The pH is preferably 6 to 8. The cultivation is usually carried out at 30 to 40°C for 15 to 60 hours, with aeration or agitation if necessary.

[0058] When the host cell is Escherichia, the cultivation is usually carried out at 15 to 43°C for 3 to 24 hours, with aeration or agitation if necessary.

[0059] When bacterial transformants are cultured, a liquid medium is particularly suitable as a medium used for culture. Carbon sources, nitrogen sources, inorganic compounds and others necessary for growth of the transformant are contained therein. Examples of the carbon sources include glucose, dextrin, soluble starch and sucrose. Examples of the nitrogen sources include inorganic or organic materials such as ammonium salts, nitrates, corn steep liquor, peptone, casein, meat extracts, soybean meal and potato extract solution. The inorganic compounds include, for example, calcium chloride, sodium dihydrogenphosphate and magnesium chloride. Yeast extract, vitamins, growth promoting factors and so on may be further added thereto.

[0060] When yeast transformants are cultured, there is used, for example, Burkholder minimum medium [K. L. Bostian et al., Proc. Natl. Acad. Sci. U.S.A. 77, 4505 (1980)] as the medium. The pH of the medium is preferably adjusted to 5 to 8. The cultivation is usually carried out at 20 to 35°C for 24 to 72 hours, with aeration or agitation if necessary.

[0061] When animal cell transformants are cultured, examples of the mediums include MEM medium containing 5 to 20% fetal calf serum [Science 122, 501 (1952)], DMEM medium [Virolology 8, 396 (1959)], RPMI1640 medium [Journal of the American Medical Association 199, 519 (1957)] and 199 medium [Proceeding of the Society for the Biological Medicine 73, 1 (1950)]. The pH is preferably 6 to 8. The cultivation is usually carried out at 30 to 40°C for 15 to 60 hours, with aeration or agitation if necessary.

[0062] When the host cell is Escherichia, the cultivation is usually carried out at 15 to 43°C for 3 to 24 hours, with aeration or agitation if necessary.

[0063] When bacterial transformants are cultured, a liquid medium is particularly suitable as a medium used for culture. Carbon sources, nitrogen sources, inorganic compounds and others necessary for growth of the transformant are contained therein. Examples of the carbon sources include glucose, dextrin, soluble starch and sucrose. Examples of the nitrogen sources include inorganic or organic materials such as ammonium salts, nitrates, corn steep liquor, peptone, casein, meat extracts, soybean meal and potato extract solution. The inorganic compounds include, for example, calcium chloride, sodium dihydrogenphosphate and magnesium chloride. Yeast extract, vitamins, growth promoting factors and so on may be further added thereto.

[0064] When yeast transformants are cultured, there is used, for example, Burkholder minimum medium [K. L. Bostian et al., Proc. Natl. Acad. Sci. U.S.A. 77, 4505 (1980)] as the medium. The pH of the medium is preferably adjusted to 5 to 8. The cultivation is usually carried out at 20 to 35°C for 24 to 72 hours, with aeration or agitation if necessary.

[0065] When animal cell transformants are cultured, examples of the mediums include MEM medium containing 5 to 20% fetal calf serum [Science 122, 501 (1952)], DMEM medium [Virolology 8, 396 (1959)], RPMI1640 medium [Journal of the American Medical Association 199, 519 (1957)] and 199 medium [Proceeding of the Society for the Biological Medicine 73, 1 (1950)]. The pH is preferably 6 to 8. The cultivation is usually carried out at 30 to 40°C for 15 to 60 hours, with aeration or agitation if necessary.

[0066] When the host cell is Escherichia, the cultivation is usually carried out at 15 to 43°C for 3 to 24 hours, with aeration or agitation if necessary.

[0067] When bacterial transformants are cultured, a liquid medium is particularly suitable as a medium used for culture. Carbon sources, nitrogen sources, inorganic compounds and others necessary for growth of the transformant are contained therein. Examples of the carbon sources include glucose, dextrin, soluble starch and sucrose. Examples of the nitrogen sources include inorganic or organic materials such as ammonium salts, nitrates, corn steep liquor, peptone, casein, meat extracts, soybean meal and potato extract solution. The inorganic compounds include, for example, calcium chloride, sodium dihydrogenphosphate and magnesium chloride. Yeast extract, vitamins, growth promoting factors and so on may be further added thereto.
utilizing a difference in isoelectric point such as isoelectro-focusing electrophoresis.

