

HUMAN TUMOR NECROSIS FACTOR AND DNA THEREFOR

This invention relates to a deoxyribonucleic acid (hereinafter referred to as "DNA") coding for a polypeptide having human physiological activity. This invention also relates to a replicable recombinant DNA containing the DNA, a microorganism or cell transformed with the replicable recombinant DNA, a human physiologically active polypeptide obtained by expressing the DNA, a substantially pure human physiologically active polypeptide having the amino acid sequence described herein, pharmaceutical compositions containing the physiologically active polypeptides as the effective ingredient, and a process for producing the human physiologically active polypeptide. More particularly, the present invention is concerned with a DNA coding for human Tumor Necrosis Factor (TNF), human TNF having an amino acid sequence deduced from the base sequence of the DNA, a process for producing human TNF from the DNA utilizing recombinant DNA technology, and the use of the product obtained by the process.

In the present specification, amino acids and peptides are represented using abbreviations, as indicated below, approved by IUPAC-IUB Commission on Biochemical Nomenclature (CBN). With respect to amino acids and the like having isomers, those represented by the following abbreviations are of the L-configuration unless otherwise specified.

Gln: glutamine residue
Asp: aspartic acid residue
Pro: proline residue
Tyr: tyrosine residue
Val: valine residue

Lys: lysine residue

Glu: glutamic acid residue

Ala: alanine residue

Asn: asparagine residue

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Leu: leucine residue

Phe: phenylalanine residue

Gly: glycine residue

His: histidine residue

Ser: serine residue

10

Thr: threonine residue

Ile: isoleucine residue

Trp: tryptophan residue

Arg: arginine residue

Met: methionine residue

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Cys: cysteine residue

Polydeoxyribonucleotides and oligodeoxyribonucleotides are represented by sequences of deoxynucleotide residues which are abbreviated as follows:

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A: 2'-deoxyadenylic acid residue

C: 2'-deoxycytidylic acid residue

G: 2'-deoxyguanylic acid residue

T: thymidylic acid residue

Unless otherwise specified, the left end of the sequence of deoxynucleotides is the 5' end.

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There are known various substances having a capacity for stimulating the reticuloendothelial system, for example,

physiologically active substances having antitumor activity which are induced by various Gram-positive bacteria and endotoxins. Specifically, Carswell et al discovered that the serum from CD-1 Swiss mice infected with bacillus Calmette-Guérin (BCG), and after two weeks, followed by intravenous injection of endotoxin has cytotoxic activity against cultured L cells and also discovered a phenomenon that it induces hemorrhagic necrosis of transplanted Meth A sarcoma in the (BALB/c x C57BL/6)F₁ mouse. They gave the name of TNF (Tumor Necrosis Factor) to the active substance in the serum [Proc. Nat. Acad. Sci. USA, Vol. 72 (No. 9), pp. 3666-3670 (1975)]. Thereafter, Ruff et al reported that the rabbit TNF prepared according to the above-mentioned method proposed by Carswell et al was purified about 2,000-fold over the serum (J. Immunol., Vol. 125 (No. 4), pp. 1671-1677 (1980)]. Further, Matthews et al reported that the rabbit TNF was purified about 1,000-fold over the serum [Br. J. Cancer, Vol. 42, pp. 416-422 (1980)]. However, in Ruff et al and Matthews et al, the tumor necrosis effect with respect to the purified TNF is not confirmed in animal experiments.

Japanese Patent Application Laid-Open Specification No. 57-140725 (1982) discloses a process for isolating and purifying a proteinaceous physiologically active substance having anti-tumor activity, which is induced by administering to a mammal such as mouse, rabbit or guinea pig at least one substance having a capacity for stimulating the reticuloendothelial

system and then injecting endotoxin from a Gram-negative bacterium into the mammal, or by adding endotoxin from a Gram-negative bacterium to a tissue culture containing activated macrophages from a mammal. In this Japanese Patent Application Laid-Open Specification, there are also disclosed the molecular weight and isoelectric point of the purified proteinaceous physiologically active substance (molecular weight, $39,000 \pm 5,000$ as measured by gel filtration and SDS-polyacrylamide gel electrophoresis; isoelectric point, $\text{pH } 3.9 \pm 0.3$ as measured by isoelectric focusing) but not any detailed structure of the proteinaceous physiologically active substance.

Meanwhile, Matthews reported that there is obtained a substance having cytotoxic activity against L cells by a process in which BCG is injected into a rabbit and mononuclear phagocytes from various tissues of the rabbit are obtained two weeks after the injection, followed by addition of endotoxin to the cell culture of the mononuclear phagocytes [Br. J. Cancer, Vol. 44 (3), pp. 418-424 (1981)]. However, in his report, the detailed structure of the obtained substance is not disclosed and, further, there is no evidence showing that the obtained substance is identical with TNF found in the serum.

Further, there are a number of printed publications reporting that factors having TNF-like bioactivity or a bioactivity similar to that of TNF. For example, Reed et al found such a factor in macrophages and the like present in human

peripheral blood [J. Immunology, Vol. 115, p. 395 (1975)],
Matthews et al in leukemia cells derived from human peripheral
blood monocytes or from a patient suffering from myelogenous
monocytic leukemia [Immunology, Vol. 44, p. 135 (1981)], D.

5 Barbara in human B cells transformed with Epstein-barr virus
[Proc. Nat. Acad. Sci. USA, Vol. 80, p. 5397 (1983)], and B.

Bharat in lymphoblastoid 1788 cell line. The above-mentioned factors are
also disclosed in Japanese Patent Application Laid-Open
Specifications Nos. 58-15921 (1983), 58-21621 (1983),

10 58-107197 (1983) and 58-225024 (1983), and British Patent
Application Laid-Open Specifications Nos. 2,106,117 and
2,117,385. However, the cells or cell lines capable of
efficiently producing such factors have not yet been found.

Further, with respect to such factors, there are many matters to
15 be elucidated such as their structures and properties.

Ito [Japanese Patent Application No. 58-251817 (1983)]
made studies on properties and structure of rabbit TNF and
rabbit-TNF producing cells. As a result, he obtained cells
capable of producing a substance having cytotoxic activity
20 against L cells by administering a substance having a capacity
for stimulating the reticuloendothelial system to a rabbit,
followed by injection of endotoxin derived from a bacterium
into the rabbit, and then obtained such a substance using those
cells. He also affirmed that the molecular weight and
25 immunological properties of the substance having cytotoxic
activity against L cells obtained using the above obtained

cells are in agreement with those of TNF obtained from rabbit serum. Meanwhile, with the progress of genetic manipulation techniques, it became possible to determine the structure of a protein so long as a DNA coding for the protein is obtained in isolated form. This is so because the structure of the isolated DNA can be determined and, then, the structure of the protein can be deduced from the structure of the DNA. Further, it became possible to produce a protein from a DNA coding for the protein utilizing a microorganism or cell culture. Ito applied the above-mentioned genetic manipulation techniques to the cells capable of producing a substance having cytotoxic activity against L cells. As a result, he succeeded in isolating a DNA coding for rabbit TNF, determining the structures of the DNA and rabbit TNF, and producing rabbit TNF using the DNA.

As is apparent from the foregoing, Ito has made a great success in producing rabbit TNF. However, it should be noted that the administration of TNF to an animal which is not the origin of the TNF has a danger that anaphylactic shock might be caused. This is so because TNF is a polypeptide and, hence, when TNF is administered to an animal which is not the origin of the TNF, the TNF functions as an antigen to produce an antibody. For this reason, when TNF is intended to be administered to a human body, the use of TNF derived from human beings is highly preferable. However, even the structure of human TNF has not been successfully elucidated. Therefore,

the determination of the structure of the DNA coding for human TNF has been strongly needed.

5 The present inventors have made extensive and intensive studies on the structure of the DNA coding for human TNF. As a result, the present inventors have surprisingly found that a human polypeptide gene and a rabbit TNF gene can be cloned by the use of rabbit cDNA as a probe, that the DNA coding for human polypeptide can be skillfully isolated and the structure thereof can be
10 determined by comparison between rabbit TNF gene, human polypeptide gene and rabbit cDNA with respect to the homology of their base sequences, that the structure of pure DNA coding for human polypeptide can be skillfully determined and such a pure DNA can be obtained, and that
15 a human polypeptide produced using the DNA coding for the human polypeptide has a cytotoxic activity against L cells.

The present invention has been made based on such novel findings.

20 The features and advantages of the present invention will be apparent from the following detailed description and appended claims taken in connection with the accompanying drawings in which:

Fig. 1 illustrates the restriction maps of plasmid inserts each containing a DNA coding for a conventional
25 rabbit physiologically active polypeptide;

Fig. 2 illustrates the flow-sheet of the method for the preparation of a recombinant DNA (pTNF-lac-1) coding for the conventional rabbit physiologically active polypeptide;

Fig. 3 illustrates the flow-sheet of the method for the preparation of another recombinant DNA (pTNF-lacUV5-1) coding for the conventional rabbit physiologically active polypeptide;

5 Fig. 4 illustrates the restriction map of the portion of a plasmid containing a gene for human physiologically active polypeptide of the present invention;

Fig. 5 illustrates the restriction map of the portion of a plasmid containing a gene for a conventional rabbit
10 physiologically active polypeptide;

Fig. 6 illustrates the flow-sheet of the method for the preparation of a recombinant DNA (pHTNF-lacUV5-1) coding for a human physiologically active polypeptide of the present invention; and

15 Fig. 7 illustrates the flow-sheet of the method for the preparation of another recombinant DNA (pHTNF-lacUV5-2) coding for the human physiologically active polypeptide of the present invention.

Essentially, according to the present invention, there is provided a human physiologically active polypeptide having an amino acid sequence represented by the following formula (I):

5 Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His Val Val
Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg
Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln
Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val
Leu Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr
10 His Thr Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn
Leu Leu Ser Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu
Gly Ala Glu Ala Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly
Val Phe Gln Leu Glu Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn
Arg Pro Asp Tyr Leu Asp Phe Ala Glu Ser Gly Gln Val Tyr Phe
15 Gly Ile Ile Ala Leu

wherein Gln stands for a glutamine residue,
Asp an aspartic acid residue, Pro a proline
residue, Tyr a tyrosine residue, Val
a valine residue, Lys a lysine residue,
20 Glu a glutamic acid residue, Ala an alanine
residue, Asn an asparagine residue, Leu
a leucine residue, Phe a phenylalanine
residue, Gly a glycine residue, His
a histidine residue, Ser a serine residue,
25 Thr a threonine residue, Ile an isoleucine
residue, Trp a tryptophan residue, Arg
an arginine residue, Met a methionine
residue, and Cys a cysteine residue.

The present invention also includes a polypeptide having an amino acid methionine attached to the N-terminus of the above-mentioned amino acid sequence and an intermediate having a partial or entire signal peptide for human TNF attached to the N-terminus of the above-mentioned amino acid sequence. It is possible to change part of the structure of a DNA coding for a polypeptide by natural or artificial mutation without significant change of the activity of the polypeptide. The present invention includes a polypeptide having a structure corresponding to homologous variant(s) of the polypeptide having the above-mentioned amino acid sequence and also having human tumor necrosis activity. All such physiologically active polypeptides are hereinafter referred to as "human TNF".

In another aspect of the present invention, there is provided a deoxyribonucleic acid comprising a base sequence coding for a human physiologically active polypeptide,

said human physiologically active polypeptide having an amino acid sequence represented by the following formula (I):

Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His Val Val
Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg
Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln
Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val
Leu Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr
His Thr Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn

Leu Leu Ser Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu
 Gly Ala Glu Ala Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly
 Val Phe Gln Leu Glu Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn
 Arg Pro Asp Tyr Leu Asp Phe Ala Glu Ser Gly Gln Val Tyr Phe
 5 Gly Ile Ile Ala Leu

wherein Gln stands for a glutamine residue,
 Asp an aspartic acid residue, Pro a proline
 residue, Tyr a tyrosine residue, Val a valine
 10 residue, Lys a lysine residue, Glu a glutamic
 acid residue, Ala an alanine residue, Asn an
 asparagine residue, Leu a leucine residue,
 Phe a phenylalanine residue, Gly a glycine
 residue, His a histidine residue, Ser
 a serine residue, Thr a threonine residue,
 15 Ile an isoleucine residue, Trp a tryptophan
 residue, Arg an arginine residue, Met
 a methionine residue, and Cys a cysteine
 residue.

In further aspect of the present invention, there is
 20 provided a deoxyribonucleic acid comprising at least one
 base sequence selected from the group consisting of a base
 sequence represented by the following formula (II) and
 a complementary base sequence to said base sequence:

TCA TCT TCT CGA ACC CCG AGT GAC AAG CCT GTA GCC CAT GTT GTA
 GCA AAC CCT CAA GCT GAG GGG CAG CTC CAG TGG CTG AAC CGC CGG
 GCC AAT GCC CTC CTG GCC AAT GGC GTG GAG CTG AGA GAT AAC CAG
 CTG GTG GTG CCA TCA GAG GGC CTG TAC CTC ATC TAC TCC CAG GTC
 5 CTC TTC AAG GGC CAA GGC TGC CCC TCC ACC CAT GTG CTC CTC ACC
 CAC ACC ATC AGC CGC ATC GCC GTC TCC TAC CAG ACC AAG GTC AAC
 CTC CTC TCT GCC ATC AAG AGC CCC TGC CAG AGG GAG ACC CCA GAG
 GGG GCT GAG GCC AAG CCC TGG TAT GAG CCC ATC TAT CTG GGA GGG
 GTC TTC CAG CTG GAG AAG GGT GAC CGA CTC AGC GCT GAG ATC AAT
 10 CGG CCC GAC TAT CTC GAC TTT GCC GAG TCT GGG CAG GTC TAC TTT
 GGG ATC ATT GCC CTG

wherein A stands for a deoxyadenylic acid
 residue, G a deoxyguanylic acid residue, C
 a deoxycytidylic acid residue and T
 15 thymidylic acid residue and wherein
 the left end and right end
 of the formula (II) represent
 5'-hydroxyl group side and 3'-hydroxyl group
 side, respectively.

