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(54) **Titre :** CODAGE GENETIQUE SYNTHETIQUE POUR L'HORMONE PARATHYROIDIENNE HUMAINE
(54) **Title:** SYNTHETIC GENE CODING FOR HUMAN PARATHYROID HORMONE

(57) **Abrégé/Abstract:**

Disclosed are (1) a DNA containing a synthetic gene for expression of human parathyroid hormone represented by the following DNA sequence: TCTGTG TCCGAGATTC AGTTAATGCA TAACCTTGGC AAACATTTGA ACTCCATGGA GCGTGTAGAA TGGCTGCGTA AGAAGTTGCA GGATGTGCAC AATTTTGTTG CCTTAGGTGC CCCATTGGCT CCTCGTGATG CTGGTTCCCA AAGACCACGT AAAAAGGAAG ACAATGTCTT AGTTGAGAGC CATGAAAAAT CCCTAGGCGA GGCAGACAAG GCCGATGTGA ATGTATTAAC TAAAGCTAAA TCCCAG (2) a method for producing the DNA described in (1), which comprises enzymatically ligating a plurality of oligodeoxynucleotides to one another to form the DNA and inserting the DNA into a vector if necessary, (3) a transformant transformed by the DNA described in (1), and (4) a method for producing human parathyroid hormone which comprises cultivating the transformant described in (3), accumulating human parathyroid hormone in a culture medium, and collecting the same, whereby hPTH can be allowed to express in large amounts in a system using E. coli as a host.



TITLE: SYNTHETIC GENE CODING FOR HUMAN PARATHYROID HORMONE

ABSTRACT

Disclosed are (1) a DNA containing a synthetic gene for expression of human parathyroid hormone represented by the following DNA sequence:

TCTGTG TCCGAGATTC AGTTAATGCA TAACCTTGGC
AAACATTTGA ACTCCATGGA GCGTGTAGAA TGGCTGCGTA
AGAAGTTGCA GGATGTGCAC AATTTTGTTG CCTTAGGTGC
CCCATTTGGCT CCTCGTGATG CTGGTTCCCA AAGACCACGT
AAAAAGGAAG ACAATGTCTT AGTTGAGAGC CATGAAAAAT
CCCTAGGCCGA GGCAGACAAG GCCGATGTGA ATGTATTAAC
TAAAGCTAAA TCCCAG

(2) a method for producing the DNA described in (1), which comprises enzymatically ligating a plurality of oligodeoxynucleotides to one another to form the DNA and inserting the DNA into a vector if necessary, (3) a transformant transformed by the DNA described in (1), and (4) a method for producing human parathyroid hormone which comprises cultivating the transformant described in (3), accumulating human parathyroid hormone in a culture medium, and collecting the same, whereby hPTH can be allowed to express in large amounts in a system using E. coli as a host.

SYNTHETIC GENE CODING FOR HUMAN PARATHYROID HORMONE

BACKGROUND OF THE INVENTION

The present invention relates to a recombinant
5 DNA technique for producing human parathyroid hormone
(hereinafter referred to as hPTH), and more specifically to
a synthetic gene corresponding to the amino acid sequence
of hPTH, DNA containing it, a host cell transformed by the
DNA, and a method for producing hPTH using the
10 transformant.

hPTH is a polypeptide hormone secreted from the
parathyroid consisting of 84 amino acids, and one of the
most important regulators for calcium metabolism. The
clinical application of hPTH to various bone diseases such
15 as hypoparathyroidism and osteoporosis has therefore been
strongly desired. However, natural hPTH exists in very
small amounts and it is also a protein having a relatively
low molecular weight. For this reason, though hPTH can be
chemically synthesized, the synthesis thereof requires
20 utilizing techniques which require a high level of skill
and which are difficult. Accordingly, the production of
hPTH using recombinant DNA techniques is desirable.

The DNA sequence of hPTH was first revealed by G. N.
Hendy et al. Proc. Natl. Acad. Sci. U.S.A. 78, 7365-7369
25 (1981). Then, two or three groups attempted to synthesize
hPTH using microorganisms to clone cDNA of hPTH and insert
it into various expression vectors. Alestrøm et al.
attempted to express and secrete cDNA-derived genes in

yeast (Japanese Patent Unexamined Publication No. 2-501108/1990). However, they did not succeed in isolating and identifying hPTH. A. Høgset et al. prepared a gene containing a Met-Gly residue at the N-terminus of hPTH from an hPTH cDNA clone, and expressed the gene in Escherichia coli Biochem. Biophys. Res. Commun. 166, 50-60 (1990). However, the resulting hPTH protein had a Met-Gly residue added to the N-terminus thereof and did not exhibit the activity of hPTH. E. Wingender et al. expressed hPTH in E. coli as a fused protein with β -galactosidase J. Biol. Chem. 246, 4367-4373 (1989). However, this protein whose unnecessary portions were removed was a molecule having Pro added to the N-terminus. A. Høgset et al. succeeded in ligating cDNA of hPTH downstream from a Staphylococcus aureus-protein A promoter and a signal peptide gene, expressing and secreting it outside the cells by E. coli, as well as isolating and identifying hPTH J. Biol. Chem. 265, 7338-7344 (1990). However, the yield is only about 1 mg/liter of culture solution, and the expression amount is insufficient from an industrial viewpoint, particularly when the loss of the product during the purification process is taken into account.

When a gene encoding a protein of a certain living organism is intended to be expressed in other hosts, the method of synthesizing that gene chemically is also used as a method for obtaining the gene, in addition to the method of using the gene derived from that living organism. In recent years, automatic synthesis techniques of DNA

oligomers have been developed. When the molecular weight of the protein is relatively low and the amino acid sequence or DNA sequence thereof is known, the method of synthesizing the gene is frequently more advantageous for
5 the following reasons:

(1) The desired gene is relatively easily obtained;
(2) A terminal sequence used when inserting the gene into a plasmid or a recognition sequence of a restriction enzyme appropriate for modification of the gene can be
10 freely introduced; and

(3) A gene sequence can be designed so that the desired gene product is highly expressed.

W. L. Sung et al. synthesized a structural gene encoding hPTH, using an amino acid codon most acceptable by
15 yeast Biochem. Cell. Biol. 64, 133-138 (1986). Also S. A. Rabban et al. expressed this gene in E. coli, J. Biol. Chem. 263, 1307-1313 (1988), however, its yield of about 200 µg/l was very low and thus not practical.

As described above, attempts have been made to express
20 hPTH in various systems using the cDNA-derived or synthetic genes. In either case, however, the yield was very low and insufficient from an industrial viewpoint.

SUMMARY OF THE INVENTION

The present invention provides an amino acid sequence
25 of hPTH, appropriate for solving the above-described problems, as well as a method for producing the gene, recombinant DNA containing the gene, a host cell transformed by the DNA and a method for producing hPTH

using the transformant.

In accordance with the present invention, there is provided (1) DNA containing a synthetic gene for expression of human parathyroid hormone represented by the following

5 DNA sequence:

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          TCTGTG TCCGAGATTC AGTTAATGCA TAACCTTGGC
          AACATTTGA ACTCCATGGA GCGTGTAAGAA TGGCTGCGTA
          AGAAGTTGCA GGATGTGCAC AATTTTGTTG CCTTAGGTGC
          CCCATTGGCT CCTCGTGATG CTGGTTCCCA AAGACCACGT
10        AAAAAGGAAG ACAATGTCTT AGTTGAGAGC CATGAAAAAT
          CCCTAGGCCGA GGCAGACAAG GCCGATGTGA ATGTATTAAC
          TAAAGCTAAA TCCCAG
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(2) a method for producing the DNA described in (1), which comprises enzymatically ligating a plurality of
15 oligodeoxynucleotides, one to another, to form the DNA and inserting the DNA into a vector if necessary, (3) a host cell transformed by the DNA described in (1), and (4) a method for producing human parathyroid hormone which comprises cultivating the transformant described in (3),
20 accumulating human parathyroid hormone in a culture medium, and collecting the same.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows an amino acid sequence and a nucleotide sequence of the synthetic gene of the present invention
25 encoding hPTH;

Fig. 2 is a schematic representation showing an example of cleavage of DNA fragments in synthesizing the hPTH gene of the present invention;

Fig. 3 is a schematic representation showing an example of DNA fragments for producing synthetic genes of the present invention encoding hPTH;

Fig. 4-1 is a schematic representation illustrating the production of a synthetic hPTH gene by ligating the respective fragments shown in Fig. 3 one to another;

Fig. 4-2 is a schematic representation illustrating the production of a synthetic hPTH gene by ligating the respective fragments shown in Fig. 3 one to another;

Fig. 5 is a schematic representation showing the construction of a plasmid for expression into which a synthetic gene of the present invention encoding hPTH is inserted;

Fig. 6 shows electrophoresis and Western blotting blot results indicating the production of hPTH in a transformant by a gene of the present invention, in which lane 1 shows a molecular weight marker, lane 2 shows a standard sample of hPTH, lane 3 shows an extract (corresponding to 10 μ l of culture solution) of a transformant induced by isopropyl- β -D-thiogalactopyranoside (IPTG), and lane 4 shows an extract (corresponding to 10 μ l of culture solution) of a transformant not induced by IPTG;

Fig. 7 shows electrophoresis and Western blot results indicating the production of hPTH in a transformant by a gene of the present invention, in which lane 1 shows a prestained molecular weight marker, lane 2 shows an extract (corresponding to 10 μ l of culture solution) of a transformant not induced by IPTG, lane 3 shows an extract

(corresponding to 10 μ l of culture solution) of a transformant induced by IPTG, and lane 4 shows a standard PTH sample;

Fig. 8 shows an elution pattern of purified hPTH obtained by reversed phase high performance liquid chromatography;

Fig. 9 shows electrophoresis and Western blot results for an hPTH sample, in which lane 1 shows a prestained molecular weight marker, lane 2 shows purified hPTH obtained by the present invention, and lane 3 shows a standard hPTH sample;

Fig. 10 shows an electrophoresis and Western blot results for an hPTH sample, in which lane 1 shows a prestained molecular weight marker, lane 2 shows purified hPTH obtained by the present invention, and lane 3 shows a standard hPTH sample; and

Fig. 11 is a graph showing the biological activity of hPTH fragments, in which *- shows a synthetic hPTH(1-34) fragment, and --- shows purified hPTH obtained by the present invention.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Sequences of synthetic genes are designed so as to satisfy various conditions to enhance the expression of the genes. One of the conditions is that codons most acceptable by cells of expression systems should be employed. Accordingly, in the hPTH genes of the present invention, codons are employed which are frequently used for the production of proteins by recombinant technology

and accepted by both E. coli and yeast. Further, consideration is given to arranging recognition sequences of restriction enzymes convenient for modification of the genes at appropriate positions when selecting the codons.