[0062] The activity of the endothelin-3 precursor protein or mature peptide thus formed can be measured by an enzyme immunoassay using a specific antibody. If the products have vasoconstrictive activity, this activity may also be measured as an index.

[0063] The cells, such as animal cells or Escherichia coli, transfected or transformed with the DNA sequence of the present invention allow large amounts of the endothelin-3 mature peptide to be produced. Hence, the production of these peptides can be advantageously achieved.

[0064] The endothelin-3 mature peptide prepared here not only can be utilized as hypotension therapeutic agents or local vasoconstrictors, but also give a clue to analysis of the mechanism of the vasoconstrictr reactions in vivo and to elucidation of antagonists to the vasoconstrictor factors, including the other endothelin peptides.

[0065] The peptide has such effects as preventing various kinds of hemorrhage, for example, gastric or esophageal hemorrhage as vasoconstrictors, and may also be useful in curing various shock symptoms. The peptides can be administered orally, locally, intravenously or parenterally, preferably locally or intravenously. The dose is 0.001 μg to 100 μg/kg, preferably 0.01 μg to 10 μg/kg. The dose is preferably dependent on weight and preferably used in the form of a solution in 1 to 10 ml of a saline solution.

[0066] The peptides of the present invention can be formed into various preparations together with additional components, such as emulsions, hydrated mixtures, tablets, solutions, powders, granules, capsules and pills. Examples of the additional components include pharmaceutically acceptable vehicles, disintegrators, lubricants, binders, dispersants, plasticizers, fillers and carriers. As to the additional components, examples of the vehicles include lactose, glucose and white sugar; those of the disintegrators include starch, sodium alginate, agar powder and carboxymethyl cellulose calcium; those of the lubricants include magnesium stearate, talc and liquid paraffin; those of the binders include syrup, gelatin solution, ethanol and polyvinyl alcohol; those of the dispersants include methyl cellulose, ethyl cellulose and shellac; and those of the plasticizers include glycerin and starch.

[0067] When nucleotides, amino acids and so on are indicated by the abbreviations in this specification and drawings, the abbreviations adopted by IUPAC-IUB Commission on Biochemical Nomenclature or commonly used in the art are employed. For example, the following abbreviations are used. When amino acids are capable of existing as optical isomers, the L-forms are represented unless otherwise specified.

DNA : Deoxyribonucleic acid
cDNA : Complementary deoxyribonucleic acid
A : Adenine
T : Thymine
G : Guanine
C : Cytosine
RNA : Ribonucleic acid
mRNA : Messenger ribonucleic acid
dATP : Deoxyadenosine triphosphate
dTTP : Deoxythymidine triphosphate
dGTP : Deoxyguanosine triphosphate
dCTP : Deoxycytidine triphosphate
ATP : Adenosine triphosphate
EDTA : Ethylenediaminetetraacetic acid
SDS : Sodium dodecyl sulfate
Gly or G : Glycine
Ala or A : Alanine
Val or V : Valine
Leu or L : Leucine
Ile or I : Isoleucine
Ser or S : Serine
Thr or T : Threonine
Cys or C : Cysteine
Met or M : Methionine
Glu or E : Glutamic acid
Asp or D : Aspartic acid
Lys or K : Lysine
Arg or R : Arginine
His or H : Histidine
Phe or F : Phenylalanine
Tyr or Y : Tyrosine
Trp or W : Tryptophan
Pro or P : Proline
Asn or N : Asparagine
Gln or Q : Glutamine

[0068] The present invention will hereinafter be described in detail with the following Reference Example and Ex-
amples.

[0069] Transformant Escherichia coli XL-1/pghET20SGl obtained in Example 2 and transformant Escherichia coli
XL-1/pghET3E1 obtained in Example 3 are deposited in Fermentation Research Institute, Agency of Industrial Science
and Technology, Ministry of International Trade and Industry, Japan (FRI) with the accession numbers FERM BP-2118
and FERM BP-2119, respectively, on October 24, 1988.

