Title: PROCESS FOR PRODUCING A HUMAN NEUTROPHIL CHEMOTACTIC FACTOR POLYPEPTIDE

Abstract

An expression vector in which a DNA encoding a human neutrophil chemotactic factor polypeptide is inserted, a transformant (a host cell transformed with the expression vector), and a process for production of the said polypeptide by using the said transformant.
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DESCRIPTION

Process for producing a human neutrophil chemotactic factor polypeptide

TECNCICAL FIELD

This invention relates to an expression vector in which a DNA encoding a human neutrophil chemotactic factor polypeptide is inserted, a transformant (a host cell transformed with the expression vector), and a process for production of the said polypeptide by using the said transformant.

BACKGROUND ART

Human neutrophil chemotactic factor (abbreviated NCF hereinafter) is a physiologically active polypeptide which is produced from human mononuclear leukocytes stimulated with lipopolysaccharide, and has biological activity specifically to attract neutrophils, and is evaluated as one of the modulating factors relating to the initial stage of the inflammatory reaction (Yoshimura, T. et al., J. Immunol., 139, 788, 1987).

As for the said NCF derived from human mononuclear leukocytes, its partial amino acid sequence has already been determined (Yoshimura, T. et al., Proc. Natl. Acad. Sci., USA, 84, 9233, 1987). The present inventors have succeeded in isolation of a cDNA encoding the human NCF based on the above partial amino acid sequence (see Referential Example 1). The base sequence of the cloned human NCF cDNA was identical in the coding region with the base sequence reported by Schmid and Weissmann (J. Immunol., 139, 250, 1987). A human NCF polypeptide was found to be a polypeptide with the low molecular weight of 8.4kD since the complete primary structure of its polypeptide was established by the genetic analysis.

As regards various physiologically active polypeptides, such as interleukin-1, interleukin-2, interleukin-3, TNFα (tumor necrosis factor), lymphotoxin (TNFβ), GM-CSF (granulocyte-macrophage colony stimulating factor), G-CSF (granulocyte colony stimulation factor) and various interferons, the production of these polypeptides having the molecular weight of more than 10kD has been succeeded by the use of recombinant DNA technology. But in the case of producing a polypeptide
with the low molecular weight by recombinant DNA technology, especially by using \textit{E. coli} as the host, it is usually produced as a fused protein with the protein derived from \textit{E. coli} in general, in order to avoid degradation in the host cells. For example, Schulz et al. reported that it is extremely difficult to produce directly a human somatomedin C polypeptide (7.7kD) consisting of 69 amino acid residues by recombinant DNA technology using \textit{E. coli} as the host, and they succeeded in the efficient production of it as a dimer polypeptide or the truncated derivatives of the dimer polypeptide by recombinant DNA technology in \textit{E. coli} (Schulz, M-F. et al., J. Bacteriol., 169, 5385, 1987). The degree of this degradation is closely related to the structure of the polypeptide, and this relationship is more likely when the molecular weight of the required polypeptide is lower, and particularly, most likely in the case of an extremely lower molecular weight polypeptide in \textit{E. coli} (Itakura, K. et al., Science 198, 1056, 1977).

**DISCLOSURE OF INVENTION**

The present inventors attempted to express directly the human NCF polypeptide with such lower molecular weight by applying recombinant DNA technology using \textit{E. coli} as the host. Consequently, it has been found unexpectedly that the said polypeptide could be produced efficiently without significant degradation in the host cells.

The object of this invention is to offer the efficient process of a human NCF polypeptide by recombinant DNA technology.

Another object of this invention is to offer a recombinant expression vector in which a DNA encoding a human NCF polypeptide is inserted and a host cell transformed with the said vector.

Other object will be understood from the following descriptions.

According to this invention, a human NCF polypeptide, particularly, a polypeptide consisting of an amino acid sequence represented by formula [1] shown in Table 1, can be produced by applying recombinant DNA technology in microorganisms or animal cells.

**Table 1**

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<tr>
<th>Ser</th>
<th>Ala</th>
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LeuCysLeuAspProLysGluAsnTrpValGlnArg
ValValGluLysPhelLeuLysArgAlaGluAsnSer

As a base sequence of a DNA encoding a polypeptide consisting
of an amino acid sequence represented by formula [I], the DNA
consisting of a base sequence represented by formula [A] shown in Table
2 and its degenerative sequence are illustrated.

Table 2

5'-AGTGAATTAGATGTGAGTCATTAGATAAGACA
TACTCCAAACCTTCCACCCAAATTATCAAAAGAA
CTGAGAGTGTTGAGAGTGGACCACACTGGGCAAC
ACAGAAATTATTGGAAGCTTCTGATGGAAGAGAG
CTCTGTCGTCGACCAGAAGAAACTGTGGGTCAGAGG
GTTGGAAAGAAGTTTTTGAGAGGCTGGAAATTCA-3'

The DNA encoding a human NCF polypeptide can be isolated, for
example, according to the method as described in Referential Example 1
and the method reported by Schmid and Weissmann as mentioned above. It
is also possible to perform the total synthesis of the said DNA
chemically. As regards the extra region or deficient region of the
resulting DNA, the required DNA can be produced by the methods of the
digestion and/or repairment such as, for example, digesting by an
appropriate restriction enzyme and ligating with chemically synthesized
oligodeoxyribonucleotides.