5 Furthermore, to construct the desired genes accurately, relatively long, unnecessary palindrome sequences and directly repeated sequences existing on DNA chains are removed as much as possible. As additional conditions to enhance the expression of the genes, the stability and
10 translation efficiency of mRNAs corresponding to structural genes should be considered. In this case, secondary structure determined by nucleotide sequences of mRNAs is believed to be of importance. Therefore, for the hPTH genes of the present invention, hairpin structures and the
15 like are searched for using a computer during the course of design, and the sequence is then modified, preferably by removing it. Thus, a novel DNA sequence most suitable for the production of hPTH as shown in Fig. 1 (SEQ ID NO:1) has been discovered. This gene sequence is about 25.4%
20 different from the above-described gene of W. L. Sung et al. in DNA sequence. In addition to the DNA sequence, the amino acid sequence is shown in Fig. 1.

The gene can be expressed as either a fused peptide or hPTH directly without forming the fused peptide.

25 In the former case, either a DNA sequence starting with an initiation codon ATG and coding for a protein other than hPTH may be arranged at the 5'-terminal side of the synthetic gene of hPTH, and it ends with a stop codon (for

example, TAA); or a DNA sequence coding for a protein other than hPTH may arranged at the 3'-terminal side of the synthetic gene of hPTH starting with an initiation codon ATG and it ends with a stop codon (for example, TAA).

5 In the latter direct expression, the initiation codon ATG and a stop codon (for example, TAA) are directly arranged on the 5'- and 3'-terminal sides, respectively, and the 5'- and 3'-termini have, for example, NdeI and BamHI cohesive ends, respectively, for insertion into a
10 vector, in addition to a sequence coding for a polypeptide of hPTH, as shown in Fig. 2 (SEQ ID NO:2).

 In synthesizing the hPTH gene of the present invention, the hPTH gene is finally cleaved into 14 fragments, for example, as shown in Fig. 2 (SEQ ID NO:2, 15 SEQ ID NO:3). In this case, care is taken so as to avoid self-linkage between fragments, e.g. a self-complimentary sequence at the 5'- or 3'-terminus. The respective fragments are shown in Fig. 3. Methods for cleaving the gene into the fragments are not limited to the above-
20 mentioned method, and various methods are available as long as the above self-linkage is avoidable.

 Fragments #1 to #14 (SEQ ID NOS: 4 to 17) can be produced by known synthesizing methods. For the fragments, except for #1 and #14, the termini are phosphorylated at
25 5'-termini with polynucleotide kinase as so required, and all of the fragments are hybridized to ligate them to one another with DNA ligase, or the phosphorylated fragments first divided into two or three groups are hybridized to

form a double stranded DNA fragment with DNA ligase, and the DNA fragments of the respective groups are further ligated to one another with DNA ligase, thereby obtaining a complete double stranded hPTH gene (refer to Fig. 4).

5 The resulting gene is ligated to a product obtained by digesting pUC19 with NdeI and BamHI to obtain a novel plasmid pU·PTH·C19, and E. coli JM109 is transformed. As to the isolated plasmid, the nucleotide sequence is determined by the Sanger method using a portion of the DNA
10 fragment as a primer. More easily, the DNA fragment obtained by NdeI-BamHI digestion is digested with AvrII, NcoI, HgiAI or AluI to give a correct restriction site, thereby confirming the existence of the desired hPTH gene.

When the synthetic gene of the present invention is
15 expressed, it is preferably used as a recombinant DNA inserted into vectors such as plasmids or bacteriophages.

Examples of such plasmids include pBR322 [Gene 2, 95 (1977)], pBR325 [Gene 4, 121 (1978)], pUC12 [Gene 19, 259 (1982)] and pUC13 [Gene 19, 259 (1982)], each derived from
20 Escherichia coli, and pUB110 derived from Bacillus subtilis [Biochemical and Biophysical Research Communication 112, 678 (1983)]. However, any other plasmids can be used as long as they are viable in the host cells. Examples of the phage vectors include λ gt11 R. Young and R. Davis, Proc.
25 Natl. Acad. Sci. U.S.A. 80, 1194 (1983). However, any other phage vectors can be used as long as they are viable in the host cells.

The plasmids thus obtained are introduced into

appropriate host cells such as Escherichia, Bacillus, yeast and animal cells.

Methods for transforming the host cells with the plasmids include, for example, the calcium chloride method
5 or the calcium chloride/rubidium chloride method described in T. Maniatis et al., Molecular Cloning , Cold Spring Harbor Laboratory, p.249 (1982).

When phage vectors are used, for example, they can be transduced into proliferated E. coli, using the in vitro
10 packaging method.

It is preferred that the above-mentioned recombinant DNA has a promoter upstream from the above-mentioned initiation codon ATG, and as the promoter, any promoter may be employed as long as it is suitable for the host cells
15 used for the production of transformants.

For example, trp promoter, lac promoter, recA promoter, λ PL promoter, lpp promoter, T7 promoter, etc. may be used for E. coli (for example, MM294, BL21, BL21/PLys, W3110, DH1 and N4830); SP01 promoter, SP02
20 promoter, penP promoter, etc. may be used for Bacillus subtilis (for example, MI114); promoter for major cell wall proteins, etc. may be used for Bacillus brevis; PH05 promoter, PGK promoter, GAP promoter, ADH promoter, etc. may be used for Saccharomyces cerevisiae (for example,
25 AH22); and SV40-derived promoter, mouse leukemia virus (MuLV)-derived LTR region promoter, etc. may be used for animal cells (for example, monkey cell COS-7 and Chinese hamster ovary cell (CHO)). In particular, it is preferred

that the promoter is the T7 promoter, the trp promoter or the λ PL promoter when the host cell is E. coli, and that an enhancer is used in addition to the above-mentioned promoter when the host cell is an animal cell.

5 The transformants thus obtained are cultivated in the known media. The media for the transformants of Escherichia coli include, for example, LB medium.

 The transformants of Escherichia coli are cultivated usually at 15 to 43°C for 2 to 24 hours, preferably at 28
10 to 40°C for 4 to 16 hours, with aeration or agitation if necessary. After cultivation, the cells are collected by methods known in the art. In the case of the transformants of Escherichia coli, the cells are suspended in buffer solutions or buffer solutions containing protein
15 denaturants such as urea and guanidine hydrochloride, and disrupted by mechanical disruption such as ultrasonic treatment, lysozyme treatment and glass bead treatment, followed by centrifugation to obtain extracted solutions containing hPTH. hPTH can be separated and purified from
20 the extracted solutions by known means. The separating and purifying means include column chromatography such as gel filtration, ion exchange chromatography using a cation exchange resin or an anion exchange resin, hydrophobic chromatography and partition adsorption chromatography, and
25 high performance liquid chromatography. The cultivation of the transformants of the Bacillus, the yeast and the animal cells and the separation and purification of hPTH from the cultures are carried out by methods known in the art.

hPTH thus obtained can be used as a therapeutic agent for various diseases caused by an abnormality of the calcium metabolism, for example, osteoporosis, hypoparathyroidism and hypertension. The dosage thereof is properly determined, taking into account the object of administration, the disease, etc. in each case, and a suitable amount is given within the range of 1 ng to 100 µg/kg of weight a day. Usually, hPTH is given parenterally in combination with pharmaceutically acceptable carriers, excipients or diluents as injections, nasotracheal agents, perirectum agents, transvaginal agents or percutaneous agents, but it may be given orally in some cases.

One example of the expression of the hPTH gene is described below (refer to Fig. 5).

A DNA fragment of 267 base pairs cut out of pU·PTH·C19 with NdeI-BamHI was inserted into a NdeI-BamHI site to obtain a vector pET·PTH·3C for expression under T7 promoter control.

E. coli BL21 was transformed using pET·PTH·3C, and growing colonies were selected based upon ampicillin resistance to obtain a strain containing a desired hPTH gene. This transformant strain was cultivated, and cells were collected by centrifugation. hPTH contained in a solution treated with 7 M guanidine was stained with Coomassie Brilliant Blue (CBB) after SDS-polyacrylamide electrophoresis (SDS-PAGE), or immunoblotting was conducted by an hPTH antibody after SDS-PAGE, thereby determining hPTH. As a result, the product was about 200 mg/l or more

(refer to Figs. 6 and 7). This expression amount is epoch-making as the direct expression of hPTH in E. coli.

In the present invention, the codon most acceptable in cells of expression systems is employed as the DNA sequence of the hPTH gene, and the gene is designed, also taking into account the secondary structure of mRNA corresponding to the DNA sequence, whereby the translation efficiency of mRNA are improved, which results in the efficient production of hPTH. Further, in the present invention, the arrangement of the recognition sites of several kinds of restriction enzymes in the structural gene makes the insertion of the gene and the confirmation of an inserting direction easy, and makes the modification of the gene easy when analogue proteins of hPTH will be synthesized in future. Moreover, the gene can be inserted into several kinds of vectors. Thus, the advantage and the diversity on gene manipulation can be exhibited.

Example 1

1. Synthesis of DNA Fragments

Fourteen DNA fragments #1 to #14 (SEQ ID NO: Nos. 4 to 17) shown in Fig. 3 were synthesized using properly protected DNA β -cyanoethylphosphoamidite, and using an automatic synthesizer (Model 380A, Applied Biosystems). As a protocol for synthesis, one specified by Applied Biosystems was used. The protected DNA oligomer-resins thus synthesized (0.2 μ mole of the resin) were heated in 2 ml of concentrated aqueous ammonia at 60°C for 6 hours. The resulting products were purified by reversed phase high

performance liquid chromatography (hereinafter referred to as HPLC) to obtain DNA oligomers only the 5'-terminal hydroxyl groups of which were protected by dimethoxytrityl groups. These DNA oligomers were treated with 2 ml of 80% acetic acid for 20 minutes to remove the terminal dimethoxytrityl groups, and the resulting products were purified by reversed phase HPLC and ion exchange HPLC. The fourteen DNA oligomers thus synthesized are as shown in Fig. 3 (SEQ. NOS: 4 to 17).