Reference Example

(1) Assay of Vascular Smooth Muscle Constrictor Activity

[0070] Porcine right coronary artery spiral specimens (0.5 X 20 mm) with the intima denuded by rubbing with a small
swab are suspended in 3 ml of Krebs-Ringer solution maintained at 37°C and saturated with a mixed gas containing
5% carbon dioxide and 95% oxygen by volume. After setting the basal tension to 2 g, the isometric tension is measured
with tension transducers.

(2) Assay of Cardiotonic Action

[0071] Instead of the porcine right coronary artery spiral specimens used in the assay described in the above item
(1), suspended guinea pig right atrium specimens are used, and the tension and the heart rate per minute are measured
according to the same procedure as described in (1).

Example 1

Preparation of DNA Probe Coding for a Portion of Endothelin-1 [Porcine Endothelin (Human Endothelin I)]

[0072] The messenger RNA sequence expected from the amino acid sequence composed of the 7th to the 16th
residues of endothelin-1,

\[\text{Met-Asp-Lys-Glu-Cys-Val-Tyr-Phe-Cys-His-Leu-Asp-Ile-Ile},\]

was used to chemically synthesize a DNA probe having the following sequence.

\[5'\text{ ATG GAC AAG GAG TGT GTC TAC TTC TGC CAT CTG GAC ATC ATC 3'}\]

[0073] The 5'-terminus of this DNA probe was phosphorylated with \(\gamma^{32}\)P-ATP by using T4 polynucleotide kinase. The
phosphorylated DNA probe was used for screening a genomic DNA library.

Example 2 (Reference example)

Isolation of Endothelin-2 Precursor Genomic DNA and Determination of Nucleotide Sequence Thereof

[0074] Escherichia coli Le392 was infected with the above human genomic DNA library (Clontech Laboratories, Inc.)
and plated, whereby phage plaques were allowed to appear. According to the report of W. Benton and R. Davis [Science
196, 180-182 (1977)], a portion of plaque DNA was transferred to a nylon filter and hybridized with the DNA probe
labeled with \(^{32}\)P in Example 1. The hybridization was carried out in the presence of 20% formamide at 42°C, and then
the filter was washed in 0.2 X SSC, 0.1% SDS at 20°C. Hybridization-positive clones were isolated. Then, a mature
code region of \( \lambda \)ghET20, one of these clones, was cut out with SacI and subcloned in plasmid pUC118. By transforming Escherichia coli XL-1 with the resulting plasmid, transformant Escherichia coli XL-1/\( \lambda \)ghET20SG1 was obtained. There is shown in Fig. 1 the simplified restriction enzyme map of a human genomic DNA fragment contained in this plasmid. In the figure, the heavy bar ( ) shows a mature endothelin-2 code region.

This mature peptide code region and the nucleotide sequence in the vicinity thereof were determined by the method of Sanger [Proc. Nat. Acad. Sci. U.S.A. 74, 5463-5467 (1977)]. The nucleotide sequence and the amino acid sequence presumed therefrom are shown in Fig. 2. The region surrounded by ( ) shows the mature peptide portion.

Fig. 3 shows, for comparison, the amino acid sequences of the mature peptides of endothelin-1, endothelin B and rat endothelin-3 which have previously been discovered, along with the amino acid sequence of the endothelin-2 mature peptide.

Example 3

Isolation of Mature Human Endothelin-3 Code Region and Determination of Nucleotide Sequence Thereof

Using methods similar to those in Example 2, hybridization-positive clones were isolated. Then, a mature code region of \( \lambda \)ghET3, one of these clones, was cut out with EcoRI and subcloned in plasmid pUC118. By transforming Escherichia coli XL-1 with the resulting plasmid, transformant Escherichia coli XL-1/\( \lambda \)ghET3EI was obtained. There is shown in Fig. 1 the simplified restriction enzyme map of a human genomic DNA fragment contained in this plasmid. In the figure, the heavy bar ( ) shows a mature human endothelin-3 code region.