20 The DNA of the present invention includes a DNA
 comprising a base sequence having ATG (A, T and G are as
 mentioned above) attached to the 5'-end of the above-
 mentioned base sequence in order to produce mature human
 TNF by means of culture of a microorganism or cell.

25 The DNA of the present invention also includes a DNA having
 a 5'-flanking DNA coding for a partial or entire signal
 peptide of human TNF.

The structure of a DNA and the structure of the polypeptide deduced therefrom may be partially changed by natural or artificial mutation without causing the main activity of the polypeptide to be changed. Hence, the DNA of the present invention may alternatively have a base sequence that codes for a polypeptide with a structure corresponding to that of a homologous variant of any of the aforementioned polypeptides.

In accordance with degeneracy of genetic code, it is possible to substitute at least one base of the base sequence of a gene by another kind of base without causing the amino acid sequence of the polypeptide produced from the gene to be changed. Hence, the DNA of the present invention may also have any base sequence that has been changed by substitution in accordance with degeneracy of genetic code. In this instance, the amino acid sequence deduced from the base sequence obtained by the above-mentioned substitution is identical with the amino acid sequence of the formula (I) as defined before.

In a further aspect of the present invention, there is provided a replicable recombinant DNA which comprises the above-mentioned deoxyribonucleic acid according to the present invention and a replicable expression vehicle. The recombinant DNA is capable, in a transformed microorganism or cell culture, of expressing a polypeptide comprising the amino acid sequence of human TNF. As the suitable vehicle, there may be mentioned, for example, pHTNF-lacUV5-1 and

pHTNF-lacUV5-2 expression vehicles.

Further, the present invention is directed to a microorganism or cell culture transformed with a recombinant DNA capable of expressing a polypeptide comprising the amino acid sequence of human TNF. Examples of such microorganism or cell culture include Escherichia coli, Bacillus subtilis, yeasts and higher animal cells.

In an even further aspect of the present invention, there is provided a method for producing the human physiologically active polypeptide of the present invention which comprises:

(a) ligating the deoxyribonucleic acid of the formula (II) as defined above to a replicable expression vehicle to obtain a replicable recombinant DNA comprising said deoxyribonucleic acid and said replicable expression vehicle;

(b) transforming cells of a microorganism or cell culture with said replicable recombinant DNA to form transformants;

(c) selecting said transformants from parent cells of the microorganism or cell culture;

(d) incubating said transformants, causing said transformants to express said deoxyribonucleic acid and produce a human physiologically active polypeptide; and

(e) isolating said human physiologically active polypeptide from the incubated transformants.

According to the method of the present invention, the above-described polydeoxyribonucleic acid of the present

invention is ligated to a replicable expression vehicle as a vector to obtain a replicable recombinant DNA containing the above-mentioned polydeoxyribonucleic acid. A micro-organism or cell culture is transformed with the thus obtained replicable recombinant DNA to obtain a transformed micro-organism or cell culture containing the recombinant DNA. The thus obtained transformant is isolated from the parent microorganism or cell culture by means of a phenotypical trait imparted with the DNA. The thus obtained transformed microorganism or cell culture is grown to effect expression of the genetic information that is encoded on the above-mentioned deoxyribonucleic acid, thereby producing a physiologically active polypeptide according to the present invention.

Furthermore, the present invention is directed to a human TNF, in mature form, secreted from host cells as a direct expression product. As a process for obtaining such a mature human TNF, there may be mentioned, for example, a process comprising constructing a DNA sequence so as to bond an amino acid sequence, known as a signal peptide, composed of from 15 to 40 amino acids that is derived from a microorganism or higher animal to the terminus of the amino acid sequence of the mature TNF.

The human TNF may be obtained as follows:

1. A bacteriophage λ /rabbit genomic library and a bacteriophage λ /human genomic library prepared by Prof. T. Maniatis, Department of Biochemistry and

Molecular Biology, Harvard University, 7 Divinity Avenue, Cambridge, Massachusetts 02138, U.S.A. are used. These materials may be prepared according to the following procedures [see Cell, 15, p.687 (1978)]:

- 5 (1) rabbit or human tissues, for example rabbit or human pancreas tissue, are reduced to frozen powder and treated to digest RNA and protein materials and provide, on precipitation, high molecular weight rabbit or human DNA;
- 10 (2) the high molecular weight DNA is partially digested for random cutting with respect to gene locus;
- (3) the resultant DNA fragments are size-fractionated giving from 15 to 20 kilo base pair (kb) fragments;
- (4) the resultant fragments of Step 3 are cloned using
15 a λ Charon 4A phage vector; and
- (5) the resultant vectors are packaged in vitro to infectious phage particles containing rDNA to obtain the above-mentioned rabbit or human genomic library.
- 20 2. The rabbit TNF cDNA obtained in Reference Example 3 is ^{32}P -labelled by P.W.J. Rigby et al's nick translation method [see J. Mol. Biol. 113, p.237 (1977)].
3. Each of the bacteriophage λ /rabbit genomic library and bacteriophage λ /human genomic library is plated to
25 virtual confluence on a lawn of bacteria and screened for hybridization with the ^{32}P -labelled rabbit TNF cDNA.

4. From the appropriate clones, the corresponding DNA is isolated, restriction mapped and analyzed by Southern hybridization [see E.M. Southern, J. Mol. Biol., 98, p.503 (1975)].

5 Restriction fragments containing rabbit or human TNF genes are subcloned into plasmid vectors and then sequenced.

10 5. The base sequence of the rabbit TNF cDNA is compared with that of the rabbit TNF gene to determine the exons (certain sequences of bases which code for the amino acid sequence of rabbit TNF) and introns (certain sequences of bases which do not code for the amino acid sequence of rabbit TNF) of the rabbit TNF gene.

15 6. Thereafter, the base sequence of the human TNF gene is compared with that of the rabbit TNF gene to determine the exons and introns of the human TNF gene.

20 7. The amino acid sequence of rabbit TNF that has been deduced from the base sequence obtained by deleting the introns of the rabbit TNF gene and combining the exons thereof is affirmed to be in agreement with that deduced from the base sequence of the rabbit TNF cDNA.

25 8. Next, the amino acid sequence of human TNF is deduced from the base sequence of the DNA coding for human TNF obtained by deleting the introns of the human TNF gene and combining the exons thereof. The amino acid sequence of the human TNF is affirmed to be partially in agreement with that of the rabbit TNF.

9. Then, the DNA coding for human TNF is tailored in vitro for insertion into an appropriate expression vehicle to form recombinant DNA containing the coding DNA. The recombinant DNA is used to transform an appropriate host cell which is, in turn, permitted to grow in a culture and to express the desired human TNF.

10. The human TNF thus produced has 155 amino acid residues in its mature form, beginning with serine. When it has a signal peptide in its presequence, the signal peptide is very hydrophobic in character.

The foregoing discloses the procedures for obtaining the human TNF gene, the base sequence of the DNA coding for human TNF and the process for producing the human TNF by the use of the DNA. However, it should be understood that the foregoing disclosure is not intended to limit the invention and that obvious changes may be made by those skilled in the art without changing the essential characteristics and the basic concept of the invention.

Due to the variable use frequency of a codon (genetic code) corresponding to each amino acid and for other reasons, a partial or entire portion of the base sequence of the DNA coding for human TNF may be substituted by an organic chemically synthesized artificial DNA without causing the amino acid sequence of the polypeptide obtained therefrom to be changed.

Presumably, the human TNF may be intracellularly produced in immature form as a prepeptide or prepropeptide,

which may be processed via an intermediate form to a mature TNF in the processing stage. The immature form of human TNF may be deduced from the base sequence of the human TNF gene. The TNF DNA comprising a DNA encoding the TNF in
5 immature or intermediate form may also be recombined with a natural or artificially synthesized DNA.

One application of this technique may be attained by inserting the methionine codon (ATG) in the 5'-end and inserting at least one stop codon selected from TAA, TAG and TGA in the
10 3'-end of the mature or intermediate or immature TNF DNA. Due to the presence of the methionine codon, the mature or intermediate or immature TNF may be produced on the mRNA synthesized with the aid of an appropriate promoter. However, the methionine residue attached to the N-terminus of the TNF is cleaved or
15 not cleaved according to the kind of the host cell employed. The purpose of inserting the stop codon is to stop translation of the mRNA transcribed from the TNF DNA at an appropriate position (C-terminus of polypeptide of the formula I).

Another application of this technique may be attained
20 by adding to the DNA a highly hydrophobic base sequence known as a "signal sequence". By this addition, it may become feasible to secrete the TNF to outside the host cell or, in the case of a gram-negative bacteria, into the space known as "periplasm".

25 When a vector in which a start codon is incorporated is employed, a fused peptide may be produced which consists of the human TNF and a peptide attributed to the vector. In this case, the fused peptide may be cleaved chemically or enzymatically. Alternatively, the fused peptide, if

the main activity of the human TNF is not adversely affected, may be used as it is.

5 The human TNF DNA may be connected, at its region upstream of the 5'-end, to the gene sequence of a promoter thereby to obtain a TNF DNA-promoter sequence which does not hinder its replication and does not cause translation of the resultant RNA to be adversely affected. The thus obtained TNF DNA-promoter sequence may be combined with a vector which is replicable in a bacterium or higher organism
10 cell to obtain a recombinant gene. The thus obtained recombinant gene may be used to transform a bacterium or higher organism cell used as a host. The thus obtained transformant may be cultured to effect expression of the TNF gene in order to produce the human TNF.

15 When Escherichia coli is used as the above-mentioned host, there may be mentioned, as the suitable host, various mutant strains of E. coli K-12, such as HB101(ATCC 33694), C600K(ATCC33955), D1210, RRI(ATCC31343), MC1061, LE392 (ATCC33572), JM101 (ATCC33876), JM83 and χ 1776 (ATCC31244).

20 When the E. coli host is employed, there may be mentioned, as the suitable vector, plasmids such as pBR322, pBR325, pBR327, pUC8, pUC9, pMB9(ATCC37019), pJB8(ATCC37074) and pKC7(ATCC37084), λ phages such as λ gt, λ B and Charon 4A, and M13 phage. To have TNF produced in the E. coli
25 cell, a promoter selected from the promoters of the E. coli and phage genes may be employed. Examples of the suitable promoter include the genes for lactose degradation enzyme

(LAC), UV5 mutant thereof, penicillinase (BLA) and tryptophan synthetase (TRP), λ phage P_L promoter and tac promoter which is a fused promoter of tryptophan synthetase and lactose degradation enzyme.

5 When Bacillus subtilis is used as the host, there may be mentioned, as the suitable host, BD170 strain (ATCC33608), BR151 strain (ATCC33677) and MI112 strain (ATCC33712). When the Bacillus subtilis host is employed, there may be mentioned, as the suitable vector, plasmids pC194(ATCC37034),
10 pUB110(ATCC37015), pSA2100(ATCC37014) and pE194. Further, when the Bacillus subtilis host is employed, there may be mentioned, as the suitable promoter, the genes for chloramphenicol acetylation enzyme (CAT), penicillinase and anti-erythromycin.

15 When a yeast is used as the host, there may be mentioned, as the suitable host, strains of Saccharomyces cerevisiae such as RH218(ATCC44076), SHY1(ATCC44769), SHY3(ATCC44771), D131A, 483 and 830. When the yeast host is employed, there may be mentioned, as the suitable vector, plasmids such as
20 YEpl3(ATCC37115), YEpl6, YRp7 and YIp5. Further, when the yeast host is employed, there may be mentioned, as the suitable promoter, the genes for acid phosphatase, alcohol dehydrogenase (ADHI), tryptophan synthetase (TRP), phosphoglycerate kinase (PGK), cytochrome B(COB) and actin.

25 When a higher organism cell culture is used as the host, there may be mentioned, as the suitable host, the cell cultures of monkey kidney, COS and mouse C127(ATCC 1616). When the higher organism cell culture host is employed, there may be mentioned, as the suitable vector, SV40.

The novel human physiologically active polypeptide of the present invention induces necrosis of tumors with no toxic effect upon the normal tissues of the living body.

5 The active polypeptide of the present invention may be formulated according to known methods to prepare pharmaceutical compositions which are useful for the inhibition of cell proliferation, e.g. malignant tumor cells proliferation. The active polypeptide may be combined in admixture
10 with a pharmaceutically acceptable carrier vehicle.

An effective amount of the active polypeptide of the present invention may be mixed with a suitable amount of vehicle in order to prepare pharmaceutically acceptable compositions suitable for effective administration to the recipient.

15 The physiologically active polypeptide of the present invention may be administered, to subjects requiring anti-tumor or antiviral treatment, as an injection, eye drop, nasal drop, inhalant, external preparation, oral administration, rectal administration or vaginal tablet. The daily
20 dose of the polypeptide of the present invention per adult may be generally in the range of from 50 to 100,000,000 units. It may be preferably in the range of from 50 to 500,000 units in the case of local administration, from 1,000 to 1,000,000 units in the case of general injection.
25 such as intravenous injection and intramuscular injection, and from 10,000 to 100,000,000 units in the case of oral administration. The daily dose may be increased or

decreased according to the direction for use and symptom of recipient.