10 2. Phosphorylation of DNA oligomers

Each of the twelve DNA oligomers #2 to #13 (SEQ ID NOS: 5 to 16) except for #1 (SEQ ID NO: 4) and #14 (SEQ ID NO: 17) which were to form the 5'-termini was reacted in 25 μ l of a phosphorylation reaction solution [10 μ l of the DNA oligomer, 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 1 mM spermidine, 10 mM dithiothreitol (hereinafter referred to as DTT), 0.1 mg/ml bovine serum albumin (hereinafter referred to as BSA), 1 mM ATP, 10 units of T4 polynucleotide kinase (Takara Shuzo)] at 37°C for 1 hour to phosphorylate the 5'-terminus. This reaction solution was treated at 65°C for 10 minutes, followed by freezing and thawing. The resulting product was subjected to the following reaction.

3. Ligation of DNA Fragments (Refer to Figs. 4-1 and 4-2)

25 3-1 A series of stages for forming a double stranded structure of an hPTH gene are as shown in Fig. 4-1. Referring to Fig. 4-1, the mark $\blacktriangleleft$ indicates that a 5'-terminal hydroxyl group is phosphorylated. For example,

the ligation of block I was carried out as follows. 7.5 μ l portions of the phosphorylated reaction solutions of the five DNA fragments [corresponding to DNA fragments #2 to #6 (SEQ ID NOS: 5 to 9)] obtained by the operation described in the above item 2 were combined with 2.5 μ g of DNA fragment #1 (SEQ NO: 4) corresponding to the 5'-terminus to 50 μ l. Then, 5 units of T4 DNA ligase (New England Biolabs) was added thereto, followed by incubation at 14°C for 5 hours. The resulting product was thereafter treated at 65°C for 10 minutes to terminate the reaction, thus obtaining block I. Blocks II and III were similarly prepared. 20 μ l portions of these block I to III were mixed and 5 units of T4 DNA ligase was added thereto, followed by incubation at 14°C for 20 hours. The resulting product was treated at 65°C for 10 minutes to terminate the reaction.

The product thus obtained was subjected to electrophoresis on a 7.5% polyacrylamide gel in a buffer (pH 8.3, 100 mM Tris-HCl, 100 mM borate, 2 mM EDTA) at 160 V for 1.5 hours. After electrophoresis, the gel was stained with 0.6 mg/l ethidium bromide (EtBr). Gel fragments containing 263-bp DNA fragments were sealed in a dialysis tube and submerged in a buffer for electrophoresis. Then, the DNA fragments were electrically eluted from the gel [J. Mol. Biol. 110, 119 (1977)]. A solution in this dialysis tube was recovered and poured on an Elutip-d^{*} column (Schleicher & Schnell) previously buffered with a solution containing 0.2 M NaCl, 20 mM Tris-

*Trade-mark

HCl (pH 7.4) and 1.0 mM EDTA to allow the DNA fragment to be adsorbed. Then, the DNA fragments were eluted with a solution containing 1.0 M NaCl, 20 mM Tris-HCl (pH 7.4) and 1.0 mM EDTA. Twice as much ethanol as the eluate was added
5 to the eluate, and the mixture was cooled to -20°C. Then, the DNA fragments were precipitated by centrifugation.

3-2 A series of stages for forming a double stranded structure of an hPTH gene can also be achieved by a process shown in Fig. 4-2. Referring to Fig. 4-2, the mark —
10 indicates that a 5'-terminal hydroxyl group is phosphorylated. 5 µl portions of the phosphorylated reaction solutions of the twelve kinds of DNA fragments [corresponding to DNA fragments #2 to #13 (SEQ NOS: 5 to 16)] obtained in the above item 2 were combined with 2 µg
15 of DNA fragments #1 (SEQ ID NO: 4) and #14 (SEQ ID NO: 17) corresponding to the 5'-terminus to 50 µl. Then, 5 units of T4 DNA ligase (Takara Shuzo) was added thereto, followed by incubation at 15°C for 20 hours.

The product thus obtained was subjected to
20 electrophoresis on a 8% polyacrylamide gel in a buffer (pH 8.3, 100 mM Tris-HCl, 100 mM boric acid, 2 mM EDTA) at 125 V for 2 hours. After electrophoresis, the gel was stained with 0.6 mg/l EtBr. Gel fragments containing 263-bp DNA fragments were sealed in a dialysis tube and submerged in a
25 buffer for electrophoresis. Then, the DNA fragments were electrically eluted from the gel. A solution in this dialysis tube was subjected to phenol treatment twice, followed by recovery of an aqueous layer (an upper layer).

Then, twice as much ethanol as the aqueous layer was added thereto, and the mixture was cooled to -70°C . The DNA fragments were thereafter precipitated by centrifugation. Thus, about 1 μg of the DNA fragments was obtained. After 5 phosphorylation with T4 polynucleotide kinase (Takara Shuzo), the DNA fragments were subjected to the following experiment 4-2.

4. Cloning of hPTH Gene (Fig. 5)

4-1 As a cloning vector, E. coli plasmid pBR322-
10 derived pUC19 [J. Messing, Gene 33 103-109 (1985)] was used. pUC19 DNA was reacted in 20 μl of a reaction solution [20 mM Tris-HCl (pH 7.6), 7 mM MgCl_2 , 150 mM NaCl, 10 mM 2-mercaptoethanol, 20 units of NdeI (New England Biolabs), 15 units of BamHI (Takara Shuzo)] at 37°C for 24
15 hours. Then, the resulting product was diluted 5 times with water, and treated at 65°C for 20 minutes to inactivate the enzyme. 5 μl of this reaction solution was mixed with about 5 equivalents of the DNA fragments obtained in the above item 3-1 to prepare 20 μl of a
20 reaction solution containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl_2 , 10 mM DTT, 1 mM spermidine, 0.1 mg/ml BSA and 1 mM ATP. Then, T4 DNA ligase (New England Biolabo) was reacted with this solution at 14°C for 15 hours to ligate the hPTH gene to the plasmid.

25 Using this reaction solution, the E. coli JM109 strain [J. Messing, Gene 33, 103-119 (1985)] was transformed according to methods known in the art. Namely, 50 μl of competent cells [D. Hanahan, J. Mol. Biol. 166, 557 (1983)]

stored at -70°C was incubated at 0°C for 15 minutes, and then 10 μl of the above-mentioned reaction solution was added thereto. The resulting solution was further incubated at 0°C for 15 minutes, and then incubated at 42°C for 1.5 minutes and further at 0°C for 2 minutes. To this reaction solution was added 200 μl of LB medium (containing 10 g of Bacto^{*}-tryptone, 5 g of a Bacto^{*}-yeast extract and 5 g of NaCl), and incubated at 37°C for 1 hour. This E. coli was seeded onto LB agar medium containing 50 $\mu\text{g/ml}$ ampicillin, 100 $\mu\text{g/ml}$ X-Gal and 0.1 mM isopropyl- β -D-thiogalactopyranoside (IPTG), and incubated at 37°C overnight. Of the resulting ampicillin-resistant colonies, 14 β -galactosidase-deficient strains were selected and plasmid DNAs of transformed strains thereof were crudely purified by the alkali method [T. Maniatis et al., Molecular Cloning, (Cold Spring Harbor Laboratory) 368-369 (1982)], followed by digestion with NcoI and BamHI, and further with NdeI and BamHI. The electrophoresis patterns of these digests on a 1.7% agarose gel revealed that one strain was a transformed strain into which the hPTH gene was correctly inserted.

4-2 The hPTH gene was also cloned by the following method. As a cloning vector, pUC19 (Takara Shuzo) was used. 0.5 μg of pUC19 DNA was reacted in 10 μl of a reaction solution [50 mM Tris-HCl (pH 7.5), 10 mM MgCl_2 , 100 mM NaCl, 1 mM dithiothreitol, 20 units of NdeI (New England Biolabs), 10 units of BamHI (Takara Shuzo)] at 37°C for 5 hours. Then, the resulting product was treated at

*Trade-mark

65°C for 15 minutes to inactivate the enzyme. 1 µl of this reaction solution was mixed with about 10 equivalents of the DNA fragment obtained in the above item 3-2, and the hPTH gene was ligated to the plasmid using a DNA ligation
5 kit (Takara Shuzo). The transformation into the E. coli JM109 strain was carried out in the same manner as with the above item 4-1. Of the resulting ampicillin-resistant colonies, 17 β-galactosidase-deficient strains were selected and plasmid DNAs of transformed strains thereof
10 were crudely purified by the alkali method, followed by digestion with NcoI and BamHI, and further with NdeI and BamHI. The electrophoresis patterns of these digests on a 1.5% agarose gel revealed that three strains were transformed strains into which the hPTH gene was correctly
15 inserted.

The cloning vectors obtained in the above items 4-1 and 4-2 were named pU·PTH·C19. 20 ml of LB medium containing 50 µg/ml ampicillin was inoculated with one loopful of E. coli JM109 recombinants having this plasmid
20 pU·PTH·C19, and cultivated at 37°C overnight with shaking. Plasmid DNA was crudely purified from this culture solution, and dissolved in 80 µl of TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA] containing 20 µg/ml RNase.

Example 2

25 Construction of Plasmid for Expression of hPTH and Production of Transformant (Fig. 5)

1) About 10 µg of pU·PTH·C19 obtained in the above item 4 of Example 1 was reacted in a reaction solution [150

mm NaCl, 20 mM Tris-HCl (pH 7.8), 7 mM MgCl₂, 10 mM
mercaptoethanol, 40 units of NdeI, 20 units of BamHI
(Takara Shuzo)] at 37°C for 5 hours. Then, 263-bp DNA
fragments were purified by 1.7% agarose gel electrophoresis
5 according to known methods. On the other hand, as a vector
for expression, pET3C [F. W. Stadler et al., Methods in
Enzymology 195, 60-89 (1990)] was used. pET3C DNA was
digested with NdeI and BamHI in the same manner as above,
and four times as much water as the resulting reaction
10 solution was added thereto, followed by heating at 65°C for
20 minutes to inactivate the enzymes.

Each of the 263-bp DNA and the plasmid DNA has single
stranded cohesive ends produced by NdeI digestion and BamHI
digestion at both ends thereof.