This mature peptide code region and the nucleotide sequence in the vicinity thereof were determined by the method of Sanger [Proc. Nat. Acad. Sci. U.S.A. 74, 5463-5467 (1977)]. The nucleotide sequence and the amino acid sequence presumed therefrom are shown in Fig. 2. The region surrounded by a frame shows the endothelin-3 mature peptide portion.

Example 4 (Reference example)

Synthesis of Endothelin-2

Human endothelin A-II was synthesized by a conventional method, using 0.7 g (0.5 m mol) of the commercially available Boc-Trp(CHO)-PAM resin (Applied Biosystems) and a peptide synthesizer (Applied Biosystems, Model 430A).

A condensation process was conducted as follows:

A Boc group on the resin was treated with 50% trifluoroacetic acid in methylene chloride to form a free terminal amino group, to which the following amino acids were condensed in turn according to the amino acid sequence of endothelin-2 from the C-terminus in the presence of dicyclohexyl carbodiimide (DCC):

- Boc-Ile, Boc-Asp(Obzl), Boc-Leu, Boc-His(Tos), Boc-Cys(Acm), Boc-Tyr(Br-z), Boc-Val, Boc-Phe, Boc-Glu(Obzl), Boc-Lys(Cl-Z), Boc-Trp(CHO) and Boc-Ser(Bzl)

890 mg of 2.53 g of the protected endothelin-2 resin thus obtained was swelled with 1 ml of anisole and 1 ml of 1,2-ethanediol, and then treated with 10 ml of hydrogen fluoride at 0°C for 60 minutes, followed by removal of excess hydrogen fluoride under reduced pressure. After washing with 5 ml of diethyl ether, the residue was extracted with trifluoroacetic acid and the resin was filtered off. After removal of trifluoroacetic acid by distillation under reduced pressure, the extract was dissolved in 50% aqueous acetic acid and applied on a dextran gel column (Sephadex® G-50, 2 X 90 cm). Peak fractions eluted with the above solvent were collected and lyophilized, thereby obtaining 180 mg of white powder. In 4 ml of 50% aqueous acetic acid was dissolved 31 mg of the powder, and 19 mg of trifluoroacetate-mercury(II) was added thereto, followed by stirring at room temperature for 16 hours. The resulting mixture was diluted with addition of 100 ml of n-butanol, 50 ml of methanol and 50 ml of water, and hydrogen sulfide gas was blown through it. Then, 5% NH₄OH was added thereto for adjustment to pH 8, and thereafter the mixture was subjected to air-oxidation for 6 hours. Acetic acid was further added thereto to give pH 3, followed by lyophilization. The lyophilized mixture was applied to a Sephadex G-50 column (2 X 90 cm) filled with 50% acetic acid, and the peak fractions were collected. The collected fractions were further fractionated by using HPLC [column: YMC (YMC Co. Ltd.), solvent: eluted by a linear gradient of 0.1% aqueous trifluoroacetic acid and acetonitrile containing 0.1% trifluoroacetic acid] to obtain 1.0 mg of the desired product.

Synthesized endothelin-2 was eluted by HPLC at 41.4 minutes, in contrast with 40.2 minutes for endothelin-1.
Column conditions

[0081]

Wakosil 5C18 (Wako Chem. Ind. Ltd., Japan) (4.6 X 250 mm)
Eluent: A (0.1% aqueous trifluoroacetic acid)
B (acetonitrile containing 0.1% trifluoroacetic acid)
Linear concentration gradient elution from A to B (50 minutes)
Flow rate: 1.0 ml/minute

Values of analysis of amino acids: Values of analysis (the number in the synthesized product)

<table>
<thead>
<tr>
<th>Asx</th>
<th>Ser</th>
<th>Glx</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.92</td>
<td>2.31</td>
<td>1.04</td>
</tr>
<tr>
<td>Cys</td>
<td>Val</td>
<td>Ile</td>
</tr>
<tr>
<td>1.30</td>
<td>1.16</td>
<td>1.35</td>
</tr>
<tr>
<td>Leu</td>
<td>Tyr</td>
<td>Phe</td>
</tr>
<tr>
<td>1.85</td>
<td>0.89</td>
<td>1.01</td>
</tr>
<tr>
<td>His</td>
<td>Lys</td>
<td>Trp</td>
</tr>
<tr>
<td>1.10</td>
<td>1.03</td>
<td>(*)</td>
</tr>
</tbody>
</table>

* No data was obtained due to acid decomposition.