The terminology "1 unit" used above means a quantity of the physiologically active polypeptide of the present invention by which 50 % of 1×10^5 cells/ml of L-M cells (American Type Culture Collection CCL 1.2) are killed. The above-mentioned quantity is measured as follows. As culture vessels, there are employed 96-well microtiter plates produced by Flow Laboratories, Inc. (U.S.A.), and L-M cells are cultured in Eagle's minimum essential medium containing 1 v/v % of bovine fetal serum [the composition of this medium is described, for example, in Tissue Culture, edited by Junnosuke Nakai et al, Asakura Shoten, Japan (1967)]. A sample (0.1 ml) serially diluted with the medium and the L-M cell suspension (0.1 ml, 1×10^5 cells/ml) are mixed into each well of the plates and the plates are incubated at 37°C for 48 hours in air containing 5 % carbon dioxide. At the end of the culture period, 20 μ l of glutaraldehyde is added to fix the cells. After fixation, the plates are washed with distilled water and allowed to dry, and 0.05 % methylene blue (0.1 ml) is added to stain the viable cells. The plates are thoroughly washed with distilled water to remove excess dye and allowed to dry. 0.36 N Hydrochloric acid is added to each well to extract the dye from stained cells. Absorbance of each well at 665 nm is measured with Titertek Multiskan produced by Flow Laboratories, Inc. The absorbance is proportional to the number of viable cells.

The above-mentioned quantity of the physiologically active polypeptide of the present invention by which 50% of 1×10^5 cells/ml of L-M are killed is obtained by plotting the dilution versus the absorbance on a graph.

5 The physiologically active polypeptide of the present invention may be suitably administered parenterally.

In the parenteral preparation, there may be incorporated as an additive, a thickener such as sucrose, glycerine, methylcellulose, carboxymethylcellulose or
10 the like and/or a pH adjusting agent such as various inorganic salts. The polypeptide may also be suitably administered in the form of a tablet. In the tablet, there may be incorporated, as an additive, a vehicle such as starch, lactose or the like.

As a result of animal experiment, it has been found that a
15 mouse tumor is completely healed by one or two injections only, in most cases. In particular, an aliquot of artificial neoplastic cells (Meth-A cells) were transplanted to the skin of each mouse. When the tumor grew to have a diameter of 6 to 7 mm, as little as 0.6 μ g of the polypeptide of the present
20 invention was injected. A week later, a scab appeared. Two weeks later, hairs began to grow, which means complete healing of the tumor. Later, pregnancy and successful birth were observed for the mice.

The present invention will be described in more detail with reference to the following Referential Examples and Working Examples, which should not be construed to be limiting the scope of the present invention.

In practicing the present invention, construction of a recombinant DNA and insertion of a recombinant DNA to a microorganism are carried out in accordance with the procedure described in the following experimental reports [Literatures (1) to (4)], unless otherwise indicated.

(1) Yasutaka Takagi, Manual For Genetic Engineering, Kodan-sha, Tokyo.

(2) Yasutaka Takagi, Experimental Method In Genetic Engineering, Kodan-sha, Tokyo.

(3) T. Maniatis, E. F. Fritsch, J. Sam Brook, Molecular Cloning, Cold Spring Harbor Laboratory, New York.

(4) Ray Wu et al., Method in Enzymology, Vol. 101, Academic Press, New York.

Abbreviations used in Referential Examples and Examples

MOPS:	morpholinopropanesulfonic acid
LB medium:	Luria-Bertani medium
DMSO:	dimethylsulfoxide
PFU:	plaque forming unit
EDTA:	ethylenediaminetetraacetic acid
SDS:	sodium dodecyl sulfate
BRL:	Bethesda Research Laboratories Inc.
DMT:	dimethoxytrityl
lac:	lactose
Tris:	tris(hydroxymethyl)aminomethane
XAR-5:	X-ray film manufactured and sold by Eastman Kodak Company, U.S.A.
1 x SSC:	0.15 M NaCl + 0.015 sodium citrate, pH7
2 x SSC:	0.30 M NaCl + 0.030 M sodium citrate, pH7
3 x SSC:	0.45 M NaCl + 0.045 M sodium citrate, pH7
5 x SSC:	0.75 M NaCl + 0.075 M sodium citrate, pH7
6 x SSC:	0.9 M NaCl + 0.09 M sodium citrate, pH7
FDSS:	50 % deionized formamide + 5 x Denhardt's + 5 x SSPE + 0.1 % SDS + 100 µg/ml denatured calf thymus DNA

SSPE: 0.18 M NaCl + 10 mM NaH₂PO₄ +
1 mM EDTA, pH7.4

SM: phage storage medium which contains 5.8 g of NaCl
2 g of MgSO₄·7H₂O, 50 ml of 1 M Tris.Cl(pH7.5) and 5
ml of 2 % gelatin per liter.

NZ-broth: medium which contains 10 g of NZ amine, 5 g of NaCl
and 2 g of MgSO₄·7H₂O (NZ amine is a Type-A
hydrolysate of casein manufactured and sold by Humko
Sheffield Chemical Division of Kraft, Inc., USA)

IPTG: isopropyl thiogalactoside

x-gal: 5-dibromo-4-chloro-3-indolylgalactoside

TAE: 0.04 M Tris-acetate (pH8.0) - 0.002 M EDTA

5 x Denhardt's solution:

an aqueous solution containing Ficoll* 1000 mg,
polyvinylpyrrolidone 1000 mg and BSA 1000 mg per
liter

bp: base pair

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Referential Example 1

(Evaluation of cytotoxic
activity against L cells)

5 The evaluation of the cytotoxic activity of the physiologically active substance prepared in the following Referential Examples and Examples against L cells is effected by measuring its cytotoxic effect on the L929 cells (American Type Culture Collection CCL 1), in accordance with the method of Ruff et al [see Lymphokines, Vol. 2, edited by E. Pick, Academic Press, N.Y., p 235 (1980) or the method described in J. Immunol, 126, p.1279 (1981)].

10 The method of evaluation of the cytotoxic activity of the physiologically active substance prepared in the Examples is explained below.

As culture vessels, there are employed 96-well microtiter plates produced by Flow Laboratories, Inc. (U.S.A.), and L929 cells are cultured in Eagle's minimum essential medium containing 1 v/v % of fetal calf serum and 5 µg/ml (final concentration) of actinomycin D [the composition of this medium is described, for example, in Tissue Culture, edited by Junnosuke Nakai et al, Asakura Shoten, Japan (1967)].

15 A sample (0.1 ml) serially diluted with the medium and the L929 cell suspension (0.1 ml, 1×10^5 cells) are mixed in each well of the plates and the plates are incubated at 37°C for 21 hours in air containing 5 % carbon dioxide. At the end of the culture period, 20 µl of a 20 % aqueous solution of glutaraldehyde is added to fix the cells. After

20 fixation, the plates are washed with distilled water and allowed to dry, and 0.05 % methylene blue (0.1 ml) is added to stain the viable cells. The plates are thoroughly

25

washed with distilled water to remove excess dye and allowed to dry. 0.36 N Hydrochloric acid is added to each well to extract the dye from stained cells. Absorbance of each well at 665 nm is measured with Titertek Multiskan (produced by Flow Laboratories, Inc., U.S.A.). The absorbance is proportional to the number of viable cells. The cytotoxic activity of the physiologically active substance, unit/ml, is defined as the reciprocal dilution of the physiologically active substance that causes 50 % cytotoxicity, and can be obtained by plotting the dilution versus the absorbance on a graph. The "1 unit" used in Referential Examples means a quantity of the rabbit TNF by which 50 % of 10^5 cells/ml of L929 cells are killed.

On the other hand, the amount of protein is determined by a method in which Coomassie* Brilliant Blue G250 is bonded to protein, according to the teaching of Bradford et al [see Anal. Biochem. Vol. 72, pp 248-254 (1976)].

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Referential Example 2

Step 1

(Preparation of TNF from rabbit serum)

5 Female rabbits, weighing 2.5 to 3 kg, are injected with 50 mg of formalin-killed Propionibacterium acnes (Corvnebacterium parvum; Wellcome Research Laboratories, England) through the ear vein. Eight days later, 100 µg of endotoxin (lipopolysaccharide from Escherichia coli 026:B6, produced by Difco Laboratories, U.S.A.) is injected again through
10 the ear vein and 2 hours later whole blood is collected from the heart. To the collected blood, heparin sodium is added in an amount of 100 units per 100 ml. The blood is then centrifuged while cooling at 5,000 rpm for 30 minutes to remove blood cells and insoluble solids. As a result,
15 a plasma (2.4 liters) having a serum TNF cytotoxic activity of 3×10^4 units/ml is obtained from 40 rabbits.

Step 2

(Partial purification of TNF from rabbit serum)

20 To the plasma (2.4 liters) obtained in Step 1 is added 24 g of cellite. The resultant is stirred for one hour, and then subjected to filtration. The filtrate is mixed with 1.2 liters of 0.04 M Tris-HCl buffer (pH 7.8), and then applied to a column of DEAE-Sephacel*CL-6B (manufactured and sold by Pharmacia Fine Chemicals, Inc. Sweden)
25 sufficiently equilibrated with 0.04 M Tris-HCl buffer (pH 7.8) containing 0.1 M NaCl. The column is washed with 0.04 M Tris-HCl buffer, and the adsorbed TNF is eluted with

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0.04 M Tris-HCl buffer (pH 7.2) containing 0.18 M NaCl.

Fractions exhibiting cytotoxic activities against L cells are concentrated by ultrafiltration. The so obtained concentrate is applied to a column of Sephacryl* S-200

5 (manufactured and sold by Pharmacia Fine Chemicals, Inc. Sweden) sufficiently equilibrated with 5 mM phosphate buffer and gel-filtered using the same buffer. The active fractions are concentrated by ultrafiltration, whereby a purified TNF having an activity of 3.5×10^6 units and a specific
10 activity of 18×10^6 units/mg is obtained.

Step 3

(Anti-TNF antibody)

The rabbit serum TNF partially purified in Step 2 is mixed with complete Freund's adjuvant (1:1), and then injected
15 subcutaneously at the back of a 12 week age BALB/c male mouse. The above operation is repeated 2 and 4 weeks after the initial injection. One week after the last injection, whole blood is collected. From the collected blood, a serum is
obtained.

20 The so-obtained serum is added to the culture medium for evaluation of the cytotoxic activity of TNF against L cells in such an amount that it is diluted 500-fold in final concentration. The cytotoxic activity of the rabbit serum TNF against L cells is evaluated in the same manner as
25 described in Referential Example 1. It is found that the rabbit serum TNF exhibits no cytotoxicity against L cells. From the above result, it can be concluded that the mouse

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serum obtained in this step contains an antibody to the rabbit serum TNF (hereinafter referred to as "anti-TNF antibody").

5 Referential Example 3

 Step 1

 (Preparation of TNF-producing cells)

 A female rabbit is injected intravenously with formalin-killed cells of Propionibacterium acnes (Coryne-
10 bacterium parvum; Wellcome Research Laboratories, England). Seven days later, the rabbit is subjected to tracheotomy, and the lung is washed with a physiological saline solution, whereby floating cells are obtained. The so obtained cells are washed with a physiological saline solution. Using as
15 a culture medium RPMI 1640 (Flow laboratories Inc., U.S.A.) containing 10 v/v % fetal calf serum, the cells are incubated at 37°C in air containing 5 % carbon dioxide. The cell culture is divided into two groups, and to one of them endotoxin derived from Escherichia coli
20 (lipopolysaccharide from Escherichia coli 026:B6, produced by Difco Laboratories, U.S.A.) is added at a concentration of 10 µg/ml. The same amount of sterile water is added to the other. The supernatant of the cell culture to which endotoxin is added exhibits cytotoxic activity against L

cells, and the activity reaches the maximum value within seven hours. Such activity is dissipated by the anti-TNF antibody, but is not dissipated by the normal mouse serum.

On the other hand, the supernatant of the cell culture to which no endotoxin is added exhibits no cytotoxicity against L cells.

Step 2

(Molecular weight of TNF)

To the cell culture prepared in Step 1 to which endotoxin is added, radioactive L-[³⁵S] methionine (1300 Ci/mmol, produced by Amersham Industries plc, England) is further added (1 mCi/ml). In accordance with the method of Laemmli [see Laemmli, U.K. (1970), Nature (London), Vol. 227, pp 680-685], the supernatant is analyzed by the SDS-polyacrylamide gel electrophoresis. The gel concentration is adjusted to 12.5 wt %. After the electrophoresis, the gel is treated with ENHANCE® (trademark of a product of New England Nuclear Inc., U.S.A.), and after drying, is exposed to X-ray film (Fuji*RX, manufactured and sold by Fuji Photo Film Co., Ltd., Japan). In the supernatant of the

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cell culture in the presence of endotoxin, it is observed that a substance having a molecular weight of about 17500 is formed.

Further, the supernatant of each cell culture prepared in Step 1 is subjected to SDS-polyacrylamide gel electrophoresis in the same manner as described above. Thereafter, the gel is shaken in 2.5 % NP 40[®] (a surface active agent sold by Calbiochem, U.S.A.) for one hour, and then in water for two hours. After shaking, each migration lane is separated by cutting, and cut into strips of 2 mm-width in a direction perpendicular to the direction of migration. Each strip is cultured with L cells, and evaluated for cytotoxic activity against L cells. In the lane on which the supernatant of the cell culture containing endotoxin is developed, cytotoxicity against L cells is observed at a position corresponding to the molecular weight of 17500. No cytotoxicity is observed at other positions.