15 Both of them were mixed with each other, and the
mixture was reacted with T4 DNA ligase (New England
Biolabs) in the presence of 50 mM Tris-HCl (pH 7.6), 10 mM
MgCl₂, 10 mM DTT, 1 mM spermidine, 0.1 mg/ml BSA and 1 mM
ATP at 14°C for 16 hours to ligate the DNAs to each other,
20 followed by transformation of the E. coli JM109 strain in
the same manner as above. Then, this E. coli was seeded
onto LB agar medium containing 50 µg/ml ampicillin, and
cultivated at 37°C for 1 day. The resulting ampicillin-
resistant colonies were selected. Plasmid DNAs of the
25 transformed strains were further digested by combinations
of restriction enzymes such as NdeI-BamHI, BglII-BamHI,
EcoRI-NdeI and AvrII-BglII. Transformed strains containing
the correct hPTH genes were selected by their patterns of

polyacrylamide electrophoresis. The plasmids for expression thus obtained were named pE-PTH, and the transformed strains were named E. coli JM109/pE-PTH.

2) Plasmid DNAs were isolated from JM109/pE-PTH
5 obtained in the above item 1), and crudely purified to transform E. coli MM294(DE3) having T7 RNA polymerase necessary for expression of a T7 promoter (European Patent Unexamined Laid-Open No. 416505). First, 10 ml of LD medium was inoculated with one loopful of E. coli
10 MM294(DE3), and cultivated at 37°C with shaking to a Klett of 60 to 180. To this culture solution were added 10% w/v polyethylene glycol, 5% v/v dimethyl sulfoxide and 50 mM MgCl₂ (pH 6.5), and a reaction solution was brought up to 100 µl with the addition of LB medium. 10 mg of the
15 plasmid DNAs was added thereto, and incubated at 4°C for 10 minutes, followed by seeding onto LB agar medium containing 50 µg/ml ampicillin. Then, cultivation was carried out overnight at 37°C.

Plasmid DNAs obtained from the resulting colonies
20 similarly with the method described above were digested with restriction enzymes, and transformed strains containing the hPTH genes were selected by their patterns of electrophoresis. These strains were named E. coli MM294(DE3)/pE-PTH.

25 E. coli MM294(DE3) pE-PTH was deposited with the Institute for Fermentation, Osaka, Japan (IFO) under the accession number IFO 15087 on August 28, 1990, and also deposited with the Fermentation Research Institute, the

Agency of Industrial Science and Technology, the Ministry of International Trade and Industry, Japan (FRI) under the accession number FERM BP-3110 on September 25, 1990.

Example 3

5 Production of hPTH

1) E. coli MM294(DE3) pE-PTH was cultivated overnight at 37°C in LB medium containing 50 µg/ml ampicillin with shaking. 100 µl of the resulting culture solution was added to 10 ml of the same medium dispensed in 200 ml
10 flasks, and cultivated at 37°C to a Klett of about 170. Then, IPTG was added thereto to a concentration of 0.1 mM. After cultivation was further continued for 2 hours, 1 ml of the culture solution was centrifuged at 15,000 rpm at 4°C for 5 minutes. The resulting cells were dissolved in
15 100 µl of a solution containing 0.5 M Tris-HCl (pH 6.8), 10% glycerol, 10% (w/v) sodium dodecyl sulfate (SDS), 0.1% (w/v) β-mercaptoethanol and bromophenol blue [U. K. Laemmli, Nature 227, 680 (1970)]. After boiling for 3 minutes, the solution was subjected to 16% SDS-
20 polyacrylamide gel electrophoresis (PAGE). After electrophoresis, the gel was stained with Coomassie Brilliant Blue. As a result, an intense band indicating the same mobility as that of a standard hPTH sample was observed (refer to Fig. 6). Further, another gel was
25 subjected to western blotting using an hPTH antibody. As a result, the same stain pattern as with the standard hPTH sample was obtained (refer to Fig. 7). Quantitative comparison with the standard sample for the stain of the

gels and western blotting revealed that hPTH is expressed in an amount of about 200 mg per liter of culture solution.

2) hPTH accumulated in E. coli was purified in the following manner. Cells from 200 ml of a culture solution
5 obtained similarly with the above-mentioned method were suspended in a buffer (5 ml) containing 8 M urea, 50 mM Tris-HCl (pH 7.5), 50 mM EDTA and 1 mM α -toluenesulfonyl fluoride, and vigorously stirred under ice cooling for about 1 hour to disrupt the cells, followed by
10 centrifugation at 15,000 rpm at 4°C for 20 minutes. The supernatant was recovered, and the precipitate was similarly extracted twice with 3 ml portions of a buffer having the same composition. The extracts were combined with the supernatant, followed by two-fold dilution. The
15 resulting solution was passed through a CM-Toyo Pearl* column (10 ml) of TSK-Gel* (Tosoh) equilibrated with 50 mM ammonium acetate buffer (pH 5) containing 4 M urea to allow a desired product to be adsorbed. The column was washed with 50 mM ammonium acetate buffer (pH 5) containing 4 M
20 urea. About 10 ml of the buffer was required for washing. When absorption at 280 nm disappeared, the column was developed by a linear gradient of 50 ml of 50 mM ammonium acetate buffer (pH 5)-50 ml of 0.5 M ammonium acetate buffer (pH 6) (flow rate: 10 ml/hour, volume of 1 fraction:
25 2 ml). Fractions 33 to 47 were collected and lyophilized. These fractions were subjected to reversed phase HPLC under the following conditions:

Column: YMC-Pack* A-325 S-5 120A ODS (1X30 cm) (Y. M.

*Trade-mark

C.)

Solvent: A linear gradient of 25% to 50% acetonitrile
containing 0.1% trifluoroacetic acid (for 30
minutes)

5 Flow rate: 3 ml/minute

The main peak (retention time: 17.0 minutes) contained the
desired product. The resulting eluate was passed through a
Bio-Rad^{*} AG1X8 (acetate form) column (Bio-Rad Laboratory)
and the washings were also combined therewith. Then,
10 acetonitrile was removed by distillation, followed by
lyophilization. 4.2 mg of desired hPTH was obtained as a
white powder.

The following analytical results revealed that this
sample was hPTH of high purity.

15 a) Reversed phase HPLC showed a single sharp peak
(refer to Fig. 8).

Column: YMC-Pack^{*} A-324 S-5 DDS 120A (10X300 mm)

Eluents: Solution A (0.1% trifluoroacetic acid)

20 Solution B (acetonitrile containing 0.1%
trifluoroacetic acid)

Linear gradient: 0 to 3 minutes (0%, B), 3 to 10
minutes (0 to 30%, B), 10 to 70
minutes (30 to 45%, B)

25 b) SDS-PAGE and western blotting after electrophoresis
also showed a single band of the same mobility as that of
the standard sample. The conditions were the same as with
Figs. 6 and 7 (refer to Figs. 9 and 10).

c) Amino acid analysis (hydrolyzed with 5.7 N

^{*}Trade-mark

hydrochloric acid in the presence of thioglycollic acid in a sealed tube under reduced pressure at 110°C for 24 hours, and values in parentheses indicate theoretical values):

Asp (10) 10.5; Thr (1) 0.96; Ser (7) 6.04; Glu (11)
5 11.73; Pro (3) 2.88; Gly (4) 4.69; Ala (7) 7.03; Val (8)
7.91; Met (2) 1.96; Ile (1) 1.06; Leu (10) 10.6; Phe (1)
1.10; Lys (9) 9.32; His (4) 4.04; Arg (5) 4.00; Trp (1)
0.97

(Recovery: 82.3%)

10 d) N-terminal amino acid sequence analysis by a gas-phase sequencer Model 470A (Applied Biosystems) revealed that the sequence from the 1-position Ser to the 15-position Leu was correct.

e) In first atom bombardment mass spectrometry
15 analysis [JMS-HX110* (Nihon Denshi) was used] after cyanogen bromide cleavage in 0.1 N hydrochloric acid, the following peaks were observed:

(The 1-position to the 7-position) - homoserine (MH+)

Found: 858.5

20 Theoretical: 858.5

(The 1-position to the 17-position) - homoserine (MH+)

Found: 1990.2

Theoretical: 1990.0

(The 9-position to the 17-position) - homoserine (MH+)

25 Found: 1102.7

Theoretical: 1102.7

No peak derived from formylhomoserine or homoserine was observed.

*Trade-mark

Also in analysis after digestion with asparagine-specific endopeptidase, the following peaks were observed:

(The 1-position to the 16-position) (MH+)

Found: 1819.9

5

Theoretical: 1819.9

(The 17-position to the 33-position) (MH+)

Found: 2168.8

Theoretical: 2168.1

(The 58-position to the 76-position) (M + Na)

10

Found: 2061.8

Theoretical: 2062.0

(The 77-position to the 84-position) (MH+)

Found: 874.4

Theoretical: 874.5

15

From the results obtained above, it was judged that hPTH obtained here did not contain Met or formyl-Met at the N-terminus thereof and had a structure starting with the 1-position Ser and ending in the C-terminal 84-position Gln.

Experimental Example

20

The biological activity of hPTH thus obtained was assayed by a modified method of Shigeno et al. [The Journal of Biological Chemistry 263. 18369-18377 (1988)]. A

culture solution (Hank's solution containing 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES),

25

0.1% bovine serum albumin and 0.5 mM isobutylmethyl-xanthine) containing 0.01, 0.1, 1, 10 or 100 nM the present analogue was added in an amount of 100 μ l to a mouse cranial bone-derived osteoblast-like cell strain, MC3T3-EI

cells, cultivated on a 96-well multiplate (Nunc^{*}), followed by reaction at room temperature for 30 minutes. After addition of 100 μ l of 0.2 N hydrochloric acid, the mixture was immersed in boiling water for 2.5 minutes, and

5 cyclic adenosine monophosphate (cAMP) produced by a PTH receptor was extracted from the cells. The total cAMP in the culture solution and the cells was assayed using a commercial radioimmunoassay kit (cyclic AMP [¹²⁵I] kit "Du Pont-Daiichi", Daiichi Kagaku Yakuhin). An increase in

10 cAMP production depending on the concentration of the human PTH partial peptide (the 1-34 positions) added as a standard was always observed. hPTH obtained in Example 1 described above also had an activity approximately similar to this activity (refer to Fig. 11).

15

The hPTH gene provided by the present invention has the novel DNA sequence and shows a high expression rate of hPTH. Further, the present invention first made it possible to allow hPTH to express in large amounts in the

20 system using E. coli as the host. Furthermore, hPTH can be produced more efficiently by the recombinant DNA technique of the present invention using the synthetic gene corresponding to hPTH, which serves to produce hPTH as a therapeutic agent or to study the biological role of hPTH

25 in vivo.