By adding a translation initiation codon ATG to the 5'-terminus
(upstream) of this DNA, ligating a DNA fragment containing a termination
codon to the 3'-terminus (downstream) of the DNA having the initiation
codon, connecting the resulting DNA with a proper promoter (e.g. \texttt{trp},
\texttt{lac}, \texttt{phoS}, PL, SV40 early promoter) and SD sequence, and then inserting
the resulting DNA having the proper promoter and SD sequence into a
proper vector (e.g. plasmid pBR322), an expression vector for production
of the human NCF polypeptide is constructed.

The base sequence from the SD sequence to the translation
initiation codon is preferably represented by formula [D].

5'-XGGAGTTTYATT-3' \hspace{1cm} \text{formula [D]}

wherein \(X\) means (A), \(x\) being 1 to 5, and \(Y\) means (A)/(T), \(y\) being 0 to
3, \(z\) being 0 or 1.

A transformant of this invention can be obtained by introducing
the expression vector constructed as above into a proper host cell, for example E. coli according to the method of Cohen et al. (Cohen, S. N., et al., Proc. Natl. Acad. Sci., USA, 69, 2110, 1972).

The human NCF polypeptide can be produced by cultivating the transformant of this invention under suitable culture conditions. The extract containing the said polypeptide can be obtained from the culture after destroying the cells, for example by lysozyme digestion and freeze-thawing, sonication or by using a French press, followed by collecting the extract by centrifugation or filtration.

The human NCF polypeptide can be purified from the extract by purification methods characterized by combination of treatment for removing nucleic acids, salting-out, anion and/or cation exchange chromatography, ultrafiltration, gel filtration, if necessary, dialysis, electrophoresis, affinity chromatography using specific antibodies, and so on.

The chemical, physicochemical and biological properties of the human NCF polypeptide will be described hereinafter in detail.

The highly purified human NCF polypeptide obtained in Example (to be referred to as the recombinant human NCF) was used for analyses as shown below.

(1) Molecular weight

Molecular weight of the recombinant human NCF was measured by SDS-polyacrylamide gel electrophoretic analysis. As molecular weight marker proteins, the standard protein kit (Pharmacia Fine Chemicals, Sweden) consisting of the following proteins was used: lysozyme (14.4kD), soybean trypsin inhibitor (20.1kD), carbonic anhydrase (30kD), ovalbumin (43kD), bovine serum albumin (67kD) and phosphorylase b (94kD).

As a result, the recombinant human NCF had a molecular weight of approximately 8–10kD.

Polymerized molecule of the recombinant human NCF due to the formation of intermolecular disulfide bond was not detected significantly.

(2) Amino acid sequence

An amino acid sequences of the recombinant human NCF were determined by the Automated Edman degradation method.

The recombinant human NCF was previously treated by reductive cleavage of disulfide bonds with 2-mercaptoethanol, followed by S-
pyridylethylataion of cysteine residues with 4-vinyl pyridine according to the method of Fullmer (Anal. Biochem., 142, 336, 1984).

Separately, several kinds of peptide fragments of the recombinant human NCF were prepared and isolated according to the following methods.

The recombinant human NCF was treated with 70% formic acid by the method of Sonderegger et al. (Anal. Biochem., 122, 298, 1982) to cleave specifically Asp-Pro peptide bond. From the resulting two peptide fragments, the C-terminal fragment was isolated by high-performance liquid chromatography using a column of SynChropak RP-P (SynChrom, Inc., USA) under the elution condition of a linear gradient of acetonitrile concentration from 0 to 50% in 0.1% trifluoroacetic acid.

And the pyridylethylated human NCF was digested with a metalloendopeptidase (EC 3.4.24; Seikagaku Kogyo, Japan) and the resulting peptide fragments were isolated by high-performance liquid chromatography under the elution condition of a linear gradient of acetonitrile concentration from 0 to 30% in 0.1% trifluoroacetic acid.

N-terminal amino acid sequences of the pyridylethylated NCF and each peptide fragment were determined with a Protein Sequencer, Model 470A (Applied Biosystems, USA) and a SP8440 UV/VIS detector (Spectra-Physics, USA).

It was found that the N-terminal amino acid sequence of the pyridylethylated recombinant human NCF was as follows:
SerAlaLysGluLeuArgCysGlnCysIleLysThrTyrSer
LysProPheHisProLysPheIleLysGluLeuArgValIle
GluSerGlyProHisCysAlaAsn

This amino acid sequence was completely identical with the amino acid sequence from N-terminal Ser to Asn at the 36th position of the human NCF polypeptide represented by formula [I] shown in Table 1. A methionine residue due to the translation initiation codon (ATG) could not be detected at the N-terminus.