[0082] The combination of the disulfide bonds was 1-15 and 3-11 cysteine groups in mature endothelin-2.

Claims

1. DNA coding for a precursor protein of human endothelin-3 represented by the following formula (4):

```
Glu Gly Ala Pro Glu His His Arg Ser Arg Arg Cys Thr Cys Phe Thr
```

2. The DNA according to claim 1, wherein said DNA has a nucleotide sequence represented by the following formula (3):

```
GAG GGG GCC CCT GAG CAC CAC CGA TCC AGG CGC TGC
ACG TGC TTC ACC TAC AAG GAC AAG GAG TGT GTC TAC
TAT GTC CAC CTG GAC ATC ATT TGG ATC AAC ACT CCC
```

3. A precursor protein of human endothelin-3 having an amino acid sequence represented by the following formula (4):

```
Glu Gly Ala Pro Glu His His Arg Ser Arg Arg Cys Thr Cys Phe Thr
```

4. A transformant containing a vector comprising the DNA of claim 1.

5. A method for preparing mature endothelin-3 which comprises:

(a) culturing the transformant according to claim 4.
(b) accumulating mature endothelin-3 in a culture medium, and
(c) collecting same.

Patentansprüche

1. DNA-Codierung für ein Vorläuferprotein von humanem Endothelin-3 der folgenden Formel (4):

```
Glu Gly Ala Pro Glu His His Arg Ser Arg Arg Cys Thr Cys Phe Thr
1      5      10    15
Tyr Lys Asp Lys Glu Cys Val Tyr Tyr Cys His Leu Asp Ile Ile Trp
 20    25    30
Ile Asn Thr Pro Glu
 35    37
(4).
```

2. DNA nach Anspruch 1, wobei die DNA eine Nucleotid-Sequenz aufweist, die durch die folgende Formel (3) dargestellt wird:

```
GAG GGG GCC CCT GAG CAC CAC CGA TCC AGG CGC TGC
ACG TGC TTC ACC TAC AAG GAC AAG GAG TGT GTC TAC
TAT TGC CAC CTG GAC ATC ATT TGG ATC AAC ACT CCC
GA
(3).
```


```
Glu Gly Ala Pro Glu His His Arg Ser Arg Arg Cys Thr Cys Phe Thr
1      5      10    15
Tyr Lys Asp Lys Glu Cys Val Tyr Tyr Cys His Leu Asp Ile Ile Trp
 20    25    30
Ile Asn Thr Pro Glu
 35    37
(4).
```

4. Transformante, enthaltend einen die DNA von Anspruch 1 umfassenden Vektor.

5. Verfahren zur Herstellung von reifem Endothelin-3, umfassend das:
   (a) Kultivieren der Transformante nach Anspruch 4,
   (b) Akkumulieren des reifen Endothelin-3 in einem Kulturmedium und
   (c) dessen Sammeln.

Revendications

1. ADN codant une protéine précurseur de l'endothéline 3 humaine représentée par la formule (4) suivante :

```
Glu Gly Ala Pro Glu His His Arg Ser Arg Arg Cys Thr Cys Phe Thr
1      5      10    15
Tyr Lys Asp Lys Glu Cys Val Tyr Tyr Cys His Leu Asp Ile Ile Trp
 20    25    30
Ile Asn Thr Pro Glu
 35    37
(4).
```
2. ADN selon la revendication 1, dans lequel ledit ADN a une séquence nucléotidique représentée par la formule (3) suivante :

\[
\begin{align*}
&GAG
ggG
gcc
cct
gag
cac
cac
cga
tcc
agg
cgc
tgc
\text{ACG}
\text{TGC}
\text{TTC}
\text{ACC}
\text{TAC}
\text{AAG}
\text{GAC}
\text{AAC}
\text{GAG}
\text{TGG}
\text{GTC}
\text{TAC}
\text{TAT}
\text{TGC}
\text{CAC}
\text{CTG}
\text{GAC}
\text{ATC}
\text{ATT}
\text{TGG}
\text{ATC}
\text{AAC}
\text{ACT}
\text{CCC}
\text{GA}
\end{align*}
\]