*Trade-mark

Sequence Listing

SEQ ID NO:1:

LENGTH:252

TYPE:nucleic acid

STRANDEDNESS:double

TOPOLOGY:linear

MOLECULE TYPE:other nucleic acid, synthesizing DNA

FEATURES:

FEATURES KEYS:Mat peptide

LOCATION:1 . . . 252

IDENTIFICATION METHOD:E

FEATURES KEYS:mutation

LOCATION:7, 8, 9, 12, 15, 19, 21, 33, 36, 43, 51, 58, 60, 82, 109, 111,
114, 117, 120, 121, 123, 129, 130, 132, 147, 150, 153, 156, 177, 196, 197,
198, 201, 204, 216, 219

IDENTIFICATION METHOD:S

SEQUENCE DESCRIPTION:

TCT GTG TCC GAG ATT CAG TTA ATG CAT AAC CTT GGC AAA CAT TTG AAC	48
Ser Val Ser Glu Ile Gln Leu Met His Asn Leu Gly Lys His Leu Asn	
1 5 10 15	
TCC ATG GAG CGT GTA GAA TGG CTG CGT AAG AAG TTG CAG GAT GTG CAC	96
Ser Met Glu Arg Val Glu Trp Leu Arg Lys Lys Leu Gln Asp Val His	
20 25 30	
AAT TTT GTT GCC TTA GGT GCC CCA TTG GCT CCT CGT GAT GCT GGT TCC	144
Asn Phe Val Ala Leu Gly Ala Pro Leu Ala Pro Arg Asp Ala Gly Ser	

35	40	45	
CAA AGA CCA CGT AAA AAG GAA GAC AAT GTC TTA GTT GAG AGC CAT GAA			192
Gln Arg Pro Arg Lys Lys Glu Asp Asn Val Leu Val Glu Ser His Glu			
50	55	60	
AAA TCC CTA GGC GAG GCA GAC AAG GCC GAT GTG AAT GTA TTA ACT AAA			240
Lys Ser Leu Gly Glu Ala Asp Lys Ala Asp Val Asn Val Leu Thr Lys			
65	70	75	80
GCT AAA TCC CAG			252
Ala Lys Ser Gln			
84			

SEQ ID NO:2:

LENGTH:263

TYPE:nucleic acid

STRANDEDNESS:double

TOPOLOGY:linear

MOLECULE TYPE:other nucleic acid, synthesizing DNA

FEATURES:

FEATURES KEYS:CDS

LOCATION:2...262

IDENTIFICATION METHOD:E

FEATURES KEYS:Mat peptide

LOCATION:5...256

IDENTIFICATION METHOD:E

FEATURES KEYS:mutation

LOCATION:11, 12, 13, 16, 19, 23, 25, 37, 40, 47, 55, 62, 64, 86, 113, 115, 118, 121, 124, 125, 127, 133, 134, 136, 151, 154, 157, 160, 181, 200, 201, 202, 205, 207, 220, 223, 259, 261, 263

IDENTIFICATION METHOD:S

SEQUENCE DESCRIPTION:

TATGTCTGTG TCCGAGATTC AGTTAATGCA TAACCTTGGC AAACATTTGA ACTCCATGGA	60
GCGTGTAGAA TGGCTGCGTA AGAAGTTGCA GGATGTGCAC AATTTTGTTG CCTTAGGTGC	120
CCCATTGGCT CCTCGTGATG CTGGTTCCCA AAGACCACGT AAAAAGGAAG ACAATGTCTT	180
AGTTGAGAGC CATGAAAAAT CCCTAGGCCA GGCAGACAAG GCCGATGTGA ATGTATTAAC	240
TAAAGCTAAA TCCCAGTAAT GAG	263

SEQ ID NO:3:

LENGTH:265

TYPE:nucleic acid

STRANDEDNESS:double

TOPOLOGY:linear

ANTISENSE:yes

SEQUENCE DESCRIPTION:

GATCCTCATT ACTGGGATTT AGCTTTAGTT AATACATTCA CATCGGCCTT GTCTGCCTCG	60
CCTAGGGATT TTTCATGGCT CTCAACTAAG ACATTGTCTT CCTTTTTACG TGGTCTTTGG	120
GAACCAGCAT CACGAGGAGC CAATGGGGCA CCTAAGGCAA CAAAATTGTG CACATCCTGC	180
AACTTCTTAC GCAGCCATTC TACACGCTCC ATGGAGTTCA AATGTTTGCC AAGGTTATGC	240
ATTAAGTGAA TCTCGGACAC AGACA	265

SEQ ID NO:4

LENGTH:30

TYPE:nucleic acid

STRANDEDNESS:single

TOPOLOGY:linear

MOLECULE TYPE:other nucleic acid, sythesizing DNA

FEATURES

FEATURES KEYS:CDS

LOCATION:2...30

IDENTIFICATION METHOD:E

SEQUENCE DESCRIPTION:

TATGTCTGTG TCCGAGATTC AGTTAATGCA 30

SEQ ID NO:5

LENGTH:34

TYPE:nucleic acid

STRANDEDNESS:single

TOPOLOGY:linear

ANTISENSE:yes

MOLECULE TYPE:other nucleic acid, sythesizing DNA

FEATURES

FEATURES KEYS:CDS

LOCATION:1...34

IDENTIFICATION METHOD:E

SEQUENCE DESCRIPTION:

AGGTTATGCA TTAAC**G**AAT CTCGGACACA GACA 34

SEQ ID:6

LENGTH:45

TYPE:nucleic acid

STRANDEDNESS:single

TOPOLOGY:linear

MOLECULE TYPE:other nucleic acid, sythesizing DNA

FEATURES

FEATURES KEYS:CDS

LOCATION:1...45

IDENTIFICATION METHOD:E

FEATURE DESCRIPTION:

TAACCTTGGC AACATTTGA ACTCCATGGA GCGTGTAGAA TGGCT 45

SEQ ID:7

LENGTH:45

TYPE:nucleic acid

STRANDEDNESS:single

TOPOLOGY:linear

MOLECULE TYPE:other nucleic acid, sythesizing DNA

FEATURES

FEATURES KEYS:CDS

LOCATION:1...45

IDENTIFICATION METHOD:E

SEQUENCE DESCRIPTION:

TTACGCAGCC ATTCTACACG CTCCATGGAG TTCAAATGTT TGCCA

45

SEQ ID:8

LENGTH:30

TYPE:nucleic acid

STRANDEDNESS:single

TOPOLOGY:linear

MOLECULE TYPE:other nucleic acid, sythesizing DNA

FEATURES

FEATURES KEYS:CDS

LOCATION:1...30

IDENTIFICATION METHOD:E

SEQUENCE DESCRIPTION:

GCGTAAGAAG TTGCAGGATG TGCACAATTT

30

SEQ ID:9

LENGTH:30

TYPE:nucleic acid

STRANDEDNESS:single

TOPOLOGY:linear

ANTISENSE:yes

MOLECULE TYPE:other nucleic acid, sythesizing DNA

FEATURES

FEATURES KEYS:CDS

LOCATION:1...30

IDENTIFICATION METHOD:E

SEQUENCE DESCRIPTION:

GCAACAAAAT TGTGCACATC CTGCAACTTC

30

SEQ ID:10

LENGTH:46

TYPE:nucleic acid

STRANDEDNESS:single

TOPOLOGY:linear

MOLECULE TYPE:other nucleic acid, sythesizing DNA

FEATURES

FEATURES KEYS:CDS

LOCATION:1...46

IDENTIFICATION METHOD:E

SEQUENCE DESCRIPTION:

TGTTGCCTTA GGTGCCCCAT TGGCTCCTCG TGATGCTGGT TCCCAA

46

SEQ ID:11

LENGTH:46

TYPE:nucleic acid

STRANDEDNESS:single

TOPOLOGY:linear

ANTISENSE:yes

MOLECULE TYPE:other nucleic acid, sythesizing DNA

FEATURES

FEATURES KEYS:CDS

LOCATION:1...46

IDENTIFICATION METHOD:E

SEQUENCE DESCRIPTION:

TGGTCTTTGG GAACCAGCAT CACGAGGAGC CAATGGGGCA CCTAAG

46

SEQ ID:12

LENGTH:41

TYPE:nucleic acid

STRANDEDNESS:single

TOPOLOGY:linear

MOLECULE TYPE:other nucleic acid, sythesizing DNA

FEATURES

FEATURES KEYS:CDS

LOCATION:1...41

IDENTIFICATION METHOD:E

SEQUENCE DESCRIPTION:

AGACCACGTA AAAAGGAAGA CAATGTCTTA GTTGAGAGCC A

41

SEQ ID:13

LENGTH:41

TYPE:nucleic acid

STRANDEDNESS:single

TOPOLOGY:linear

ANTISENSE:yes

MOLECULE TYPE:other nucleic acid, sythesizing DNA

FEATURES

FEATURES KEYS:CDS

LOCATION:1...41

IDENTIFICATION METHOD:E

SEQUENCE DESCRIPTION:

TTTTCATGGC TCTCAACTAA GACATTGTCT TCCTTTTAC G 41

SEQ ID:14

LENGTH:42

TYPE:nucleic acid

STRANDEDNESS:single

TOPOLOGY:linear

MOLECULE TYPE:other nucleic acid, sythesizing DNA

FEATURES

FEATURES KEYS:CDS

LOCATION:1...42

IDENTIFICATION METHOD:E

SEQUENCE DESCRIPTION:

TGAAAAATCC CTAGGCGAGG CAGACAAGGC CGATGTGAAT GT 42

SEQ ID:15

LENGTH:42

TYPE:nucleic acid

STRANDEDNESS:single

TOPOLOGY:linear

ANTISENSE:yes

MOLECULE TYPE:other nucleic acid, sythesizing DNA

FEATURES

FEATURES KEYS:CDS

LOCATION:1...42

IDENTIFICATION METHOD:E

SEQUENCE DESCRIPTION:

GTAAATACAT TCACATCGGC CTTGTCTGCC TCGCCTAGGG AT 42

SEQ ID:16

LENGTH:29

TYPE:nucleic acid

STRANDEDNESS:single

TOPOLOGY:linear

MOLECULE TYPE:other nucleic acid, sythesizing DNA

FEATURES

FEATURES KEYS:CDS

LOCATION:1...28

IDENTIFICATION METHOD:E

SEQUENCE DESCRIPTION:

ATTAATAAAA GCTAAATCCC AGTAATGAG 29

SEQ ID:17

LENGTH:27

TYPE:nucleic acid

STRANDEDNESS:single

TOPOLOGY:linear

ANTISENSE:yes

MOLECULE TYPE:other nucleic acid, sythesizing DNA

FEATURES

FEATURES KEYS:CDS

LOCATION:6...27

IDENTIFICATION METHOD:E

SEQUENCE DESCRIPTION:

GATCCTCATT ACTGGGATTT AGCTTTA

27

27580-68

39

CLAIMS:

1. DNA containing a synthetic gene encoding the human parathyroid hormone represented by the following DNA sequence:

TCTGTG TCCGAGATTC AGTTAATGCA TAACCTTGGC

5 AAACATTTGA ACTCCATGGA GCGTGTAGAA TGGCTGCGTA

AGAAGTTGCA GGATGTGCAC AATTTTGTTG CCTTAGGTGC

CCCATTGGCT CCTCGTGATG CTGGTTCCCA AAGACCACGT

AAAAAGGAAG ACAATGTCTT AGTTGAGAGC CATGAAAAAT

CCCTAGGCGA GGCAGACAAG GCCGATGTGA ATGTATTAAC

10 TAAAGCTAAA TCCCAG.

2. A method for producing the DNA of claim 1, which comprises enzymatically ligating a plurality of oligodeoxynucleotides one to another to form the DNA and inserting the DNA into a vector if necessary.