20

Step 3

(Extraction of mRNA)

The cell culture as prepared in Step 1 is incubated for 2 hours after addition of endotoxin, followed by centrifugation to collect cells. Extraction of cytoplasmic RNA from

the collected cells and extraction of mRNA from the cytoplasmic RNA are effected in accordance with the method of Chirgwin et al [see Chirgwin, J.M. et al, Biochemistry, Vol. 18, p. 5294 (1979)]. 4 ml of a 4 M guanidine thiocyanate solution is added to 3×10^8 cells, and the mixture is pulverized by means of a homogenizer (Model: AM-7, manufactured and sold by Nihon Seiki Seisakusho, Japan). The residues are removed by centrifugation, and 2.4 g of cesium chloride is dissolved therein. The mixture is carefully poured into a polyallomer tube in which 2.5 ml of 5.7 M cesium chloride and 0.1 M EDTA solution (pH 7.5) has been loaded in advance, and then subjected to ultracentrifugation at 30,000 rpm for 12 hours at 20°C using Beckman* SW41 rotor (manufactured and sold by Beckman Instrument, U.S.A.). After removal of the supernatant, the pellet is dissolved in 1 ml of 10 mM Tris-HCl buffer (containing 5 mM EDTA and 1 w/v % SDS). The resulting solution is extracted with a 4:1 by volume mixture of chloroform and 1-butanol. To the aqueous phase, 0.05 volume of 2 M sodium acetate and 2.5 volumes of ethanol are added, and allowed to stand at -20°C for 2 hours or more, thereby to precipitate RNA. The precipitate is collected by centrifugation, dried, and then dissolved in 500 μ l of sterile water. As a result, a cytoplasmic RNA solution is obtained.

The above-obtained RNA solution is heated at 68°C for 2 minutes, and thereafter, chilled quickly. 500 μ l of 2-fold concentration 10 mM Tris-EDTA buffer (pH 7.4) (containing

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1 mM EDTA, 0.1 w/v % SDS and 0.5 M lithium chloride) is added to the solution, and the mixture is applied to a 200 mg oligo dT-cellulose (manufactured and sold by Bethesda Research Laboratories, Inc., U.S.A.) column, and washed with 10 ml of the same buffer (one-fold concentration) as described above. The material retained by the column is eluted with 2 ml of an elution buffer containing 10 mM Tris-HCl buffer pH 7.4, 1 mM EDTA and 0.1 w/v % SDS. To the eluate, is added 0.05 volume of sodium acetate solution and 2.5 volumes of ethanol, and the mixture is cooled at -20°C to precipitate. The precipitate is collected by centrifugation, and applied to the oligo dT-cellulose column, and the fractions adsorbed onto the oligo dT-cellulose are collected. 85 µg of mRNA is recovered as determined by the ultraviolet spectrum analysis.

15

Step 4

(Size fractionation of mRNA)

20

880 µg of mRNA prepared by the same method as described in Step 3 is dissolved in 250 µl of water, and the resulting solution is layered onto a 10 ml 5-25 % linear sucrose density gradient. The sucrose density gradient is prepared by means of ISCO 570 gradienter (manufactured and sold by ISCO Inc., U.S.A.), using Tris buffer solutions [containing 25 mM Tris-HCl (pH 7.2), 2 mM EDTA and 1 w/v % SDS] respectively containing 5 % sucrose and 25 % sucrose.

25

30

Using Beckman* SW41 rotor, ultracentrifugation is effected at 40000 rpm for 12 hours at 4°C, and fractions each of 400 µl are recovered by means of a fraction recovering apparatus (manufactured and sold by Beckman Instrument, U.S.A.), and then ethanol precipitated. The precipitated fractions are centrifuged, and dissolved in sterile water.

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Step 5

(Experiment on translation of mRNA)

Translation of mRNA using oocytes of *Xenopus laevis* (Hamamatsu biological teaching materials) is conducted according to the procedure described in the experimental reports (for example, Hiroshi Teraoka, Mikio Itsuki and Kentaro Tanaka, "Protein, Nucleic acid, Enzyme", Genetic Engineering, extra edition., 1981, p 602). *Xenopus laevis* is procured from Hamamatsu biological teaching materials. Fractionated mRNA obtained in Step 4 is dissolved in sterile water to have a concentration of 1 $\mu\text{g}/\mu\text{l}$, and the solution is injected into oocytes in such a small amount as 50 nl per cell. Cells are then cultured for 24 hours in a Barth's solution [containing 7.5 mM Tris-HCl (pH 7.6), 88 mM NaCl, 1 mM potassium chloride, 0.33 mM calcium nitrate, 0.41 mM calcium chloride, 0.82 mM magnesium sulfate, 2.4 mM sodium bicarbonate, 18 U/ml penicillin G and 18 $\mu\text{g}/\text{ml}$ streptomycin] which contains 1 mg/ml bovine serum albumin. Oocytes are crushed, in the culture liquid, by means of a glass bar. The culture liquid is then centrifuged, and the supernatant is evaluated for the cytotoxic activity against L cells. mRNA which will be translated to give a polypeptide having maximum activity sediments as 16 S in size. This activity is eliminated by the anti-TNF antibody obtained in Step 3 of Referential Example 2, but is not eliminated by the normal mouse serum.

Step 6

(Preparation of transformants)

Using 5 μ g of the fractionated mRNA obtained in Step 4, a double stranded DNA is prepared in accordance with procedure described in Literature (1), from page 96. As the reverse transcriptase, use is made of a product of Life Science, Inc., U.S.A. The double stranded DNA is size-fractionated on a 3.5 % polyacrylamide gel, and 330 ng fraction of about 1000 to 2000 bp is obtained. In accordance with the procedure described in Literature (1), 7 ng of this fraction is extended with deoxyC residues using terminal deoxynucleotidyl transferase (manufactured and sold by Bethesda Research Laboratories, Inc., U.S.A.) and annealed with 56 ng of plasmid pBR322 which has been digested with PstI and extended with deoxyG residues. The so-annealed mixture is inserted into E. coli K-12 strain (HB101, ATCC 33694) to transform the strain. As a result, 12000 transformants are obtained.

Step 7

(Partial amino acid sequence of rabbit TNF)

Rabbit TNF partially purified in Referential Example 2 (activity: 5×10^7 units) is subjected to SDS-polyacrylamide gel electrophoresis for purification as in Step 2. Part of the gel is dyed with Coomassie* Brilliant Blue. A band at the position corresponding to the molecular weight of 17000 is cut out from the gel, and extracted with 1 % ammonium bicarbonate. About 180 μ g of TNF is recovered as protein.

150 μ g of the recovered TNF is dissolved in 75 μ l of 1 % ammonium bicarbonate, followed by addition of 3 μ g of TPCK

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trypsin (manufactured and sold by Worthington Biochemical, U.S.A.). The mixture is incubated at 37°C for 4 hours. The mixture is then fractionated by means a high-performance liquid chromatography column comprising Cosmosil 5C8 (manufactured and sold by Nakarai Chemical, Ltd., Japan) as the packing material, thereby to obtain fragments digested with trypsin.

The highly purified TNF and the trypsin-digested fragments thereof are then subjected to desalting by means of Sephadex* G-25 column, and then freeze-dried. According to the method of R.M. Hewick et al (see J. Biol. Chem., Vol. 256, pp 7990-7997, 1981), the purified TNF and the trypsin-digested fragments are each subjected to Edman Degradation from the N-terminal. PTH-amino acid liberated in each step is analyzed by the customary method by means of a high-performance chromatography model SP8100 (manufactured and sold by Spectra physics, U.S.A.) using Solpacks ODS (manufactured and sold by E.I. Du pont, U.S.A.) as the column. As a result, it is found that the TNF has the following N-terminal amino acid sequence: Ser-Ala-Ser-Arg-Ala-Leu-Ser-Asp-Lys-Pro-Leu-Ala-His-Val-Val-Ala-Asn-Pro-Gln-Val-Glu-Gly-Gln-Leu-Gln-

One of the trypsin-digested fragments has the following N-terminal amino acid sequence.

Glu Thr Pro Glu Glu Ala Glu Pro Met Ala

Step 8

(Synthesis of oligodeoxynucleotide probe)

Oligodeoxynucleotides complementary to the base sequence of the mRNA which is deduced from the amino acid sequence

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of rabbit TNF obtained in Step 7 of Referential Example 3 is synthesized according to the improved phosphotriester method which has already been reported by the present inventor in H. Ito et al, "Nucleic Acid Res." 10, 1755-1769 (1982).

5 In preparing oligodeoxynucleotides, 128 oligodeoxynucleotides estimated from the amino acid sequence of rabbit TNF are classified into five groups, namely groups of 16, 16, 32, 32 and 32 and are synthesized as mixtures of oligodeoxynucleotides of the respective groups. The obtained oligodeoxynucleotides of the respective groups are deprotected
10 according to the customary method and purified by column chromatography using Sephadex G-50 (manufactured and sold by Pharmacia Fine Chemicals, Inc., Sweden); electrophoresis on a 20 % by weight polyacrylamide gel containing 7 M of
15 urea and column chromatography using DE52 (manufactured and sold by Whatman Ltd., U.S.A.). The thus obtained oligodeoxynucleotides of the respective groups are dialyzed against 0.1 mM Tris-EDTA buffer solution.

Each of the purified oligodeoxynucleotides of the
20 respective groups is labelled using T₄ polynucleotide kinase (manufactured and sold by Bethesda Research Laboratories, Inc., U.S.A.) and γ -³²P-adenosine triphosphate according to the customary method and then purified by column chromatography using DE52 (manufactured and sold by Whatman Ltd., U.S.A.).
25 The radioactive material is incorporated into each of oligodeoxynucleotides of the respective groups in an amount

of about 3×10^8 cpm/ μ g. The oligodeoxynucleotide probes each obtained in the form of a mixture of the respective group are designated as shown in Table 1.

Part of the amino acid sequence of the rabbit TNF, the base sequence of the mRNA estimated from the amino acid sequence of the rabbit TNF and the base sequences of synthetic oligodeoxynucleotide probes of the respective groups are shown in Table 1.

Table 1.

Amino acid sequence	Carboxyl terminal...	Ala	Met	Pro	Glu	Ala	Glu	Glu...	Amino terminal
m RNA	3'	XCG	GTA	XCC	YAG	XCG	YAG	YAG	5'
Probe MH	5'	GC	CAT	MGG	MTC	GGC	MTC	MTC	3'
Probe MI	5'	GC	CAT	NGG	MTC	GGC	MTC	MTC	3'
Probe MJ	5'	GC	CAT	ZGG	MTC	AGC	MTC	MTC	3'
Probe MK	5'	GC	CAT	ZGG	MTC	CGC	MTC	MTC	3'
Probe ML	5'	GC	CAT	ZGG	MTC	TGC	MTC	MTC	3'

Note: X represents a ribonucleic acid residue of A,C,G or U.

Y represents a ribonucleic acid residue of A or G.

M represents a deoxyribonucleic acid residue of T or C.

N represents a deoxyribonucleic acid residue of A or G.

Z represents a deoxyribonucleic acid residue of

A, C, G or T.

mRNA of the cells producing TNF which is obtained according to Step 3 of Referential Example 3 is treated with a solution containing 1 M of glyoxal, 10 mM of NaH_2PO_4 and 50 % by volume dimethyl sulfoxide at 50°C for 60 minutes and then subjected to fractionation using electrophoresis on a 1.1 % by weight agarose gel. The fractionated mRNA is transferred on a filter of an electrophoresis type transfer blotting apparatus (manufactured and sold by Bio Rad, U.S.A.) according to the manual of the maker. Then the mRNA on the filter of the apparatus is treated with a 5 x Denhardt's solution containing a 5 x SSC solution and 150 $\mu\text{g/ml}$ of denatured salmon spermatozoa DNA at 65°C for two hours and, then treated with a 5 x Denhardt's solution containing 1×10^7 cpm/ml of the labelled oligodeoxynucleotides and a 5 x SSC solution at 50°C for two hours. The above-obtained filter is washed with a 6 x SSC solution successively four times at room temperature, 40°C, 50°C and 60°C. An XAR-5 X-ray film (manufactured and sold by Eastman Kodak Company, U.S.A.) is exposed to the radiation from the filter. As a result, it is found that the oligodeoxynucleotides designated by Probe MJ are most strongly hybridized with the mRNA, showing that the oligodeoxynucleotide having a base sequence which is completely complimentary to the mRNA is contained in the oligodeoxynucleotides designated by Probe MJ.

Step 9

(Cloning of TNF gene of rabbit)

In accordance with the procedure described in Literature (2), page 162, the transformants obtained in Step 6 of Referential Example 3 are transferred onto a cellulose filter and the DNA of the transformants is hybridized with the labelled oligodeoxynucleotide (Probe MJ) selected in Step 8 of Referential Example 3 under the same conditions as in Step 8 of Referential Example 3 (colony hybridization).

In the just above procedure, 49 colonies which are strongly hybridized with the labelled oligodeoxynucleotides (Probe MJ) are selected and further fixed onto another nitrocellulose filter. Then, using 49 colonies, further hybridization is carried out to select nine colonies which are more strongly hybridized with the labelled oligodeoxynucleotides (Probe MJ).

In accordance with the rapid plasmid separating procedure described in Literature (1), page 6, about 5 µg plasmid is obtained from each of the nine colonies. Each of the obtained plasmids is cleaved using restriction enzymes, PstI, TaqI, RsaI and PvuII (each manufactured and sold by Bethesda Research Laboratories, Inc., U.S.A.) according to the procedure described in the manual of the maker, followed by electrophoresis effected on a 1 % by weight agarose gel. Then, fragments obtained by cleavage by the respective restriction enzymes are compared with respect to length thereof.