(3)

3. Protéine précurseur de l'endothéline 3 humaine ayant une séquence d'acides aminés représentée par la formule (4) suivante :

\[
\begin{align*}
&\text{Glu}
\text{Gly}
\text{Ala}
\text{Pro}
\text{Glu}
\text{His}
\text{His}
\text{Arg}
\text{Ser}
\text{Arg}
\text{Arg}
\text{Cys}
\text{Thr}
\text{Cys}
\text{Phe}
\text{Thr}
\text{Tyr}
\text{Lys}
\text{Asp}
\text{Lys}
\text{Glu}
\text{Cys}
\text{Val}
\text{Tyr}
\text{Tyr}
\text{Cys}
\text{His}
\text{Leu}
\text{Asp}
\text{Ile}
\text{Ille}
\text{Trp}
\text{Ile}
\text{Asn}
\text{Thr}
\text{Pro}
\text{Glu}
\end{align*}
\]

(4)

4. Organisme génétiquement modifié contenant un vecteur comprenant l'ADN de la revendication 1.

5. Procédé de préparation d'une endothéline 3 mature comprenant

(a) la culture de l'organisme génétiquement modifié selon la revendication 4,
(b) l'accumulation de l'endothéline 3 mature dans un milieu de culture et
(c) la récolte de celui-ci.
Fig. 1

pg3ET20Scr 1

5' -> endothenlin-2 3'
SacI
|___| SacI
|___| AccI

0.3 kb

50 bp

pg3ET3E1

5' ->
EcoRI
|___| BamH I HindII

6.2 kb

1 kb
Fig. 2

Endothelin-2
CATGCCCAAGGGACCCACCTTTGGCTTGCCGCGTTGCTCTGCAAGCTCCTGCTTGCTGGCAGCAAGGAGGTGGGTCTACTTCTGGCACATCTGGAACACTCCTGA
HisAlaGlnGlyThrHisLeuArgLeuArgCysSerCysSerSerTrpLeuAspLysGluCysValTyrPheCysHisLeuAspIleIleTrpValAsnThrProGlu

Endothelin-1
AGTCCACCTGCGGCTTCGCGGTTCCAAGGGCTTGCTCTGCTGTCCCTGATGGAATAAGGAGGTGGGTCTACTTCTGGCACATCTGGAACACTCCTCGA
SerProProTrpArgLeuArgArgSerLysArgSerCysSerSerLeuMetAspLysGluCysValTyrPheCysHisLeuAspIleIleTrpValAsnThrProGlu

Human endothelin-1 (The present invention)
GAGGGGGCGTGGCGACCAACCGATCGAGGGGGCTGCAACGTCAAGGACAGGAGGTGGGTCTACTTCTGGCACATCTGGAACACTCCTCGA
GluGlyAlaProGluHisHisArgSerArgArgCysThrCysPheThrTyrLysAspLysGluCysValTyrTyrCysHisLeuAspIleIleTrpIleAsnThrProGlu

Rat endothelin-3
CACCGACCTCGGGCTGCAACGTCACTTATAGGACAGGAGGTGGGTCTACTTCTGGCACATCTGGAACACTCCTCGA
HisArgProArgArgCysThrCysPheThrTyrLysAspLysGluCysValTyrTyrCysHisLeuAspIleIleTrpIleAsnThrProGlu
Fig. 3

Endothelin-2

Cys Ser Cys Ser Ser Trp Leu Asp Lys Glu Cys Val Tyr Phe Cys His Leu Asp Ile Ile Trp

Endothelin-1

Cys Ser Cys Ser Ser Leu Met Asp Lys Glu Cys Val Tyr Phe Cys His Leu Asp Ile Ile Trp

Endothelin-8

Cys Ser Cys Asn Ser Ser Trp Leu Asp Lys Glu Cys Val Tyr Phe Cys His Leu Asp Ile Ile Trp

Endothelin-3

Cys Thr Cys Phe Thr Tyr Lys Asp Lys Glu Cys Val Tyr Tyr Cys His Leu Asp Ile Ile Trp