15 3. A vector containing the DNA of claim 1, wherein the synthetic gene is inserted into a control region of an Escherichia coli T7 promoter.

4. A host cell transformed by the DNA claimed in claim 1 or the vector of claim 3.

20 5. A method for producing human parathyroid hormone which comprises cultivating the transformant claimed in claim 4, accumulating human parathyroid hormone in a culture medium, and collecting the same.

- 40 -

6. The DNA according to claim 1, which comprises an initiation codon ATG at the 5'-terminal side and a stop codon at the 3'-terminal side of the DNA sequence shown in claim 1 so that the human parathyroid hormone may be expressed directly without a fused peptide when the DNA is inserted into an appropriate expression vector and a host cell transformed with the expression vector is cultivated.

7. The DNA according to claim 6, wherein the stop codon is TAA.

8. The DNA according to claim 7, shown as
SEQ ID NO: 2.

9. A vector containing the DNA of claim 1, identified as plasmid pU•PTH•C19.

10. An expression vector comprising the DNA as defined in claim 1, 6, 7 or 8 and a promoter upstream from the DNA.

11. The expression vector according to claim 10, wherein the promoter is one for E. coli selected from the group consisting of trp promoter, lac promoter, recA promoter, λ PL promoter, lpp promoter and T7 promoter, one for Bacillus subtilis selected from the group consisting of SP01 promoter, SP02 promoter and penP promoter, a promoter for major cell wall proteins for Bacillus brevis, one for Saccharomyces cerevisiae selected from the group consisting

- 41 -

of PH05 promoter, PGK promoter, GAP promoter and ADH promoter.

12. The expression vector according to claim 10, wherein the promoter is one for animal cells selected from the group consisting of SV40-derived promoter and mouse leukemia virus (MuLV)-derived LTR region promoter and the vector also comprises an enhancer.

13. The expression vector according to claim 11, wherein the promoter is the one for E. coli.

14. The expression vector according to claim 13, identified as vector pET•PTH•3C.

15. The expression vector according to claim 13, identified as pE-PTH.

16. A host cell transformed with the expression vector of claim 10.

17. A host cell of E. coli, Bacillus subtilis, Bacillus brevis or Saccharomyces cerevisiae transformed with the expression vector of claim 11.

18. An E. coli cell transformed with the expression vector of claim 13 or 14.

- 42 -

19. The cell according to claim 18, identified as E. coli MM294 (DE3) pE-PTH (FERM BP-3110).

20. A method for producing human parathyroid hormone, which comprises:

cultivating the cell of claim 16, 17 or 18 under such conditions that the human parathyroid hormone is expressed, and

separating and purifying the human parathyroid hormone.

FETHERSTONHAUGH & CO.
OTTAWA, CANADA

PATENT AGENTS

F i g . 1

TCT. GTG. TCC. GAG. ATT. CAG. TTA. ATG. CAT. AAC. CTT. GCC. AAA. CAT. TTG. AAC. TCC. ATG. GAG
 Ser-Val-Ser-Glu-Ile-Gln-Leu-Met-His-Asn-Leu-Gly-Lys-His-Leu-Asn-Ser-MET-Glu

CGT. GTA. GAA. TGC. CTC. CGT. AAG. AAG. TTG. CAG. GAT. GTG. CAC. AAT. TTT. GTT. GCC. TTA. GGT. GCC
 Arg-Val-Glu-Trp-Leu-Arg-Lys-Lys-Leu-Gln-Asp-Val-His-Asn-Phe-Val-Ala-Leu-Gly-Ala

CCA. TTG. GCT. CCT. CGT. GAT. GCT. GGT. TCC. CAA. AGA. CCA. CGT. AAA. AAG. GAA. CAC. AAT. GTC. TTA
 Pro-Leu-Ala-Pro-Arg-Asp-Ala-Gly-Ser-Gln-Arg-Pro-Arg-Lys-Lys-Glu-Asp-Asn-Val-Leu

GTT. GAG. AGC. CAT. GAA. AAA. TCC. CTA. GCC. GAG. GCA. GAC. AAG. GCC. GAT. GTG. AAT. GTA. TTA. ACT
 Val-Glu-Ser-His-Glu-Lys-Ser-Leu-Gly-Glu-Ala-Asp-Lys-Ala-Asp-Val-Asn-Val-Leu-Thr

AAA. GCT. AAA. TCC. CAG
 Lys-Ala-Lys-Ser-Gln

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Fig. 2

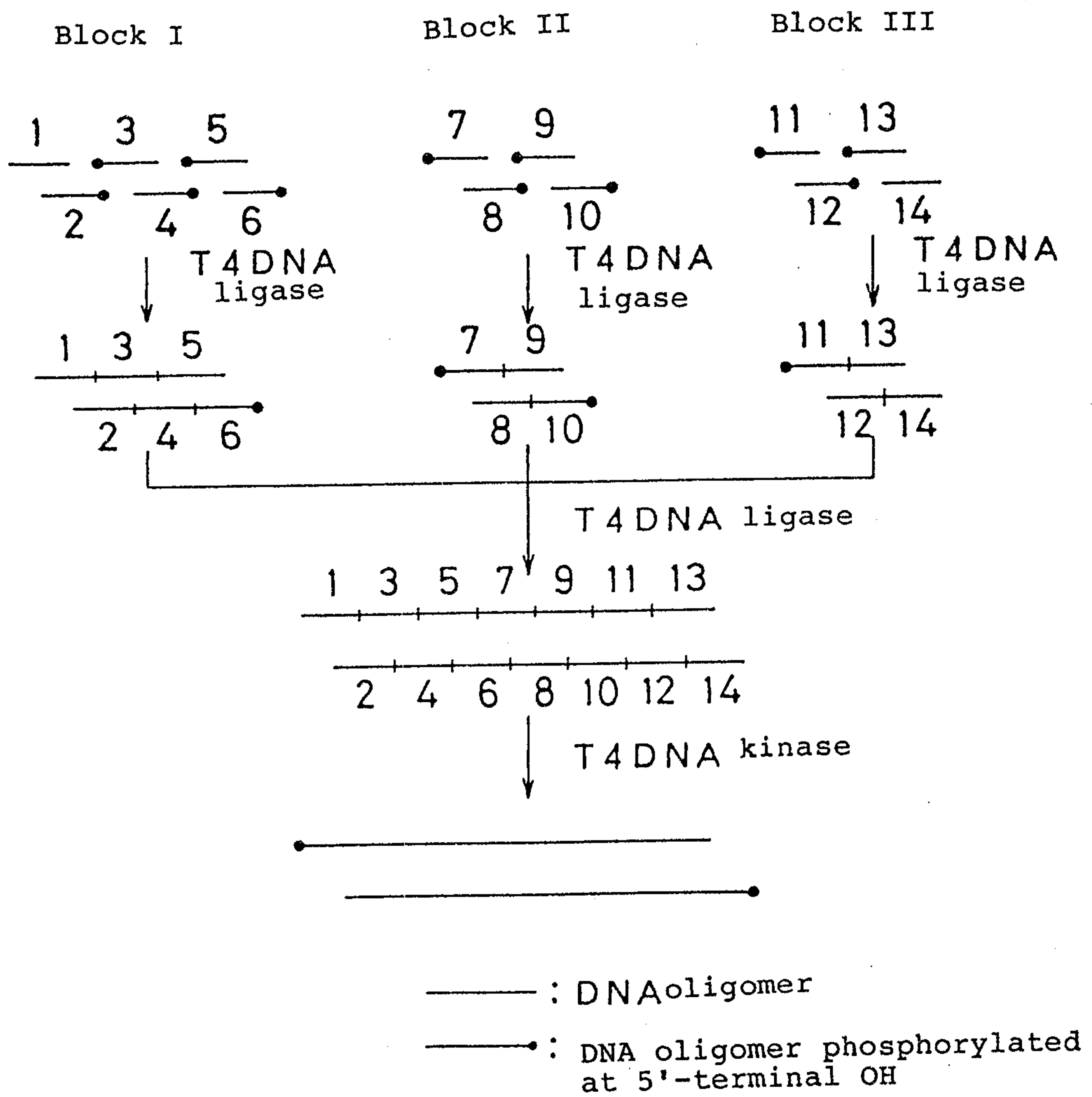
10	20	30	40	50	60
TATGTCGTG	TCCGAGATTC	AGTTAATGCA	TAACCTTGGC	AAACATTGA	ACTCCATGGA
ACAGACAC	AGGCTCTAAG	TCAATTACGT	ATTGGAACCG	TTTGTAACCT	TGAGGTACCT
Nde I	Hinf I				Nco I
70	80	90	100	110	120
GCGTGTAGAA	TGGCTGGTA	AGAAGTTGCA	GGATGTGCAC	AATTGTGTG	CCTTAGGTGC
CGCACATCTT	ACCGACGCAT	TCTTCAACGT	CCTACACCGT	TTAAAACAAC	GGAATCCACG
			HgiA I		Dde I
130	140	150	160	170	180
CCCATTTGGCT	CCTCGTGATG	CTGGTTCCCA	AGACCACCGT	AAAAAGGAAG	ACAAATGTCCT
GGGTAACCGA	GGAGCACTAC	GACCAAGGGT	TTCTGGTGCA	TTTTTTCCTTC	TGTTACAGAA
Bgl I					Dde I
190	200	210	220	230	240
AGTTGAGAGC	CATGA AAAAT	CCCTAGGGCA	GGCAGACAAG	GCCGATGTGA	ATGTAATTAAC
TCAACTCTCG	GTA CTTT TTA	GGGATCCGCT	CCGTCTGTTC	CGGCTACACT	TACATAATTG
		Avr II		HaeIII	
250	260	270			
TAAAGCTAAA	TCCCAGTAAT	GAG			
ATTTCGATT	AGGGTCATTA	CTCCTAG			
Alu I		BamH I			