The results suggest that all the nine strains corresponding to the nine colonies have the base sequence of the fragment

obtained by cleavage by PvuII and RsaI and consisting of about 50 bp and that most of the nine strains have the base sequence of the fragment obtained by cleavage by RsaI and consisting of about 200 bp. In other words, the results suggest that the nine strains have partially common base sequences. The results of analysis by the restriction enzymes are shown in Fig. 1.

Seven strains containing plasmids designated in Table 2 below are separately cultivated in 2 ml of LB medium containing 10 µg/ml of tetracycline until the optical density of the solutions shows the values shown in Table 2 below, followed by centrifugation to obtain respective strains. Each of the obtained strains is separately added into 2 ml of physiological saline and disrupted by sonication. The obtained solutions are subjected to centrifugation and the cytotoxic activity against L cells of the obtained supernatants is determined. The results are shown in Table 2 below. As a blank test, the same procedures as mentioned above are repeated using a strain containing plasmid pBR322. The results are also shown in Table 2 below.

Table 2

Plasmid	Number of annealed base pairs	OD ₆₀₀	Cytotoxic activity against L cells (unit/ml)
pB 2-2	1400	1.369	35
pB 2-3	800	1.605	< 10
pB 2-7	1060	1.364	< 10
pR 9	1550	1.618	< 10
pR 12	1400	1.458	15
pR 18	1850	1.438	< 10
pR 25	1350	1.514	< 10
pBR322	0	1.677	< 10

The cytotoxic activity against L cells is eliminated by anti-TNF antibody but is not eliminated by normal mouse serum. This shows that all of the above-mentioned nine colonies have plasmids which contain oligodeoxynucleotides coding for TNF.

Step 10

(Determination of base sequence of DNA coding for rabbit TNF)

E. coli strains containing plasmids pB2-7 and pR 18 are cultivated in one liter of M9 medium described in Literature

(3), page 440 and containing 10 µg/ml of tetracycline. Then, in accordance with procedure described in Literature (3), page 90, each of the plasmids is isolated in an amount of about 150 µg.

The base sequence of the insert of each plasmid is determined according to the Maxam-Gilbert chemical procedure described in Maxam et al "Method in Enzymology", 55, P 490 (1980), Academic Press. The thus determined base sequence is found to be in agreement with the partial amino acid sequences determined in Step 7 of Referential Example 3. Thus, the whole sequence of TNF of rabbit is considered to be elucidated.

Step 11

In this step, construction of a plasmid is carried out using the recombinant plasmid pR12 to obtain direct expression of TNF in E. coli using lac as a promoter. The procedures are illustratively shown in Fig. 2. First 10 µg of plasmid pR12 is digested with 10 units of Apal (manufactured and sold by Bethesda Research Laboratories, Inc., U.S.A.) at 37°C for two hours and electrophoresed on a 4 % by weight polyacrylamide gel to isolate 630 bp fragments. About 1 µg of the fragment is isolated from the gel by electroelution. In the same manner as in Step 8 of Referential Example 3, two oligodeoxynucleotides shown in Fig. 2, namely 5'-GATCCATGTCAGCTTCTCGGGCC-3' and

5'-CGAGAAGCTGACATG-3' are synthesized. Then, each 5' end of the oligodeoxynucleotides (about 100 pmole) is phosphorylated using T_4 polynucleotide kinase in accordance with the method described in Literature (3), page 122. After completion of the reaction, the reaction mixture is extracted with phenol and then with chloroform. Then the obtained synthetic oligomers are mixed with 0.5 μ g of the ApaI 630 bp fragment and ethanol precipitated. The fragment is ligated with the synthetic oligomers at 4°C overnight using 10 units of T_4 DNA ligase in accordance with the procedure described in Literature (1), page 37. After completion of the reaction, the reaction mixture is ethanol precipitated and digested with 20 units of BamHI at 37°C for three hours, followed by electrophoresis effected on a 4 % by weight polyacrylamide gel to recover 670 bp fragment by electroelution. One μ g of commercially available plasmid pUC-8 (catalog No. 4916, manufactured and sold by P-L Biochemicals, Inc., U.S.A.) is digested with BamHI and extracted with phenol and then with chloroform, followed by ethanol precipitation to obtain a vector. 0.5 μ g of the obtained vector is ligated with the above-obtained fragment having BamHI sites on its both ends and containing about 670 bp coding for TNF using T_4 DNA ligase. In accordance with the procedure described in Literature (4), page 20, E. coli is transformed using the above-obtained vector and cultivated on an agar medium containing 1 mM of IPTG and 0.004 % (w/v) of X-gal to obtain about 200 white

colonies. Plasmid DNA is prepared from 100 of these transformants and digested with BamHI. As a result, it is found that 15 plasmids contain the intended BamHI fragment (about 670 bp). In order to examine the direction of insertion, the above 15 plasmids are digested with EcoRI having only one recognition site on its pUC-8 and PvuII having only one recognition site on its about 670 base pair fragment part and electrophoresed on a 6 % by weight polyacrylamide gel. As a result, it is determined that 7 plasmids have the intended fragment consisting of about 140 bp and that the direction of transcription of the lac promoter on pUC-8 is in agreement with that of the oligodeoxynucleotides coding for TNF.

DNA sequence analysis shows that these seven plasmids have the same sequence and have the desired nucleotide sequence at the junctions between the lac promoter, synthetic DNA and cDNA.

Construction of further plasmids is carried out using the recombinant plasmid pR17 in order to obtain direct expression of TNF in E. coli using lac UV5 as a promoter. The procedures are illustratively shown in Fig. 3. First, 10 μ g of the plasmid pR17 is digested with 10 units of ApaI (manufactured and sold by Bethesda Research Laboratories, Inc, U.S.A.) at 37°C for two hours and electrophoresed on a 4 % by weight polyacrylamide gel to isolate a fragment consisting of about 630 bp. About 1 μ g of the fragment is isolated from the gel by electroelution. In the same manner as in

Step 8, two oligodeoxynucleotides shown in Fig. 3, namely 5'-AATTCATGTCAGCTTCTCGGGCC-3' and 5'-CGAGAAGCTGACATG-3' are synthesized. Then, each 5' end of the two oligodeoxynucleotides (about 100 pmole) is phosphorylated using T₄ polynucleotide kinase in accordance with the method described in Literature (3), page 122. After completion of the reaction, the reaction mixture is extracted with phenol and then with chloroform. Then the synthetic oligomers are mixed with 0.5 µg of the previously obtained ApaI fragment (about 630 bp) prepared from the plasmid pR17 and ethanol precipitated. The fragment is ligated with the synthetic oligomers at 4°C overnight using 10 units of T₄ ligase in accordance with procedure described in Literature (1), page 37. After completion of the reaction, the reaction mixture is ethanol precipitated and digested with 20 units of EcoRI at 37°C for three hours, followed by electrophoresis effected on a 4 % by weight polyacrylamide gel to recover a fragment (about 670 bp) by electroelution.

In accordance with the procedure described in F. Fuller, "Gene", 19, pp 42-54 (1982), plasmid pOP95-15 is prepared.

One µg of pOP95-15 is digested with EcoRI and extracted with phenol and then with chloroform, followed by ethanol precipitation to obtain a vector. Using T₄ DNA ligase, 0.5 µg of the obtained vector is ligated with the fragment (about 670 bp) obtained by ligating the synthetic oligonucleotide with the oligonucleotide coding for TNF. In accordance with the procedure described in Literature (4),

page 20, E. coli JM101 (ATCC 33876) is transformed using the above-obtained vector and cultivated on a medium containing 1 mM of IPTG and 0.004 % (w/v) of X-gal to obtain about 150 white colonies. Plasmid DNA is prepared from 100 of these colonies and digested with EcoRI. As a result, it is found that 12 plasmids contain the intended EcoRI fragment (about 670 bp). In order to examine the direction of insertion, the above 12 plasmids are digested with PvuII and PstI and electrophoresed on a 1.5 % by weight agarose gel. As a result, it is determined that four plasmids have the desired fragments (about 1280 bp and about 2600 bp) and that the direction of transcription of the lac UV5 promoter is in agreement with that of the oligodeoxynucleotides coding for TNF.

Base sequence analysis shows that these four plasmids have the same sequence and that the lac UV5 promoter, the synthetic oligodeoxynucleotide and cDNA are properly combined with each other. The obtained plasmids are designated pTNF-lacUV5-1.

Step 12

(Purification of TNF produced by E. coli)

E. coli strains containing plasmids obtained in Step 11 are cultivated in 50 ml of LB medium containing of ampicillin at 37°C overnight. Then the strains are ampicillin at 37°C overnight. Then the strains are ampicillin and further cultivated at 37°C for three hours.

Isopropyl- β -D-thiogalactopyranoside (manufactured and sold by Sigma Chemical Company, Inc., U.S.A.) is added to it to a final concentration of 1 mM. Further cultivation is carried out for six hours, followed by cooling. Then strains are collected by centrifugation. In the same manner as described in Step 11, the strains are added into 5 liters of 0.04 M Tris-HCl buffer solution (pH 7.8) and disrupted by sonication to obtain a strain protein solution. The obtained solution has cytotoxic activity against L cells of 5×10^7 units/l.

The obtained solution is purified in the same manner as in Step 2 of Referential Example 2 to obtain 1.2×10^6 units of TNF. The specific activity of the TNF is 6.8×10^7 units/mg.

Step 13

(Evaluation using transplanted Meth A sarcoma in mouse)

2×10^5 Meth A Sarcoma cells are transplanted intradermally in the abdominal area of a BALB/c mouse and, 7 days later, mice with tumors of 7 to 8 mm in diameter and with no spontaneous central necrosis are selected for evaluation. A sample (0.2 ml) of TNF obtained in Step 12 of Referential Example 3 and diluted with physiological saline solution is injected through the tail vein. The activity of the sample is

evaluated after 24 hours according to the following criterion.

- (-): no change
- (+): slight hemorrhagic necrosis
- (++): moderate hemorrhagic necrosis (central necrosis extending over approximately 50 % of the tumor surface)
- (+++): marked hemorrhagic necrosis (massive necrosis leaving a small viable rim along the tumor periphery)

20 Days after the injection of the sample, observations are made on the involution of tumors and recovery rate is determined according to the following equation.

$$\text{Recovery rate} = \frac{\text{Number of mice which had been completely recovered from tumor}}{\text{Number of mice used for test}}$$

The results are shown in Table 3.

Table 3

Injected amount of rabbit TNF produced by <u>E. coli</u> units/mouse	Number of mice used for test	Evaluation for activity of samples (after 1 day)				Recovery rate (after 20 days)
		-	+	++	+++	
2×10^5	5	0	0	1	4	5/5
Reference (physiological saline)	5	5	0	0	0	0/5

Example 1

Step 1 (Transformation of E. coli K12 Strain MC1061 with pR18, pB2-7 and pB2-2 Plasmids)

5 Colonies of E. coli K12 strain MC1061 are transformed with each of the pR18, pB2-7 and pB2-2 plasmids, which are obtained in Reference Example 3, according to the customary procedures. Specifically, colonies of E. coli K12 strain MC1061 are cultured in LB medium until the optical density
10 of the culture broth becomes 0.3 at 550nm. 50ml of the grown E. coli culture is harvested, washed with a 25ml mixture containing 10mM MOPS(pH7.0) and 10mM RbCl, and re-suspended in a 25ml mixture containing 0.1M MOPS(pH6.5) , 50mM CaCl₂ and 10mM RbCl. The resulting suspension is cooled
15 on ice for 30 min , centrifuged and suspended in a mixture of 2ml of the above-mentioned mixture containing 0.1M MOPS(pH6.5), 50mM CaCl₂ and 10mM RbCl and 30 μ l of DMSO. To a 200 μ l aliquot of the resulting suspension is separately added 10 μ l of each of the plasmid DNA solutions. Each of
20 the resulting mixtures is cooled on ice for 30 min, and then heat-shocked at 44°C for 60 seconds. Immediately thereafter, 5ml of the LB medium pre-warmed at 37°C is added to each of the heated mixtures, followed by incubation at 37°C for one hour. The obtained culture broths are each
25 subjected to centrifugation to form cell pellets. The supernatant is discarded, and LB medium is added and stirred to resuspend each of the cell pellets. Each of the resulting

suspensions is inoculated to an LB agar plate containing 30 µg/ml tetracycline, followed by incubation at 37°C overnight. As a result, colonies of tetracycline-resistant transformants transformed, each, with pR18, pB2-7 and pB2-2 plasmids are obtained.