It was also found that the N-terminal amino acid sequence of the acid-cleaved C-terminal fragment was as follows:
ProLysGluAsnTrpValGlnArgValValGluLysPheLeu
LysArgAlaGluAsnSer

This amino acid sequence was identical with the amino acid sequence from the 53rd position from the N-terminus to the C-terminal
Ser of the human NCF polypeptide represented by formula [I] shown in Table 1.

The amino acid sequences of the two peptide fragments prepared by digestion with metalloendopeptidase were determined as follows:

LysGluLeuArgValIleGluSerGlyProHisCysAlaAsn
ThrGluIleIleVal

and

LysLeuSerAspGlyArgGluLeuCysLeuAspPro

These amino acid sequences were identical with the amino acid sequence of 19 residues from the 23rd position to the 41st position from the N-terminus and the amino acid sequence of 12 residues from the 42nd position to the 53rd position from the N-terminus of the human NCF polypeptide represented by formula [I] shown in Table 1.

Consequently, it has been confirmed that the amino acid sequence of the recombinant human NCF polypeptide is completely identical with the sequence deduced from a base sequence encoding the human NCF.

(3) Extinction coefficient

The recombinant human NCF polypeptide was lyophilized without any carrier component. Contents of water and ash in this lyophilized product were determined to be 6.01% and 0.52%, respectively.

Extinction coefficient value of the recombinant human NCF was calculated to be 8.25 at 280 nm under the conditions of 1% aqueous solution and 1cm optical path length.

(4) Chemotactic activity

Neutrophil chemotactic activity was measured in a multiwell chemotaxis Boyden chamber (Neuro Probe, Inc., USA) as reported by Harvath, L. et al. (J. Immunol. Methods, 37, 39, 1980). Recombinant NCF was serially diluted in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1% bovine serum albumin (BSA). Normal human neutrophils were purified from Buffy coat collected from healthy donors (National Institutes of Health, Blood Bank, USA) by separating on Ficoll-Hypaque followed by lyses contaminating red blood cells with ACK-lyses buffer. The purity of neutrophils were more than 95% by staining cells with Giemsa solution. The viability was also more than 95% as judged by a trypan blue dye exclusion test. The neutrophil cells were incubated at a cell density of one million cells/ml in DMEM supplemented with 1% BSA for 40 minutes at 37°C. The migrated
neutrophils which adhered onto a membrane (3 μm: Nuclepore Corp., USA) were fixed with methanol and stained with Giemsa solution. At 0.1 ng/ml significant migration was observed by examining with microscope. At 10 ng/ml maximal migration was observed. The migrated cells were identified to be neutrophils morphologically.

For simplification of the description, the following abbreviations are used in the present specification and claims.

\[
\begin{align*}
A: & \quad \text{adenine} \\
C: & \quad \text{cytosine} \\
G: & \quad \text{guanine} \\
T: & \quad \text{thymine} \\
I: & \quad \text{Inosine} \\
dATP: & \quad \text{deoxyadenosine triphosphate} \\
dCTP: & \quad \text{deoxycytidine triphosphate} \\
dGTP: & \quad \text{deoxyguanosine triphosphate} \\
dTTP: & \quad \text{deoxythymidine triphosphate} \\
ATP: & \quad \text{adenosine triphosphate} \\
DNA: & \quad \text{deoxyribonucleic acid} \\
cDNA: & \quad \text{complementary DNA} \\
kbp: & \quad \text{kilobase pairs} \\
SD sequence: & \quad \text{Shine-Dalgarno sequence} \\
kD: & \quad \text{kilodaltons} \\
SDS: & \quad \text{sodium laurylsulfate} \\
\text{[3H]-thymidine: tritiated thymidine} \\
\end{align*}
\]

In the present specification and claims, the nucleotide sequence shown by a single strand is the nucleotide sequence of a sense strand, and the left end is a 5'-terminus and the right end is a 3'-terminus. In the amino acid sequence, the left end is an N-terminus, and the right end is a C-terminus.

The following Example and Referential Examples illustrate this invention more specifically.

It should be understood however that the invention is in no way limited to these examples.

BRIEF DESCRIPTION OF DRAWINGS

For a better understanding of the following Example and Referential Examples, Schemata 1 to 3 are attached to the present specification.
Schema 1 shows a process of constructing an expression plasmid pHNP101 (Example); 
Schema 2 shows a process of constructing an expression vector pEP205 (Referential Example 2); 
Schema 3 shows a process of constructing an expression plasmid pHIPH383a (Referential Example 3).

BEST MODE FOR CARRYING OUT THE INVENTION

Example

Production of human NCF polypeptide

Human NCF polypeptide having an amino acid sequence represented by formula [I] in Table 1, was produced by the following methods.