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Fig. 3

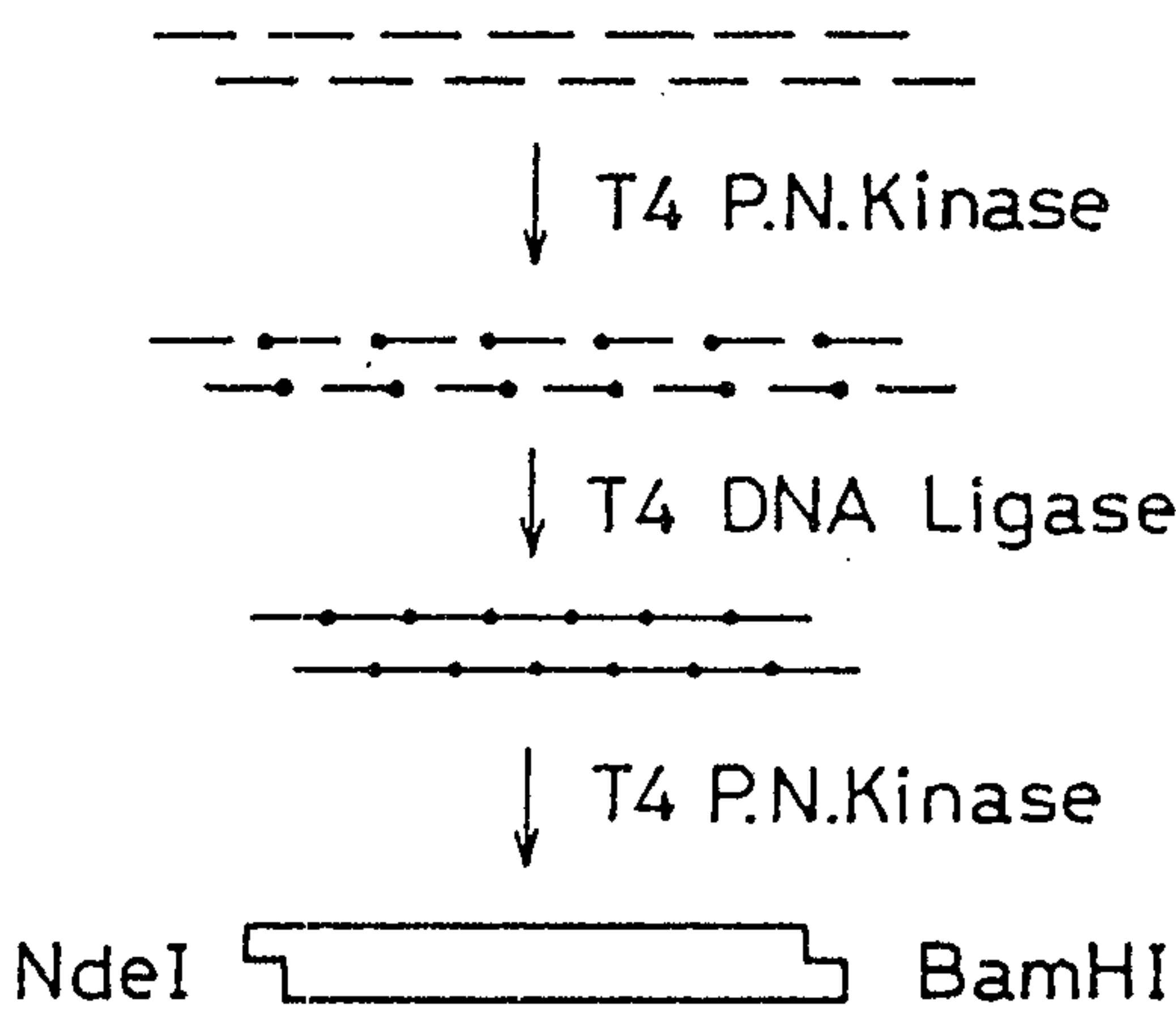
#1	5'TATGTCTGTGTC	CGAGATTCA	GTTAATGCA3'
#2	3'ACAGACACAGGCT	CTAAGTCAAT	TACGTATTGGA5'
#3	5'TAACCTTGGCAAA	CAATTTGAAC	TCCATGGAGCCGTAGAA
#4	3'ACCGTTTGTA	AAACTTGAGGT	ACCCTCGCACATCTTACCGACGCATT5'
#5	5'GCGTAAGAA	AGTTGCAGGAT	GTGCACAATT3'
#6	3'CTTCAACGTC	CTACACGTGT	TAAACAACACG5'
#7	5'TGTTGCCCTTAG	GTGCCCCCAT	TGGCTCCTCGTGATGCTGGTTCCCAA3'
#8	3'GAATCCACGGGT	AAACCGAGGAG	CACCTACGACCAAGGTTTCTGTGT5'
#9	5'AGACCACGTA	AAAAAGGAAG	ACAAATGTCTTAGTTGAGAGCCA3'
#10	3'GCATTTTTCCT	TTCTGTACAGAA	TCAACTCTCGGTACTTT5'
#11	5'TGAAAAATCCC	CTAGGCGAGGC	AGCAGACAAGGCCGATGTGAA
#12	3'TAGGGATCCG	CTCCGCTCTGT	TCCGGCTACACTTACATAATTG5'
#13	5'ATTAACTAA	AGCTAAATCCC	CAGTAATGAG3'
#14	3'ATTTCGATTT	AGGGTCA	TTACTCCTAG5'

Fig. 4-1



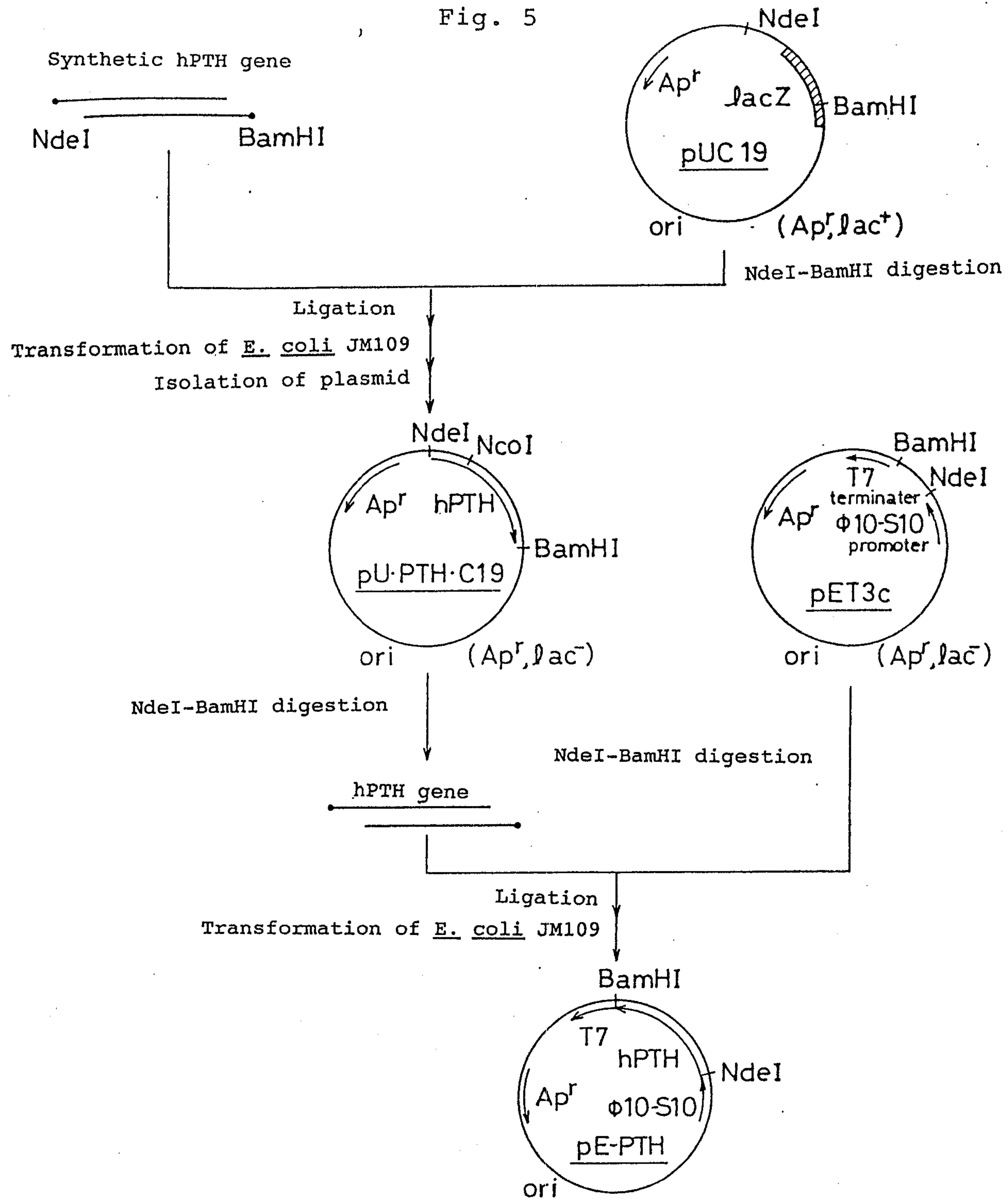
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Fig. 4-2