Step 2 (Preparation of pB2-7 and pR18 Plasmid DNAs)

Each of the transformants respectively transformed with pB2-7 and pR18 plasmids which are obtained in Step 1 is subjected to (1) growth of the transformant and amplification of the plasmid; (2) harvesting and lysis of the transformant, and (3) purification of the plasmid DNA, in accordance with the procedures as described at pages 88-96 of T. Maniatis, E. F. Fritsch and J. Sambrook, "Molecular Cloning", published by Cold Spring Harbor Laboratory, U.S.A. Illustratively stated, each of the transformants is inoculated into LB medium containing 30 µg/ml tetracycline and incubated at 37°C with vigorous shaking. This step is repeated to attain growth of the transformant and amplification of the plasmid. The transformant culture is harvested by centrifugation at 4000g for 10 min. at 4°C. The supernatant is discarded. The resulting pellet is washed in 100 ml of ice-cold STE [0.1M NaCl, 10mM Tris·Cl (pH 7.8) and 1mM EDTA], and subjected to lysis by boiling in a solution of 20 mg/ml lysozyme-10 mM Tris·Cl, pH 8.0. The viscous product is transferred to an ultracentrifuge tube, and centrifuged at 25,000 rpm for 30 min at 4°C to

obtain a DNA solution. The volume of the DNA solution is measured. For every milliliter, exactly 1g of solid cesium chloride is added and mixed gently until all of the salt is dissolved. 0.8 ml of a solution of ethidium bromide (10mg/ml in H₂O) is added for every 10ml of cesium chloride solution. The final density of the solution is 1.55g/ml, and the concentration of ethidium bromide is approximately 600 µg/ml. The cesium chloride solution is transferred to a tube suitable for centrifugation, and the remainder of the tube is filled with light paraffin oil. Centrifugation is conducted at 45,000 rpm for 36 hours at 20°C to obtain two bands of DNA, the upper band thereof consisting of linear bacterial DNA and nicked circular plasmid DNA and the lower band thereof consisting of closed circular plasmid DNA. The lower band of DNA is collected into a glass tube through a hypodermic needle inserted into the side of the tube. The ethidium bromide is removed, and the aqueous phase is dialyzed against TAE. The plasmid DNA solution is treated with RNase, and extracted with an equal volume of equilibrated phenol. The aqueous phase is layered on a column of Bio-Gel* A-150 equilibrated in TAE (pH8.0) and 0.1 % SDS. The DNA in the column is washed, and a reservoir of TE with 0.1 % SDS is applied to collect fractions. The fractions are precipitated with ethanol to obtain a pure plasmid DNA.

* Registered Trade Mark

By conducting the above procedures, 250 µg of pure pB2-7 plasmid DNA and 134 µg of pure pR18 plasmid DNA are obtained.

Step 3

(Nick Translation of Pure pB2-7 and pR18
Plasmid DNAs)

5

From the pure pB2-7 plasmid DNA obtained in Step 2, 40 µg is taken, digested with PstI restriction enzyme and subjected to electrophoresis through 4 % acrylamide gel. After electrophoresis, the DNA is stained and the desired band is cut out to
10 isolate a PstI insert.

Using 500 ng of the isolated PstI insert, nick translation is carried out in the manner as described in Maniatis, T. et al, proc. Natl. Acad. Sci. U.S.A., 72, 1184 (1975). For the nick translation, the Nick Translation Kit produced and sold by
15 Bethesda Research Laboratories Inc., U.S.A. is employed, and 80 pmole of radioactive dCTP is applied in a 25-µl reaction system (at 400 Ci/mmole). To a mixture consisting of:

- 2.5 µl Solution A (dNTP's solution)
- 2.5 µl Solution B (500 ng of test DNA viz. PstI insert)
- 20 5 µl hot dCTP (3200 Ci/mmole)
- 1.3 µl cold dCTP (65 pmole, 50 pmole/µl dCTP)
- 11.2 µl Solution E (E₂O)
- 22.5 µl (total)

is added 2.5 μ l of Solution C (DNaseI, DNA Polymerase I), and reacted at 15°C for 60 min. Then, Solution D (stop buffer) is added to the resulting mixture to stop the reaction. Further, carrier tRNA is added, subjected to ethanol precipitation twice and dissolved in 500 μ l of water. The specific activity per μ g DNA is 9.3×10^7 cpm.

With respect to the pure pR18 plasmid DNA obtained in Step 2, also, the above-described procedures are carried out to effect the nick translation. The specific activity per μ g DNA is 7×10^7 cpm.

Step 4

(Preparation of RsaI Insert Fragment of pR18 Plasmid DNA)

80 μ g of the pR18 plasmid DNA is digested with RsaI restriction enzyme, and subjected to electrophoresis through 4 % polyacrylamide gel. The following desired bands of inserts are cut out and purified by means of the BND column:

about 640 bp 3.77 μ g (recovery 52 %)

about 175 bp 1.77 μ g (recovery 50 %).

The above about 640 bp insert is designated as 3'-fragment of pR18 (meaning 3'-untranslated region of pR18), and the above about 175 bp insert is designated as pR18-cfr (meaning coding region of pR18).

Moreover, the above procedures are repeated using PstI and MstII restriction enzymes instead of the RsaI restriction enzyme to obtain the following band:

about 450 bp 3.65 μ g (recovery 60%)

The above insert is designated as 5'-fragment of pR18.

Step 5

(Isolation of the Human Genomic TNF Gene)

The ^{32}P -labelled plasmid pB2-7 insert obtained in Step 3 of Example 1 is used as a hybridization probe to screen 10^6 plaques of bacteriophage Charon 4A/human genomic library prepared by insertion into the Charon 4A EcoRI ligation site [Blattner et al, "Science" 196, 161 (1977)] of sized fragments from partially digested human DNA [Maniatis et al, "Cell" 15, 687 (1978)]. The plaque hybridization method of Benton and Davis [Benton and Davis, "Science", 196, 180 (1977)] is used. Since not all of the bacteriophage in the starting culture contain the necessary genetic material for preparing human TNF, a probe which has a base sequence complementary to the rabbit TNF gene is used. DNA of phage plaques having the desired genetic material incorporated the radioactive probe and are identified by their radioactivity. Nine hybridizing plaques are isolated from the library.

The procedures and conditions used are as follows.

1) Number of plaques:

$\sim 1 \times 10^6$ plaques ($\sim 4 \times 10^4$ plaques/ ϕ 150 mm plate x 25)

2) Transfer to nitrocellulose filters:

[see Benton and Davis, Science, 196, 186 (1977)]

3) Hybridization:

Addition of 1.25×10^5 cpm/ml of pB2-7 insert

probe prepared in Step 3 of Example 1, 42°C , 19.5 hr

4) Washing:

2 x SSC - 0.1 % SDS at room temp.

Immersion 10 min. x 4

↓
1 x SSC - 0.1 % SDS at 50°C

Immersion 30 min. x 2

5) Exposure:

XAR-5 (Eastman Kodak Company, U.S.A.)

-80°C, 2 intensifying screens, 39 hr

In the above screening, 12 candidate strains are obtained.

10 In the same manner as mentioned above, second screening is carried out to obtain nine strains containing the intended fragment. Using these strains, third screening is carried out in the same manner as mentioned above to obtain nine strains containing the intended fragment. Using the obtained strains,
15 fourth screening is carried out to confirm that the nine strains contain the intended fragment. The obtained nine bacteriophages containing the intended fragment are designated HG-1-HG-9, respectively.

Step 6

(Isolation of Rabbit Genomic TNF Gene)

Substantially the same procedure as described in Step 5 of Example 1 are repeated except that 10^6 plaques of bacteriophage Charon 4 A/rabbit genomic library which is prepared using digested rabbit DNA [Maniatis et al, Cell, 15, 687 (1978)] instead of digested human DNA. 6.7×10^5 plaques of bacteriophage Charon 4A/rabbit genomic library are used instead of 10^6 plaques of the bacteriophage Charon 4A/human genomic library. Thus, there is obtained two bacteriophage strains (RG-1 and RG-2) containing the rabbit genomic TNF gene.

Step 7

(Southern blotting analysis of human clones)

Using the bacteriophages HG-3, HG-6 and HG-7 obtained in Step 5 of Example 1, DNA of each bacteriophage is obtained according to the following procedures.

6×10^{10} cells of E. coli LE392 (host cell) are suspended in 18 ml of SM and 3×10^9 PFU of bacteriophage HG-3 is added, thus allowing the E. coli to be infected at 37°C for 20 minutes. Then, the obtained mixture is added in 3 liters of NZ-broth and subjected to shaking culture at 37°C for 23 hours. 60 ml of CHCl_3 is added to the mixture and further subjected to shaking culture for 30 minutes. NaCl is added to the mixture to a final concentration of 1 M, the mixture is allowed to stand for 15 minutes, followed by centrifugation to obtain

supernatant. Then, polyethylene glycol (molecular weight: about 6000) is added to the mixture so that the concentration of polyethylene glycol becomes 10 % (w/v), and allowed to stand for 22 hours at 4°C. Bacteriophages are collected by centrifugation. The obtained bacteriophages are suspended in 28 ml of SM and an equal volume of CHCl_3 is added. After stirring by means of Vortex for 30 seconds, the mixture is subjected to centrifugation to obtain aqueous phase. SM is added to the aqueous phase so that the total amount becomes 30 ml. 26.4 g CsCl is added to the obtained mixture and dissolved gently, followed by ultracentrifugation (45000 rpm, 20 hours) to obtain bacteriophages in the form of a band. The obtained mixture containing bacteriophages is dialyzed against 10 mM NaCl - 50 mM Tris (pH8) - 10 mM MgCl_2 . Then, EDTA, Proteinase K and SDS are added to the mixture so that the concentrations of them are 20 mM, 50 $\mu\text{g/ml}$ and 0.5 % (w/v), respectively. Then the mixture is treated at 65°C for one hour and extracted with phenol, a mixture of phenol and CHCl_3 (1:1 by volume) and then with CHCl_3 . The obtained aqueous phase is dialyzed against 10 mM Tris (pH8) - 1 mM EDTA. The ultraviolet absorption measurement of the obtained aqueous phase shows that pure DNA of the bacteriophage HG-3 is obtained.

Substantially the same procedures as described with respect to the preparation of DNA of the bacteriophage HG-3 are repeated to obtain DNAs of bacteriophages HG-6 and HG-7.

Thus, there are obtained 2920 µg of HG-3, 1100 µg of HG-6 and 819 µg of HG-7.

In accordance with the Southern method [E.M. Southern, J.Mol.Biol., 98, 503 (1975)], Southern blotting analysis of the obtained DNAs is performed. The procedures and conditions are as follows.

1) DNA:

HG-3	825 ng	each
HG-6	935 ng	each
HG-7	685 ng	each

2) Digestion with various restriction enzymes:

10 units BamHI, 10 units EcoRI,
10 units BamHI + 10 units EcoRI
10 units HindIII,
10 units HindIII + 10 units EcoRI
10 units PvuII
37°C, 3 hr

3) Electrophoresis:

0.8 % Agarose gel
TAE
28 V, 15.5 hr

4) Transfer to nitrocellulose filters:

[see E.M. Southern, J.Mol.Biol., 98,
503 (1975)]

5) Pre-hybridization:

30 ml FDSS
42°C, 6 hr

6) Hybridization

5'- fragment (1×10^5 cpm/ml) of pR18

(prepared in Step 4 of

Example 1)

42°C, 14 hr

7) Washing:

2 x SSC - 0.1 % SDS at room temp.

Immersion 10 min. x 4



1 x SSC - 0.1 % SDS at 50°C

Immersion 30 min. x 2

8) Exposure:

XAR-5 (Eastman Kodak Company, U.S.A.)

-80°C, 2 intensifying screens, 14 hr

The results of hybridization are shown in Table 4.

Table 4

Enzyme	Clone (bacterio- phage)	Hybridizing fragment size with Probe (pR18)	
		5' end	3' end
<u>Bam</u> HI	HG-3	6.7 kb	←
	-6	11.2 kb	←
	-7	9.2 kb	←
<u>Bam</u> HI + <u>Eco</u> RI	HG-3	2.9 kb	←
	-6	"	←
	-7	"	←
<u>Eco</u> RI	HG-3	"	←
	-6	"	←
	-7	"	←
<u>Hind</u> III + <u>Eco</u> RI	HG-3	"	←
	-6	"	←
	-7	"	←
<u>Hind</u> III	HG-3	9.7 kb	←
	-6	4.1 kb	←
	-7	9.7 kb	←
<u>Pvu</u> II	HG-3	2.2 kb	0.9 kb
	-6	1.9 kb	0.9 kb
	-7	2.2 kb	0.9 kb

NOTE: The symbol " ← " means same fragment hybridizes.

Step 8

(Southern blotting analysis of rabbit clones)

Substantially the same procedures as in Step 7 of Example 1 are repeated except that each of the bacteriophages RG-1 and RG-2 is used instead of each of the bacteriophages HG-3, HG-6 and HG-7. Thus, there is performed Southern blotting analysis. As a result, it is found that pR18 5'-fragment is hybridized with a single band fragment of fragments which are obtained by cleavage of RG-1 and RG-2 with each of BamHI, EcoRI, BglII, HinIII and BamHI + EcoRI.

Step 9

(Construction of bacterial clones containing human genomic TNF gene)

The method of Landy et al [Biochemistry, Vol. 13, 2134 (1974)] is used to obtain DNA of HG-3 as obtained in the above Step 5. 33 µg of the resulting HG-3 DNA is digested with 80 units of EcoRI at 37°C for 3 hours. The digest is electrophoresed on 1 % low melting agarose gel (conditions: 1 x TAE, 20 V, 14.5 hr). The 2.9 kb band is isolated from the agarose gel as described by T. Maniatis [Molecular Cloning, Cold Spring Harbor Laboratory, p 377 (1982)]. Specifically, the cut-out gel of the 2.9 kb band portion is heated at 65° for 15 min. The EcoRI-cleaved HG-3 fragment having a length of 2.9 kb (hereinafter often referred to as "HG-3/EcoRI 2.9 kb fragment") is recovered from the melted gel by extracting 3 times with phenol and then 3 times with another extraction solvent, followed by precipitation with ethanol containing ammonium acetate. Thus, there is obtained 637 ng (yield: about 30 %) of HG-3/EcoRI 2.9 kb fragment.

255 ng of the above-obtained fragment is ligated to 56.5 ng of EcoRI-cleaved pUC 13 [J. Messing, Methods in Enzymology, Vol. 101, 20 (1983)] using 2.5 units of T₄ ligase at 4°C for 20 hours.