(1) Construction of an expression plasmid pHNP101

From the recombinant plasmid pUC19-1.7-5 in which human NCF cDNA was inserted as mentioned in Referential Example 1, DNA fragment encoding the polypeptide corresponding to the amino acids from the 18th position to the 97th position from the N-terminus of human NCF precursor polypeptide (corresponding to the base sequence from the base No. 52 to No. 291 in Table 3) was isolated by digestion with restriction endonucleases PstI and EcoRI. This DNA fragment was then cloned into a phage vector M13mp18 (Takara Shuzo Co., Japan) at a region between the restriction endonuclease cleavage site of PstI and that of EcoRI in the polylinker sequence. By using the resultant recombinant phage DNA, an specific base sequence being 5'-TTAAATTG-3' was inserted between the codon corresponding to Arg at the 27th position from N-terminus of the human NCF precursor polypeptide and the codon corresponding to Ser at the 28th position, by the technique of site-directed mutagenesis according to the method of Kunkel et al. (Methods in Enzymol., 154, 367-382, 1987). The site-directed mutagenesis was carried out using a Mutagenic in vitro mutagenesis kit according to the instruction manual (Bio-Rad Labs., USA).

E. coli JM105 was infected with the recombinant phage DNA, and then it was cultivated to collect the recombinant phage. Then, E. coli CJ236 was infected with the recombinant phage obtained as above and cultivated in 2xTY medium [composition: 1.6% tryptone, 1% yeast extract, 0.5% sodium chloride] supplemented with uridine (1 μg/ml) and chloramphenicol (20 μg/ml) at 37°C for 5 hours. The single-stranded phage DNA containing uracils was isolated from the culture medium.
Separately, a mutagenic oligodeoxyribonucleotide primer consisting of 33 bases represented by the following formula [E] was chemically synthesized.

5'-GTTTTGCACAGTTAAATTAGTGCTAAAG-3'

formula [E]

The 5'-end of the mutagenic primer was previously phosphorylated. The phosphorylated primer was annealed with the single-stranded phage DNA containing uracils prepared as above in an annealing buffer [20 mM Tris-HCl buffer (pH 7.4) containing 2 mM magnesium chloride and 50 mM sodium chloride] by incubating at 70°C for 10 minutes, followed by cooling down to 30°C at a rate of 1°C per minute. Then, the primer was extended with T4 DNA polymerase in a synthesis buffer [10 mM Tris-HCl buffer (pH 7.4) containing 0.4 mM each deoxynucleoside triphosphate (dTTP, dATP, dCTP, dGTP), 0.75 mM ATP, 3.75 mM magnesium chloride and 1.5 mM dithiothreitol] to synthesize a complementary strand and the ends were ligated with T4 DNA ligase by sequential incubating on ice for 5 minutes, at 25°C for 5 minutes and at 37°C for 90 minutes. The reaction was stopped by freezing at -20°C. The circular double-stranded DNA (heteroduplex) was introduced into E. coli JM105 cells, and they were cultivated to isolate the mutated double-stranded replicative form DNA. The nucleotide sequence of the merged DNA was confirmed by sequencing the single-stranded DNA isolated from the culture medium.

The resultant mutated double-stranded DNA was digested with restriction endonucleases DraI and EcoRI in order to isolate a DNA fragment containing most of the coding region for human NCF polypeptide. The isolated DNA fragment is, hereinafter, referred to as the NCF(DraI-EcoRI)-fragment.

This NCF(DraI-EcoRI)-fragment was ligated by T4 DNA ligase with a chemically synthesized oligodeoxynucleotide adaptor represented by the following formula [F].

5'-AATTGATATGAC 3'-GATACGACAGCT  formula [F]

The resultant ligated DNA fragment is referred to as the NCF(DraI-XhoI)-fragment. Separately, an expression plasmid pEP205 as mentioned in Referential Example 2 was digested with restriction endonucleases DraI and XhoI, and the resulting larger DNA fragment including an ampicillin-
resistance gene and a replication origin (hereinafter referred to as the EP205 vector-DNA fragment) was isolated, and this EP205 vector-DNA fragment was ligated by T4 DNA ligase with the NCF(DraI-XhoI)-fragment previously prepared in order to construct an expression plasmid pHNPI01 for producing human NCF (see Schema 1).

(2) Transformation of *Escherichia coli*

The resulting expression plasmid pHNPI01 was introduced into *E. coli* HB101 by the following manner.

*E. coli* HB101 was inoculated in the LB broth [composition; 1% tryptone, 0.5% yeast extract, 1% sodium chloride (pH 7.5)], and cultivated overnight at 30°C. One milliliter of the resulting culture was inoculated in 100 ml of LB broth and further cultivated at 30°C until the turbidity at 600 nm of the culture reached approximately 0.6. After standing for 30 minutes in ice water, the cells were collected by centrifugation. They were resuspended in 50 ml of 50 mM calcium chloride, and allowed to stand for 60 minutes in ice water. Then, the cells were collected by centrifugation and again suspended in 10 ml of 50 mM calcium chloride containing 20% glycerol.

To this cell suspension, the expression plasmid pHNPI01 was mixed and incubated sequentially in ice water for 20 minutes and at room temperature for 60 minutes. Then, the LB broth was added to the cell suspension, and it was cultivated under shaking at 37°C for 60 minutes. An aliquot of the resulting cell suspension was seeded on the LB agar (1.5% agar) plates containing 25 μg/ml of ampicillin. After cultivation at 37°C overnight, ampicillin-resistant colonies were selected to obtain transformants. One of the transformants was named *E. coli* HB101/pHNPI01 and it was used for production of the human NCF polypeptide.

(3) Production of human NCF polypeptide

*E. coli* HB101/pHNPI01 obtained in section (2) was cultivated in the LB broth overnight at 37°C. The culture was inoculated in 100-fold volumes of the nutrient medium [composition; 1.5% sodium phosphate, dibasic 12-water, 0.3% potassium phosphate, monobasic, 0.1% ammonium chloride, 2 mg/liter vitamin B1, 0.5% casamino acid, 2 mM magnesium sulfate, 0.1 mM calcium chloride, 1% tryptone, 0.5% yeast extract, 1% sodium chloride and 0.4% glycerol] and then, 3-indoleacrylic acid was added to give a final concentration of 20 μg/ml. The cultivation was done at 35°C for 28 hours. The cells were collected by centrifugation, and suspended in 50 mM Tris-HCl buffer (pH 8.0) containing 0.1% lysozyme.
and 30 mM sodium chloride. The suspension was allowed to stand in ice water for 30 minutes. Further, freezing in a dry ice/ethanol bath and thawing at 37°C were repeated to disrupt the cells. After adding 1/50 volume of 10% ethyleneimine polymer, a clarified cell-extract was obtained by centrifugation. To this cell-extract, ammonium sulfate was added to give a 70% saturation, and the formed precipitate was collected by centrifugation. The precipitate was dissolved in distilled water and it was dialyzed against 5 mM phosphate buffered saline (pH 6.5) (hereinafter referred to as PBS). The dialysate was applied onto a column of Sephacryl S-200 (Pharmacia, Sweden), and the fractions containing polypeptides having about 6 to 10kD molecular weight were collected and pooled. The molecular sizes of polypeptides in each eluate were measured by SDS-polyacrylamide gel electrophoretic analysis. The pooled fraction was dialyzed against 20 mM phosphate buffer (pH 6.5) (hereinafter referred to as PB). Then, the dialysate was applied onto a column of CM-Sepharose CL-6B (Pharmacia, Sweden) previously equilibrated with PB. The column was washed with PB, and eluted with a linear gradient of sodium chloride molarity from 0 to 0.5 M in PB. The fractions containing the human NCF polypeptide were collected and pooled, and concentrated by ultrafiltration. Further, the concentrate was subjected to gel filtration on Toyopearl HW-55 column (TOSOH Co., Japan) to obtain the highly purified human NCF polypeptide.

By SDS-polyacrylamide gel electrophoretic analysis, any impurity was not detected in the highly purified human NCF polypeptide preparation.

This human NCF preparation was used for chemical, physicochemical and biological analyses as shown previously.

Referential Example 1
Cloning of cDNA encoding human NCF

The cDNA library was constructed by insertion of cDNA synthesized from normal human monocyte polyadenylated mRNA obtained from the monocyte stimulated with 10 µg/ml lipopolysaccharide for 6 hrs, into the EcoRI site of the phage vector lambda gt10. One half million individual plaques were screened for hybridization with [32P]-labeled two kinds of chemically synthesized oligonucleotide probes represented by the following formulae:

\[ 5'\text{-AAAAGGTTACATATGT} \text{TITIAT-3'} \]

and
In the first screening, 13 putative positive clones were obtained. From these positive clones, one clone (termed r-MDNCF 2-1) was selected in the second screening by using the another probe. The phage DNA in r-MDNCF 2-1 was subcloned into the pUC19 plasmid.

The resulting recombinant plasmid was termed pUC19-1.7-5. The cloned cDNA in a recombinant plasmid pUC19-1.7-5 contains a nucleotide sequence encoding human NCF shown in Table 3.

Table 3.
Nucleotide Sequence of Human NCF Precursor cDNA and Its Deduced Amino Acid Sequence

| MetThrSerLysLeu AlaValAlaLeuLeu 10 | ATGACTTCCAGCTG GCCGTGGCTCTCTTG 30 |
| AlaAlaPheLeuIle SerAlaAlaLeuCys 20 | GCAGCCTTCTGTATT TCTGCAGCTCTGTG 60 |
| GluGlyAlaValLeu ProArgSerAlaLys 30 | GAAGGTGCAGTTTGG CCAAGGAGTGCTAAA 90 |
| GluLeuArgCysGln CysIleLysThrTyr 40 | GAACCTAGATGTCAG TGCATAAAGACATAC 120 |
| SerLysProPheHis ProLysPheIleLys 50 | TCCAAACCTTTCCAC CCCAAATTTTACAAA 150 |
| GluLeuArgValIle GluSerGlyProHis 60 | GAACGTGAGTAGTATT GAGAGTGACCACAC 180 |
| CysAlaAsnThrGlu IleIleValLysLeu 70 | TGCGCACAACACAGAA ATATTGTAAAGCTTT 210 |
| SerAspGlyArgGlu LeuCysLeuAspPro 80 | TCTGATGGAAGAGAC CTCTGCTGGACCC 240 |
| LysGluAsnTrpVal GlnArgValValGlu 90 | AAGGAAAECTGGGTTC CAGAGGTGTGGGAG 270 |
| LysPheLeuLysArg AlaGluAsnSer 99 | AAGTTTTTGAAGAGC GCTGAGAATTCA 297 |
Referential Example 2