E. coli K 12 strain JM83 is transformed using the above-obtained ligation product. Specifically, E. coli K12 strain JM83 is cultured in LB medium until the optical density of the culture broth becomes 0.3 at 550 nm. 50 ml of the grown E. coli K12 strain JM83 culture is collected, washed with

a 25 ml of 10 mM MOPS (pH 7.0)-10 mM RbCl, and resuspended into a 25 ml of 0.1 M MOPS (pH 6.5)-50 mM CaCl₂-10 mM RbCl.

The suspension is cooled on ice for 30 min., centrifuged and resuspended in a mixture of 2 ml of 0.1 M MOPS (pH 6.5)-

5 50 mM CaCl₂-10 mM RbCl and 30 µl of DMSO. To 203 µl of the suspension is added 10 µl of an aqueous ligation product solution containing 10 ng of the ligation product. The mixture is cooled on ice for 30 min. and then heated at 40°C for

10 60 seconds. Immediately thereafter, 5 ml of LB broth pre-warmed at 37°C is added to the heated mixture, followed by incubation at 37°C for one hour. The obtained culture broth is subjected to centrifugation and the supernatant is removed.

An LB medium is added to the resulting cell pellet and then inoculated on an LB plate containing 30 µg/ml ampicillin and

15 40 µg/ml X-gal. Colonies containing E. coli K12 strain JM83 which have been transformed with the plasmids having the insert are white, while those containing E. coli K12 strain JM83 which have been transformed with plasmid only are blue. The obtained white colonies are inoculated again on LB plate containing

20 30 µg/ml ampicillin and 40 µg/ml X-gal for the purpose of confirmation.

From the above-obtained white colonies ten colonies (bacterial clones) are selected and screened by using a mini-prep technique.

5 Specifically, each colony is cultured overnight in LB medium containing 30 µg/ml ampicillin. The grown cells

are collected and suspended in a solution containing 2 mg/ml lysozyme-50 mM glucose-10 mM EDTA-25 mM Tris·HCl (pH 8.0).

The suspension is allowed to stand at room temperature for 5 minutes, followed by addition of 200 µl of 0.2 N NaOH-1 % SDS.

- 5 After slowly stirring, the suspension is allowed to stand at room temperature for 2 min. Thereafter, 150 µl of 3 M sodium acetate (pH5.2) is added, allowed to stand at -20°C for 10 min., followed by centrifugation for 15 min. to recover the resulting supernatant. To the supernatant is added 900 µl of cold ethanol, followed by centrifugation for 5 min. to obtain the resulting precipitate. The obtained precipitate is washed with 70 % ethanol and dried to get a plasmid DNA. In the above-mentioned method, ten plasmid DNAs are obtained.

- Each plasmid DNA is dissolved in 10 mM Tris-0.1 mM EDTA(pH8.0), digested with EcoRI and subjected to electrophoresis for restriction analysis. The conditions for digestion and electrophoresis are as follows.

Digestion: plasmid DNA solution, one-fifth of the amount as prepared above; EcoRI, 3 units; 37°C; 1.5 hr

Electrophoresis: 1 % agarose gel; 1 x TAE; 120 V; 2 hr

- The above restriction analysis shows that eight of ten clones are positive. That is, the eight clones have 2.9 kb fragment. From the eight positive clones one clone is selected and designated as E. coli K12 strain JM 83 (pHGE) (ATCC 39656).

Substantially the same procedures as in the above Step 2 are repeated to prepare 1.89 mg of pHGE DNA,

except that E. coli K12 strain JM83 (pHGE) is used instead of E. coli harboring pB2-7 and pR18.

Step 10

(Subcloning of EcoRI-cleaved RG-1)

5 30 µg of RG-1 as prepared in the above Step 6 is digested with EcoRI. From the resulting fragment mixture the fragment having a length of about 3.5 kb is recovered in substantially the same manner as in the above step 9, except that the above prepared fragment mixture and 0.8 %
10 low melting agarose gel are used. There is obtained 1.0 µg of EcoRI-cleaved RG-1 fragment (about 3.5 kb). The above-obtained EcoRI-cleaved RG-1 fragment (3.5 kb) is ligated to EcoRI-digested pUC13 in substantially the same manner as in the above step 9, except that the above-obtained
15 EcoRI-cleaved fragment (3.5 kb) is used instead of EcoRI-cleaved HG-3 fragment (2.9 kb).

 The transformation of E. coli K12 strain JM83, screening of bacterial clones, digestion of clones and electrophoresis are effected in substantially the same manner as in the above
20 Step 9, except that the above-obtained ligation product is used. The obtained clone is designated as E. coli K12 strain JM83 (pRGE) (ATCC 39655).

 Substantially the same procedures as in the above Step 2 are repeated to prepare 1.70 mg of pRGE DNA, except that E. coli K12 strain JM83 (pRGE) is used instead of pB2-7 and pR-18.

25 Step 11

(Restriction enzyme analysis of pHGE plasmid DNA)

The restriction enzyme analysis of pHGE DNA as obtained

in the above Step 9 is effected according to the method as described in Maniatis [Molecular Cloning, Cold Spring Harbor Laboratory, 98 (1982)].

The procedures and conditions used are as follows.

5

- 1) Digestion of pHGE DNA with EcoRI:

18.6 μ g pHGE DNA

64 units EcoRI

37°C, 2 hr

- 2) Ethanol precipitation: precipitate

10

- 3) Addition of distilled water to precipitate:

Preparation of 1 μ g/ μ l EcoRI-cleaved pHGE soln.

- 4) Digestion with various restriction enzymes:

1 μ g pHGE/EcoRI

15

Restriction enzyme: 5 units PvuII, 5 units PvuII + 10 units RsaI, 10 units RsaI, 4 units MstII, 3 units AvaI, 9 units PstI

37°C, 2 hr

- 5) Electrophoresis:

2 % Agarose gel, 1 x TAE,

20

28V, 14.5 hr

- 6) Transfer to nitrocellulose filter:

[see E.M. Southern, J. Mol. Biol., 98,503 (1975)]

- 7) First pre-hybridization:

25

30 ml FDSS

42°C, 6 hr

- 8) First hybridization:

5'-fragment (5×10^4 cpm/ml) of pR18

(prepared in the above Step 4)

42°C, 14 hr

9) Washing:

2 x SSC - 0.1 % SDS at room temp.

Immersion 10 min. x 4



1 x SSC - 0.1 % SDS at 50°C

Immersion 30 min x 2

10) Exposure:

XAR-5 (Eastman Kodak Company, U.S.A.),
-80°C, 2 intensifying screens, 17.5 hrs

11) Washing out:

0.5 M NaOH - 1.5 M NaCl (Immersion: 1 min.)

0.5 M Tris - 1.5 M NaCl (Immersion: 1 min.)



3 x SSC (Immersion: 1 min.)

12) Exposure:

Effectuated in the same manner as in the above 10),
except that exposure time is 19 hrs.

13) Second pre-hybridization:

In the same manner as in the above 7)

14) Second hybridization:

pB2 - 7 insert (prepared in the above Step 3),

42°C, 16.5 hrs

15) Washing:

In the same manner as in the above 9)

16) Exposure:

In the same manner as in the above 10),
except that exposure time is 19.5 hrs.

17) Washing out:

In the same manner as in the above 11)

18) Exposure:

In the same manner as in the above 10), except
that exposure time is 20 hrs.

19) Third pre-hybridization:

In the same manner as in the above 7).

20) Third hybridization:

3' - fragment (4.5×10^5 cpm/ml) of pR18
(prepared in the above Step 4), 42°C, 15 hr.

21) Washing:

In the same manner as in the above 9).

22) Exposure:

In the same manner as in the above 10).

The results of the restriction enzyme analysis are shown
in Fig. 4.

Step 12

(Restriction enzyme analysis of pRGE plasmid DNA)

In substantially the same manner as in the above Step 11,
the restriction enzyme analysis of pRGE plasmid DNA prepared
in the above Step 10 is effected, except that pRGE plasmid
DNA is used instead of pHGE plasmid DNA. The restriction

map of pRGE DNA insert obtained is shown in Fig. 5.

Step 13

(Determination of base sequences of rabbit
TNF gene and human TNF gene)

Substantially the same procedures as in the above
Step 2 are repeated, except that E. coli K12 strain JM83
(pHGE) obtained in the above Step 9 and E. coli K12 strain
JM83 (pRGE) obtained in the above Step 10 are used instead
of E. coli K12 strain MC1061 having pB2-7 and E. coli K12
strain MC1061 having pR18. Thus, 150 µg of each of pRGE
plasmid DNA and pHGE plasmid DNA is obtained.

The base sequences of pRGE and pHGE are determined
according to the Maxam-Gilbert method [Maxam et al, Methods
in Enzymology, Vol. 55, 490 (1980) published by Academic
Press].

The base sequence of pR-18 determined in Referential
Example 3 is compared with that of pRGE as determined above
to elucidate the structure, including exon and intron, of
rabbit TNF gene. The structure of pRGE DNA insert is shown
in Fig. 5. Subsequently, the base sequence of pRGE
is compared with that of pHGE to investigate the homology
and consensus sequence around the boundary between intron
and exon. Thus, the structure, including exon and intron,
of human TNF gene is elucidated. The structure of human
TNF gene is shown in Fig. 4.

The above-obtained base sequence coding for rabbit TNF and human TNF will be shown below. In the base sequences, the upper row shows the base sequence coding for rabbit TNF (R) and the lower row the base sequence coding for human TNF (H).

5

R	TCA	GCT	TCT	CGG	GCC	CTG	AGT	GAC	AAG	CCT	CTA	GCC	CAC	GTA	GTA
H	TCA	TCT	TCT	CGA	ACC	CCG	AGT	GAC	AAG	CCT	GTA	GCC	CAT	GTT	GTA

R	GCA	AAC	CCG	CAA	GTG	GAG	GGC	CAG	CTC	CAG	TGG	CTG	AGC	CAG	CGT
H	GCA	AAC	CCT	CAA	GCT	GAG	GGG	CAG	CTC	CAG	TGG	CTG	AAC	CGC	CGG

10

R	GCG	AAC	GCC	CTG	CTG	CGC	AAC	GGC	ATG	AAG	CTC	ACG	GAC	AAC	CAG
H	GCC	AAT	GCC	CTC	CTG	GCC	AAT	GGC	GTG	GAG	CTG	AGA	GAT	AAC	CAG

R	CTG	GTG	GTG	CCG	GCC	GAC	GGG	CTG	TAC	CTC	ATC	TAC	TCC	CAG	GTT
H	CTG	GTG	GTG	CCA	TCA	GAG	GGC	CTG	TAC	CTC	ATC	TAC	TCC	CAG	GTC

R	CTC	TTC	AGC	GGT	CAA	GGC	TGC	CGC	TCC	...	TAC	GTG	CTC	CTC	ACT
H	CTC	TTC	AAG	GGC	CAA	GGC	TGC	CCC	TCC	ACC	CAT	GTG	CTC	CTC	ACC

15

R	CAC	ACT	GTC	AGC	CGC	TTC	GCC	GTC	TCC	TAC	CCG	AAC	AAG	GTC	AAC
H	CAC	ACC	ATC	AGC	CGC	ATC	GCC	GTC	TCC	TAC	CAG	ACC	AAG	GTC	AAC

R	CTC	CTC	TCT	GCC	ATC	AAG	AGC	CCC	TGC	CAC	CGG	GAG	ACC	CCC	GAG
H	CTC	CTC	TCT	GCC	ATC	AAG	AGC	CCC	TGC	CAG	AGG	GAG	ACC	CCA	GAG

R	GAG	GCT	GAG	CCC	ATG	GCC	TGG	TAC	GAG	CCC	ATC	TAC	CTG	GGC	GGC
H	GGG	GCT	GAG	GCC	AAG	CCC	TGG	TAT	GAG	CCC	ATC	TAT	CTG	GGA	GGG

R	GTC	TTC	CAG	TTG	GAG	AAG	GGT	GAC	CGG	CTC	AGC	ACC	GAG	GTC	AAC
H	GTC	TTC	CAG	CTG	GAG	AAG	GGT	GAC	CGA	CTC	AGC	GCT	GAG	ATC	AAT

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R	CAG	CCT	GAG	TAC	CTG	GAC	CTT	GCC	GAG	TCC	GGG	CAG	GTC	TAC	TTT
H	CGG	CCC	GAC	TAT	CTC	GAC	TTT	GCC	GAG	TCT	GGG	CAG	GTC	TAC	TTT

R	GGG	ATC	ATT	GCC	CTG
H	GGG	ATC	ATT	GCC	CTG

Note: the symbol "... " means that this portion in the base sequence of the DNA coding for rabbit TNF is null and, therefore, two codons adjacent to this symbol at its both sides are directly connected.

Step 14

(Synthesis of oligodeoxynucleotides)

To a stainless steel 500 μ l reaction vessel with stainless steel filters at each end is added 20 mg of a polystyrene resin to which a nucleoside (2.0 μ M) is connected via a succinate linkage. The resin is treated with zinc bromide (1 M) in dichloromethane isopropanol (85:15) to remove the dimethoxytrityl (DMT) protecting group, washed with dimethylformamide, pyridine, and acetonitrile, and dried with a stream of nitrogen. To the dried resin is added a solution of DMT-nucleotide (20 μ M) and mesitylenesulfonylnitrotriazole (60 μ M) in 200 μ l pyridine. The coupling reaction is allowed to proceed at 45°C for 20 minutes. This cycle of deprotection and coupling is repeated for successive nucleotides until the desired oligodeoxynucleotide is assembled on the resin. The resin is then treated to remove the oligodeoxynucleotide therefrom and purified as described by Ito, Ike, Ikuta, and Itakura (Nuc. Ac. Res. 10:1755 (1982)).