Construction of an expression vector pEP205

Plasmid pBR322 was digested with restriction endonucleases *AvaI* and *PvuII*, and the resulting larger DNA fragment (about 3.7 kbp in size) was isolated. After filling-in its cohesive ends to blunt-ends with *E. coli* DNA polymerase I (Klenow fragment) in the presence of dGTP, dATP, dCTP and dTTP, both ends were ligated by T4 DNA ligase to construct a new plasmid vector (designated pBRS6), which was deleted a copy number regulatory gene region located near the replication origin of the plasmid pBR322.

The plasmid vector pBRS6 was digested with restriction endonucleases *EcoRI* and *PstI*, and a smaller DNA fragment containing an upstream region of the ampicillin-resistance gene (about 0.75 kbp in size) was isolated. The resultant DNA fragment is referred to as the Amp(*PstI*-EcoRI)-fragment.

This Amp(*PstI*-EcoRI)-fragment was cloned in a phage vector M13mp18 as mentioned in Example. By using the resultant recombinant phage DNA, one base (T) in the nucleotide sequence of the Amp(*PstI*-EcoRI)-fragment was changed to another base (C) by the site-directed mutagenesis according to the method as mentioned in Example, in order to eliminate the specific nucleotide sequence (AAATTT) recognizable with the restriction endonuclease *DraI*.

The single-stranded phage DNA containing uracils was isolated from the culture medium of *E. coli* C0236 infected with the above recombinant phage DNA. As a mutagenic primer, the oligodeoxuryribonucleotide represented by the following formula [G] was chemically synthesized.

\[ 5'\text{CAGAACTTTGAAAGTGCTC-3'} \]

The phosphorylated primer was annealed with the uracil-containing DNA template. According to the method described in Example section (1), the desired mutated double-stranded DNA was isolated.

The resultant mutated double-stranded DNA was digested with restriction endonucleases *PstI* and *EcoRI* in order to isolate a DNA fragment corresponding to the Amp(*PstI*-EcoRI)-fragment as mentioned above, but not containing the restriction endonuclease *DraI* cleavage recognition sequence [hereinafter referred to as the mutated Amp(*PstI*-EcoRI)-fragment]. The mutated Amp(*PstI*-EcoRI)-fragment was ligated with the larger DNA fragment isolated from the vector pBRS6 by digestion with
restriction endonucleases EcoRI and PstI, in order to construct a new vector which was eliminated the DraI cleavage recognition sequence in the ampicillin resistance gene of the plasmid vector pBRS6. This new vector is designated pBRS601.

Further, this new vector pBRS601 was digested with restriction endonuclease DraI, and the resulting larger DNA fragment was isolated. The larger DNA fragment was ligated with SmaI linker (Takara Shuzo Co., Japan) by T4 DNA ligase to construct a new plasmid vector. This resulting new plasmid vector is a derivative of plasmid pBRS6 and is not containing any recognition sequences for the restriction endonuclease DraI. This new plasmid vector is designated pBRS602.

The nucleotide sequence of the SmaI linker is shown below.

5’-CCCGGG-3’

Furthermore, this new vector pBRS602 was digested with restriction endonuclease AatII and SalI, and the resulting larger DNA fragment was isolated [hereinafter referred to as the pBRS602(AatII-SalI)-fragment].

Separately, an expression plasmid pHIPH383a for producing human interleukin-1α as mentioned in Referential Example 3, was digested with restriction endonucleases AatII and SalI, and the resulting DNA fragment containing E. coli tryptophan promoter sequence and the coding region for human interleukin-1α was isolated. This resulting DNA fragment is referred to as the trp promoter/IL1α-DNA fragment. This trp promoter/IL1α-DNA fragment was ligated with the pBRS602(AatII-SalI)-fragment by T4 DNA ligase to construct a new expression plasmid (see Schema 2).

This new expression plasmid is designated pEP205.

Referential Example 3
Construction of an expression plasmid pHIPH383a

The cloned cDNA encoding human interleukin-1α precursor polypeptide was isolated according to the method described in European Patent Publication No. 0188920.

From the recombinant plasmid pHL4 containing human interleukin-1α precursor cDNA (Furutani, Y., et al., Nucleic Acids Res., 13, 5869, 1985), the cDNA insert was isolated by digestion with restriction endonuclease PstI, and further digested with restriction endonucleases EcoRI and BstNI, to isolate a DNA fragment (411 bp in size) containing a middle portion of the coding region for the mature human interleukin-1α.
The isolated DNA fragment is corresponding to the nucleotide sequence from base No. 398 to No. 808 in Table 5 shown in European Patent Publication No. 0188920.

This DNA fragment was sequentially ligated by T4 DNA ligase with chemically synthesized oligodeoxyribonucleotide adaptors represented by the following formulae [H] and [I]. The resulting DNA fragment is referred to as the SD-IL1-fragment.

The synthetic oligodeoxyribonucleotide adaptor [H] was prepared by sequential ligation of the following five kinds of DNA fragments represented by formulae [a]-[e].

\[
\begin{align*}
5' & -AAGTAGCATGCAAGTTCACTGAC \\ 3' & -TTGACATTCGCTCAAGTGCATT  \\
5' & -GTAAAGAGGAGTTAAA \\ 3' & -TCTCTCTCAATTTATATAC  \\
5' & -TTATGTCATACACCTTTTAG \\ 3' & -AGTAGGGAAAAATCGAAGG  \\
5' & -CTTCTGAGGAATGTGAAATACACATTTA \\ 3' & -ACTCGTTACACTTTATGTTGAAATACTC \\
\end{align*}
\]

and

\[
\begin{align*}
5' & -TGAGGATCATCAAATACG \\ 3' & -CTAGTAGTTATAGCCTTA  \\
\end{align*}
\]

A base sequence of the formula [I] was as follows:

\[
\begin{align*}
5' & -AGGCCTGATGACTCGA \\ 3' & -CGCAGTACTGAGCTCTAG  \\
\end{align*}
\]

Separately, an expression vector pEP302 (Furutani, Y., et al., Nucleic Acids Res., 13, 5869, 1985) was digested with restriction endonucleases HpaI and BamHI, and the resulting larger DNA fragment containing E. coli tryptophan promoter sequence and an ampicillin resistance gene, was isolated (hereinafter referred to as the EP302 vector-DNA fragment).

The EP302 vector-DNA fragment was ligated by T4 DNA ligase with the SD-IL1-fragment prepared as above to construct an expression plasmid pHIPH383a for producing the mature human interleukin-1α polypeptide (see Schema 3).
INDUSTRIAL APPLICABILITY

Neutrophils infiltrate into the foci of bacterial infection, inflammation site and around malignant tumor cells, and play important role in homeostatic defence mechanism. Neutrophils are attracted and further activated by NCF or by the combination of NCF with interleukin-1. For example, the use of the combination of NCF and interleukin-1 are expected as a drug for treatment of certain bacterial infectious diseases or cancers.
CLAIMS

1. A process for producing a human neutrophil chemotactic factor polypeptide by cultivating cells transformed with an expression vector in which a DNA encoding the said polypeptide is inserted.

2. A process according to claim 1, wherein the human neutrophil chemotactic factor polypeptide is a polypeptide consisting of an amino acid sequence represented by formula [I].

   SerAlaLysGluLeuArgCysGlnCysIleLysThr
   TyrSerLysProPheHisProLysPheIleLysGlu
   LeuArgValIleGluSerGlyProHisCysAlaAsn
   ThrGluIleIleValLysLeuSerAspGlyArgGlu
   LeuCysLeuAspProLysGluAsnTrpValGlnArg
   ValValGluLysPheLeuLysArgAlaGluAsnSer

   formula [I]

3. A process according to claim 1, wherein the DNA encoding a human neutrophil chemotactic factor polypeptide is the DNA comprising a base sequence represented by formula [A], or its degenerative sequence.

   5'-AGTGCTAAAGAACCTTAGATGTCAGTCATAAAGACA
   TACTCCAAACCTTTCCACCCCCAATTATCTCAAGAA
   CTGAGAGTGATTGAGAGTGGACCACACTGCGCAAC
   ACAGAAATTATTGTAAAGCCTTTCTGATGGAAAGAGAG
   CTCTGTCTGGACCCCAAGGAAAAACTGGGTGCAGAGG
   GTTGAGGAGAAGTTTGGAGAGGCTGAGAAATTC–3'

   formula [A]

4. A process according to claim 1, wherein the transformed cell is Escherichia coli.

5. A process according to claim 1 characterized by using a recombinant plasmid pHNPI01 as the expression vector.

6. A DNA having a base sequence represented by formula [A] shown in claim 3 and a base sequence represented by formula [B] in the upstream of the base sequence represented by the said formula [A].

   5'-XGGAGGTTTATG-3'

   formula [B]

   wherein X means (A)x, x being 1 to 5, and Y means (A)y(T)z, y being 0 to 3 and z being 0 or 1.

7. A DNA having a base sequence represented by formula [A] shown in claim 3 and a base sequence represented in formula [C] in the upstream of the base sequence represented by the said formula [A].
8. A DNA claimed in claim 6 or 7, which is further connected with a DNA having a promoter activity to control the transcription in *Escherichia coli* in the upstream of the DNA claimed in claim 6 or 7.