Thus, the following oligodeoxynucleotides are obtained.

- 1) 5'-AATTCATGTCATCTTCTCGAACCCCGAGTGACAA-3'
- 2) 3'-GTACAGTAGAAGAGCTTGGGGCTCACTGTTTCGG-5'
- 3) 5'-GCCTGTAGCCCATGTTGTAGCAAACCCTCAAGC-3'
- 4) 3'-ACATCGGGTACAACATCGTTTGGGAGTTCGACT-5'

Step 15

(Construction of M13mp9-HGE containing the human minigene for TNF)

Plasmid pHGE (10 µg) is digested with EcoRI (20 units). After electrophoresis on a 1 % low-melting agarose gel, the 2.9 kb fragment is eluted. This fragment is inserted into EcoRI fragment from the replicative form of M13mp9 phage. The M13mp9 phage is selected because it is especially suited for receiving sections of DNA. The product transfects to E. coli JM103 [BRL (Bethesda Research Laboratories, Inc., U.S.A.) User Manual/M13mp7 Cloning/'Dideoxy' sequencing, 1980]. The product is designated M13mp9-HGE.

Step 16

(Deletion of Intron 3, using M13mp9-HGE single strand DNA and Deleter E3-4)

The single strand DNA of M13mp9-HGE is prepared by the method of BRL User Manual/M13mp7 cloning/'Dideoxy' sequencing, 1980. Oligodeoxynucleotide 4)

3'-ACATCGGGTACAACATCGTTTGGGAGTTCGACT-5'

prepared in Step 14 is used as a deleter for the intron 3. The deleter for the intron 3 is designated "E3-4".

The deleter E3-4 has a base sequence which is complementary to the base sequence of the bases before (Exon 3) and after (Exon 4) the intron 3 which is to be deleted. Deletion of the intron 3 is effected, in accordance with the teaching of Wallace et al, Science 209:1396 (1980), as follows.

E3-4 (164 ng, 15 pmole) is phosphorylated using T4 kinase and ATP (3mM) and added to the template M13mp9-HGE (1.65 µg, 0.5 pmole). The reaction mixture is

heated at 65°C, cooled to room temperature for 5 minutes,
and finally cooled in ice water. To dATP, dCTP, dGTP,
dTTP and ATP (0.4 mM), is added Klenow fragment (5 units), T4
ligase (10 units) in Hin buffer [Wallace et al, Nuc. Ac. Res. 9;
3647 (1981)], 10 mM Tris HCl (pH 7.2), 2 mM MgCl₂ and 1mM
β-mercaptoethanol. The reaction mixture (final volume 50 µl) is
incubated for 30 minutes at 4°C and then for 30 minutes at room
temperature. The DNA from the oligonucleotide-primed reaction
is used to transfect E. coli JMI03 in accordance with the
procedure of BRL User Manual/M13mp7 cloning/'Dideoxy'
sequencing, 1980. Plaques obtained in this way are picked to YT
plates [J.H. Miller, p. 433, Experiments in Molecular Genetics,
Cold Spring Harbor Laboratory (1972)]. The colonies obtained
are hybridized at 55°C for 2 hours with ³²P-labelled E3-4. For
this step, the deleter is used as a probe to identify sequences
of DNA having the corresponding complementary base sequence
after the intron has been deleted. Phage are isolated from
those colonies which hybridize with the deleter.

The resultant phage are plated and plaques are picked to YT
plates. The clones are allowed to hybridize at 55°C for 2 hours
with ³²P-labelled E3-4. Positive clones are obtained and the
phage DNA is sequenced to select those phage in which intron 3
is completely deleted. One such phage is designated mp9-BGE Δ
3-1.

Step 17

(Construction of pHTNF-lacUV5-2).

The replicative form of mp9-HGE Δ 3-1 is digested with EcoRI. The EcoRI fragment is isolated and cloned to
5 EcoRI-cleaved pBR327 to yield the plasmid pHGE Δ 3-1.

Construction of further plasmid is carried out using plasmid pHGE Δ 3-1 in order to obtain such plasmid Δ 3-1 as will directly express TNF in E. coli using lac UV5 as a promoter. The procedures are illustratively shown in Fig. 7. First,
10 10 μ g of plasmid pHGE Δ 3-1 is digested with 10 units of AvaI and EcoRI (manufactured and sold by Bethesda Research Laboratories, Inc., U.S.A.) at 37°C for two hours and electrophoresed on a 4 % by weight polyacrylamide gel to isolate fragments. About 1 μ g of fragment is isolated from the
15 gel by electroelution. In the same manner as in Step 14, two oligodeoxynucleotides shown in Fig. 7, namely 5'-AATTCATGTCATCTTCTCGAACC-3' and 5'-TCGGGGTTCGAGAAGATGACATG-3' are synthesized. Then, each 5' end of the two oligodeoxynucleotides (about 100 pmole) is phosphorylated using T4 polynucleotide kinase in accordance with the method described
20 in Literature (3), page 122. After completion of the reaction, the reaction mixture is extracted with phenol and then with chloroform. Then the so-obtained synthetic oligomers are mixed with 0.5 μ g of the previously obtained AvaI-EcoRI fragment from plasmid
25 pHGE Δ 3-1 and ethanol precipitated. These fragments are ligated at 4°C overnight using 10 units of T4 ligase in accordance with the procedure described in Literature (1),

page 37. After completion of the reaction, the mixture is ethanol precipitated, followed by electrophoresis effected on a 4 % by weight polyacrylamide gel to recover fragment by electroelution.

5 In accordance with the procedure described in F. Fuller, "Gene", 19, pp. 42-54 (1982), plasmid pOP95-15 is prepared.

One μ g of pOP 95-15 is digested with EcoRI and extracted with phenol and then with chloroform, followed by ethanol precipitation to obtain a vector. Using T4 DNA ligase, 0.5 μ g
10 of the obtained vector is ligated with the above-obtained fragment. In accordance with the procedure described in Literature (4), page 20, E. coli JM101 (ATCC 33876) is transformed using the above-obtained vector and cultivated on an agar medium containing 1 mM of IPTG and
15 0.004 w/v % x-gal to obtain about 100 white colonies.

Plasmids DNA is prepared from these transformants and digested with EcoRI to identify those plasmids containing the intended EcoRI fragment. In order to examine the direction of
insertion, those plasmids are digested with PvuII and PstI and
20 electrophoresed on a 1.5 % by weight agarose gel to select plasmids yielding fragments of about 1280 base pairs and about 2600 base pairs indicating that the direction of transcription of the lac UV5 promoter is in agreement with those of the oligodeoxynucleotides coding for TNF.

Base sequence analysis shows that these 2 plasmids have the same sequence and that the lac UV5 promoter, the synthesized oligodeoxynucleotide and DNA are properly combined with each other. The obtained plasmid is designated pHTNF-lacUV5-2.

5 E. coli containing pHTNF-lacUV5-2 is cultured in a conventional nutrient medium. Bioassay of the product for TNF activity indicates almost the same activity which is obtained with a plasmid pTNF-lacUV5-1 containing the rabbit TNF gene under control of the lac promoter.

Example 2

Using the plasmid pHGE and oligodeoxynucleotides 1 to 4) obtained by the procedure described in Steps 1 to 14 of Example 1 pHTNF-lacUV5-1 is prepared in accordance with the procedure illustrated in Fig. 6

The microorganisms and the novel plasmids were placed on deposit in the American Type Culture Collection in Rockville, Maryland, U.S.A. on April 6, 1984 by despositing samples of the microorganisms containing the plasmids. The microorganism E. coli k-12 strain JM83 (pRGE) was given the ATCC accession number 39655. The microorganism E. coli k-12 strain JM83 (pHGE) was given the ATCC accession number 39656.

The invention being thus described, it will be obvious that the same may be varied in many ways. Such variations are not to be regarded as a departure from the scope of the invention, and all such modifications as would be obvious to one skilled in the art are intended to be included within the scope of the following claims.

CLAIMS:

1. A human physiologically active polypeptide having an amino acid sequence represented by:

Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His Val
Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn
Arg Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg
Asp Asn Gln Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile
Tyr Ser Gln Val Leu Phe Lys Gly Gln Gly Cys Pro Ser Thr
His Val Leu Leu Thr His Thr Ile Ser Arg Ile Ala Val Ser
Tyr Gln Thr Lys Val Asn Leu Leu Ser Ala Ile Lys Ser Pro
Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala Lys Pro Trp
Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu Lys
Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu
Asp Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala
Leu

wherein Gln stands for a glutamine residue, Asp and aspartic acid residue, Pro a proline residue, Tyr a tyrosine residue, Val a valine residue, Lys a lysine residue, Glu a glutamic acid residue, Ala an alanine residue, Asn an asparagine residue, Leu a leucine residue, Phe a phenylalanine residue, Gly a glycine residue, His a histidine residue, Ser a serine residue, Thr a threonine residue, Ile an isoleucine residue, Trp a tryptophan residue, Arg an arginine residue, Met a methionine residue, and Cys a cysteine residue.

2. A deoxyribonucleic acid coding for a polypeptide as claimed in claim 1.
3. A deoxyribonucleic acid, comprising at least one base sequence selected from a base sequence represented by:



TCA TCT TCT CGA ACC CCG AGT GAC AAG CCT GTA GCC CAT GTT
 GTA GCA AAC CCT CAA GCT GAG GGG CAG CTC CAG TGG CTG AAC
 CGC CGG GCC AAT GCC CTC CTG GCC AAT GGC GTG GAG CTG AGA
 GAT AAC CAG CTG GTG GTG CCA TCA GAG GGC CTG TAC CTC ATC
 TAC TCC CAG GTC CTC TTC AAG GGC CAA GGC TGC CCC TCC ACC
 CAT GTG CTC CTC ACC CAC ACC ATC AGC CGC ATC GCC GTC TCC
 TAC CAG ACC AAG GTC AAC CTC CTC TCT GCC ATC AAG AGC CCC
 TGC CAG AGG GAG ACC CCA GAG GGG GCT GAG GCC AAG CCC TGG
 TAT GAG CCC ATC TAT CTG GGA GGG GTC TTC CAG CTG GAG AAG
 GGT GAC CGA CTC AGC GCT GAG ATC AAT CGG CCC GAC TAT CTC
 GAC TTT GCC GAG TCT GGG CAG GTC TAC TTT GGG ATC ATT GCC
 CTG

and a complementary base sequence to said base sequence

wherein A stands for a deoxyadenylic acid residue, G a deoxyguanylic acid residue, C a deoxycytidylic acid residue and T a thymidylic acid residue and wherein the left end and right end of the formula (II) represent 5'-hydroxyl group side and 3'-hydroxyl group side, respectively, or comprising a base sequence which is obtained by substituting at least one base of said base sequence in accordance with the degeneracy of the genetic code.

4. A replicable recombinant DNA which comprises a deoxyribonucleic acid according to claim 2 or 3 and a replicable expression vehicle.
5. A microorganism or cell culture transformed with a replicable recombinant DNA according to claim 4.
6. A method for producing a human physiologically active polypeptide which comprises:
 - (a) ligating to a replicable expression vehicle a deoxyribonucleic acid comprising a base sequence coding



for a human physiologically active polypeptide,

said human physiologically active polypeptide having an amino acid sequence represented by:

Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His Val
Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn
Arg Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg
Asp Asn Gln Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile
Tyr Ser Gln Val Leu Phe Lys Gly Gln Gly Cys Pro Ser Thr
His Val Leu Leu Thr His Thr Ile Ser Arg Ile Ala Val Ser
Tyr Gln Thr Lys Val Asn Leu Leu Ser Als Ile Lys Ser Pro
Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala Lys Pro Trp
Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu Lys
Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu
Asp Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala
Leu

wherein Gln stands for a glutamine residue, Asp an aspartic acid residue, Pro a proline residue, Tyr a tyrosine residue, Val a valine residue, Lys a lysine residue, Glu a glutamic acid residue, Ala an alanine residue, Asn an asparagine residue, Leu a leucine residue, Phe a phenylalanine residue, Gly a glycine residue, His a histidine residue, Ser a serine residue, Thr a threonine residue, Ile an isoleucine residue, Trp a tryptophan residue, Arg an arginine residue, Met a methionine residue, and Cys a cysteine residue.

to obtain a replicable recombinant DNA comprising said deoxyribonucleic acid and said replicable expression vehicle;

(b) transforming cells of a microorganism or cell culture with said replicable recombinant DNA to form transformants;

(c) selecting said transformants from parent cells of the microorganism or cell culture;



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(d) incubating said transformants, causing said transformants to express said deoxyribonucleic acid and produce a human physiologically active polypeptide; and

(e) isolating said human physiologically active polypeptide from the incubated transformants.

7. A human physiologically active polypeptide as claimed in Claim 1, obtained by recombinant DNA techniques.

8. The microorganism E. coli k-12 strain JM83 (pHGE).

9. The plasmid pHGE.

10. A process for preparing a physiologically active polypeptide which comprises cultivating a microorganism or cell culture transformed with the deoxyribonucleic acid according to Claim 2 or 3, and recovering said polypeptide.

11. A polypeptide having an amino acid sequence which is a variant of the sequence defined in Claim 1, said variant having human tumor necrosis activity.

12. The use of polypeptide according to Claim 1, 7 or 11 for manufacture of a medicament having anti-tumor activity.

13. A pharmaceutical composition comprising a polypeptide as claimed in Claim 1, 7 or 11 and at least one pharmaceutically acceptable diluent or excipient.