9. A DNA according to claim 8, wherein the promoter is a promoter of tryptophan operon.

10. A purification method of a human neutrophil chemotactic factor polypeptide produced by the methods claimed in any one of claims 1 to 5, which is characterized by the combination of following steps in the treatment of the said polypeptide; the destruction of the transformants, treatment for removing nucleic acids, salting-out, anion and/or cation exchange chromatography, ultrafiltration, and gel filtration.
Schema 1

Construction of an expression plasmid pHNP101

Human NCFcDNA clone: pUC19-1.7-5
   ↓ digested with PstI and EcoRI
PstI-EcoRI-DNA fragment
   ↓ cloned into M13mp18 vector
Recombinant phage
   ↓ infected E. coli CJ236
   ↓ cultivated in uridine containing medium
Uracil containing DNA template
   ↓ annealed a mutagenic 33-mer primer [E]

```
AAATT
T
T
G
GGTTTGGCAAGG AGTGGCTAAAG
TCCACGTCAAAACGGTTC-TCACGATTTC
```

Uracil containing DNA template

   ↓ extended the primer with T4 DNA polymerase
   ↓ ligated the ends with T4 DNA ligase
Heteroduplex
   ↓ transformed E. coli JM105
Mutated Double-Stranded DNA
   ↓ digested with DraI and EcoRI
NCF(DraI-EcoRI)-fragment

--- to be continued ---
Schema 1 (continued)

NCF(DraI-EcoRI)-fragment

Expression Vector pEP205
digested with DraI and XhoI

EP205 Vector-DNA fragment

Expression Plasmid pHPN101
Schema 2

Construction of an expression vector pEP205

Plasmid pBR322
  ↓
Larger DNA (3.7kbp)-fragment
  ↓
  filled in the cohesive ends to blunt-ends with DNA polymerase I (Klenow fragment)
  ↓
  ligated with T4 DNA ligase
  ↓
Plasmid Vector pBRS6
  ↓
digested with EcoRI and PstI
  ↓
Amp(PstI-EcoRI)-fragment
  ↓
  cloned into M13mp18 vector
  ↓
  infected E. coli JM105
  ↓
Recombinant phage
  ↓
  infected E. coli CJ236
  ↓
  cultivated in uridine containing medium
  ↓
Uracil containing DNA template
  ↓
annealed a mutagenic oligonucleotide primer [G]
  ↓

CAGAACTTAAAGTGCTC

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Uracil containing DNA template
  ↓
  extended the primer with T4 DNA polymerase
  ↓
  ligated the ends with T4 DNA ligase
  ↓
Heteroduplex

- to be continued -
Schema 2 (continued)

Heteroduplex
  transformed E. coli JM105
Mutated Double-Stranded DNA
  digested with PstI and EcoRI
Mutated Amp(PstI-EcoRI) fragment
  ligated with T4 DNA ligase
Plasmid Vector pBRS601
  digested with DraI
Larger DNA fragment
  [SmaI linker]
  5'-CGCGGG-3'
  3'-GGGGCC-5'
  ligated with T4 DNA ligase
Plasmid Vector pBRS602
  digested with AatII and SallI
pBRS602(AatII-SallI)-fragment
Expression Plasmid
  pHIPH3B3a
  digested with AatII and SallI
  trp promoter/IL1α-DNA fragment
  ligated with T4 DNA ligase
Expression Vector pEP205
Schema 3

Construction of an expression plasmid pHIPH383a

Human IL-1αcDNA: pHL4
  ↓
  digested with PstI
  ↓
cDNA insert
  ↓
digested with EcoRI
  and BstNI
  ↓
EcoRI-BstNI-fragment
  ↓
ligated with T4 DNA ligase
  ↓
Expression Vector pEP302
  ↓
digested with HpaI and BamHI
  ↓
EP302 Vector-DNA fragment
  ↓
ligated with T4 DNA ligase
  ↓
Expression Plasmid pHIPH383a
INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC: C 12 N 15/00, C 12 P 21/02, C 07 H 21/04

II. FIELDS SEARCHED

Minimum Documentation Searched 

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Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *

III. DOCUMENTS CONSIDERED TO BE RELEVANT *

<table>
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<th>Citation of Document, ** with indication, where appropriate, of the relevant passages ***</th>
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<td>Proc. Natl. Acad. Sci. USA, vol. 84, December 1987, T. Yoshimura et al.: &quot;Purification of a human monocyte-derived neutrophil chemotactic factor that has peptide sequence similarity to other host defense cytokines&quot;, pages 9233-9237, see the whole article cited in the application</td>
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* Special categories of cited documents: 10
  "A" document defining the general state of the art which is not considered to be of particular relevance
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  "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
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*** "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

**** "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"A" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search
26th July 1989

Date of Mailing of this International Search Report
22. 08. 89

International Searching Authority
EUROPEAN PATENT OFFICE

Signature of Authorized Officer

Form PCT/ISA/210 (second sheet) (January 1985)
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