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Fig. 5



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Fig. 6

1 2 3 4

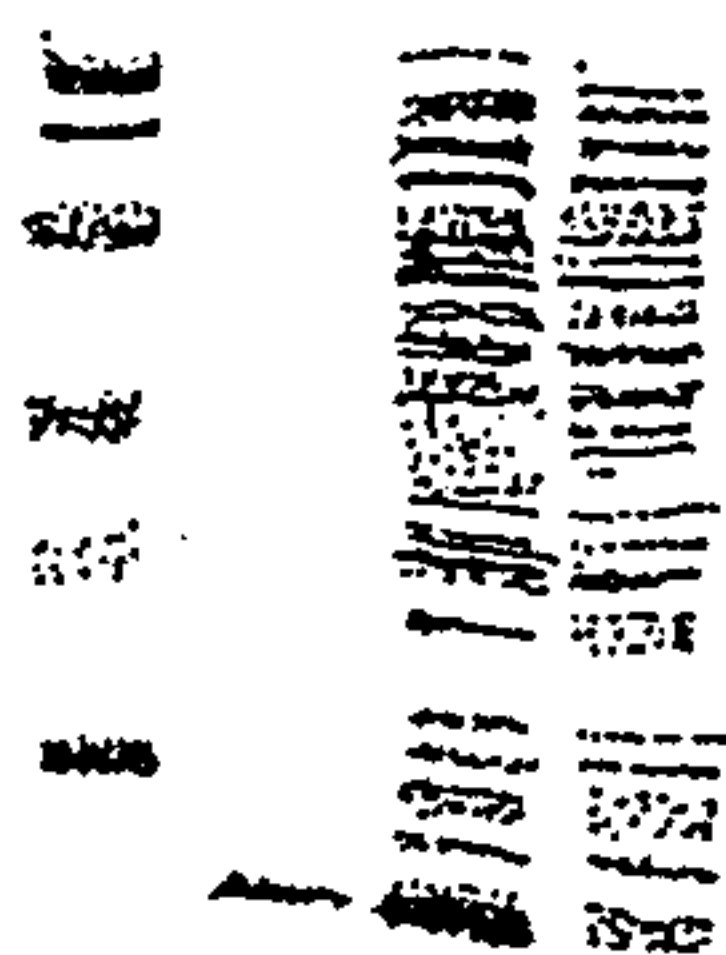
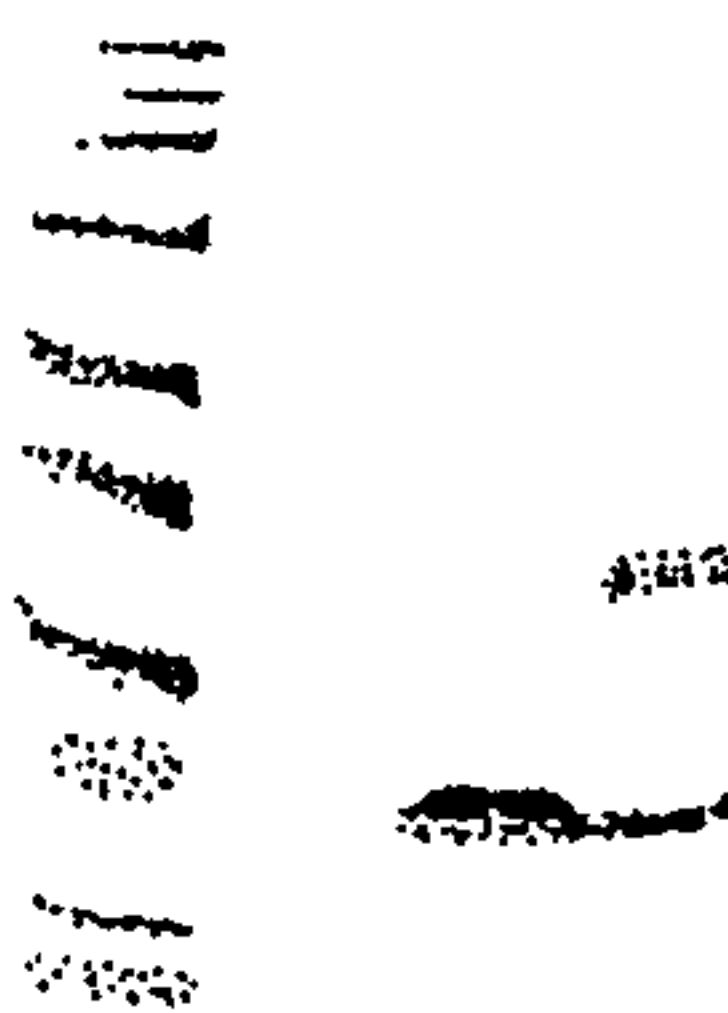


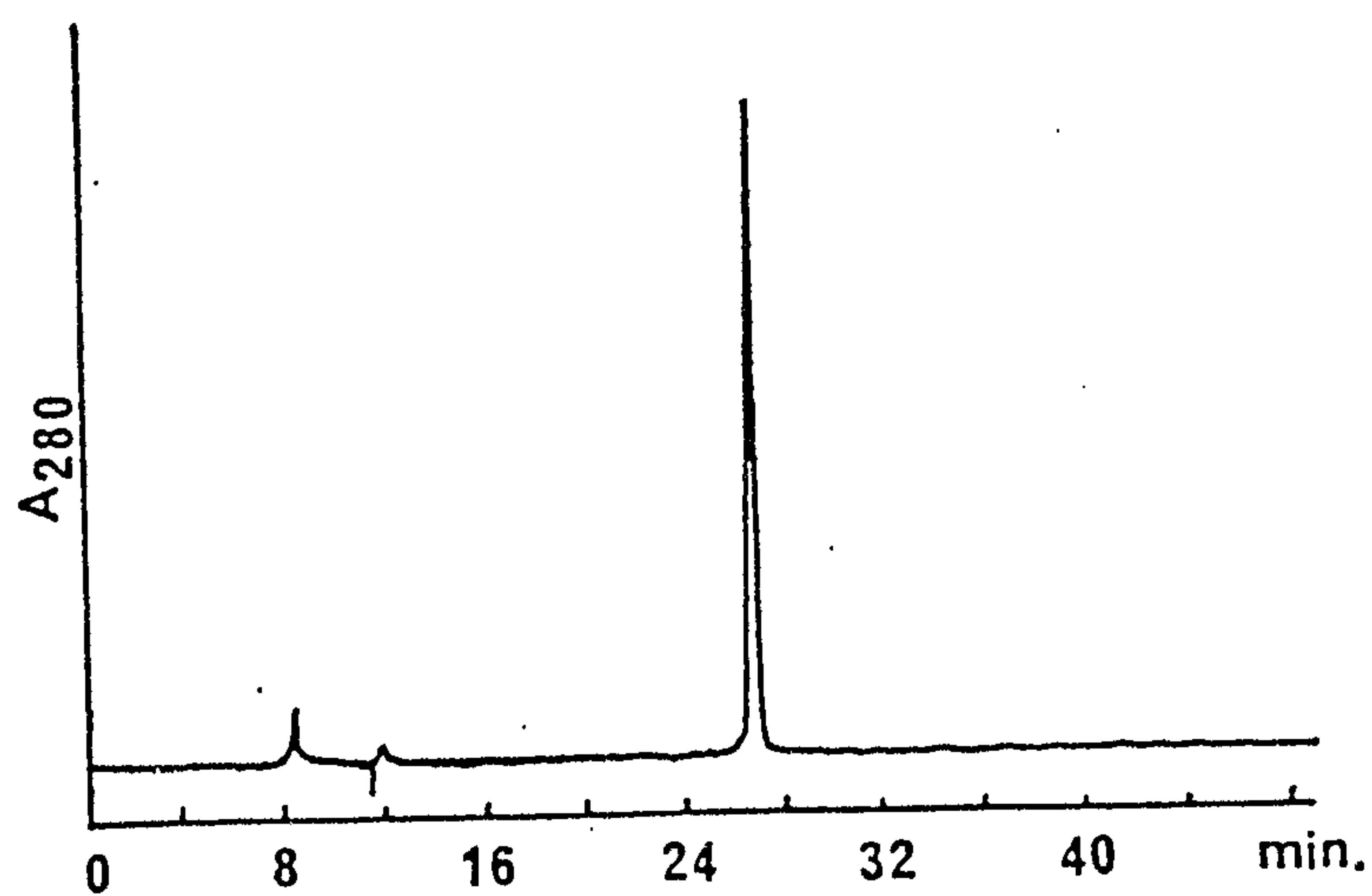
Fig. 7

1 2 3 4



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Fig. 8

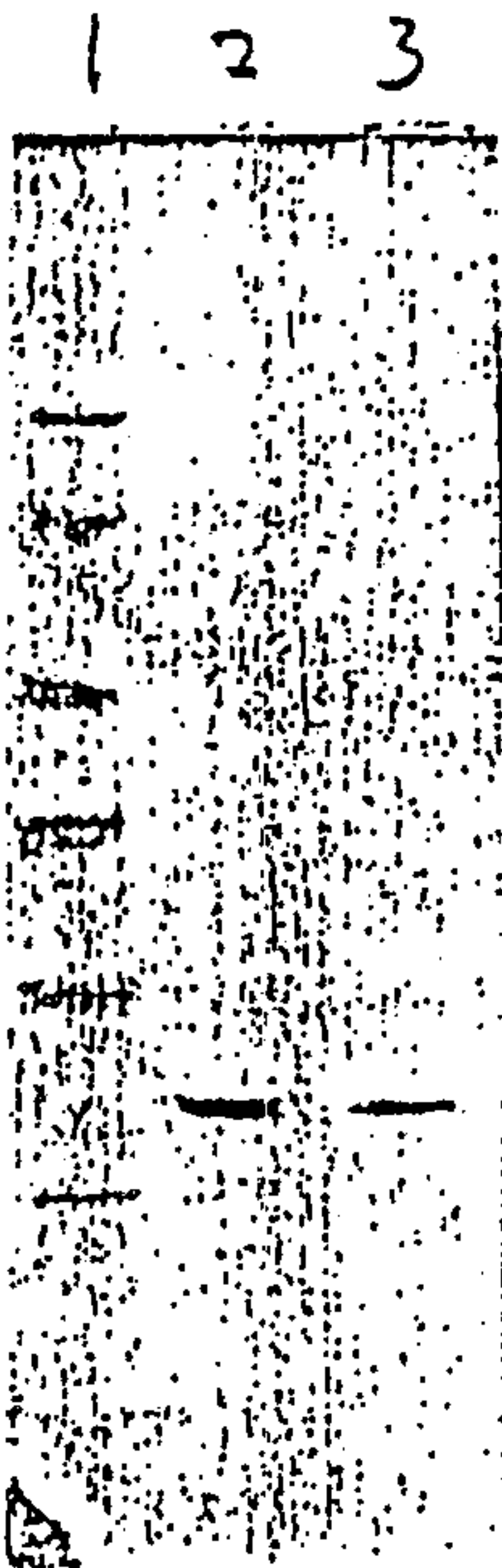


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Fig. 9

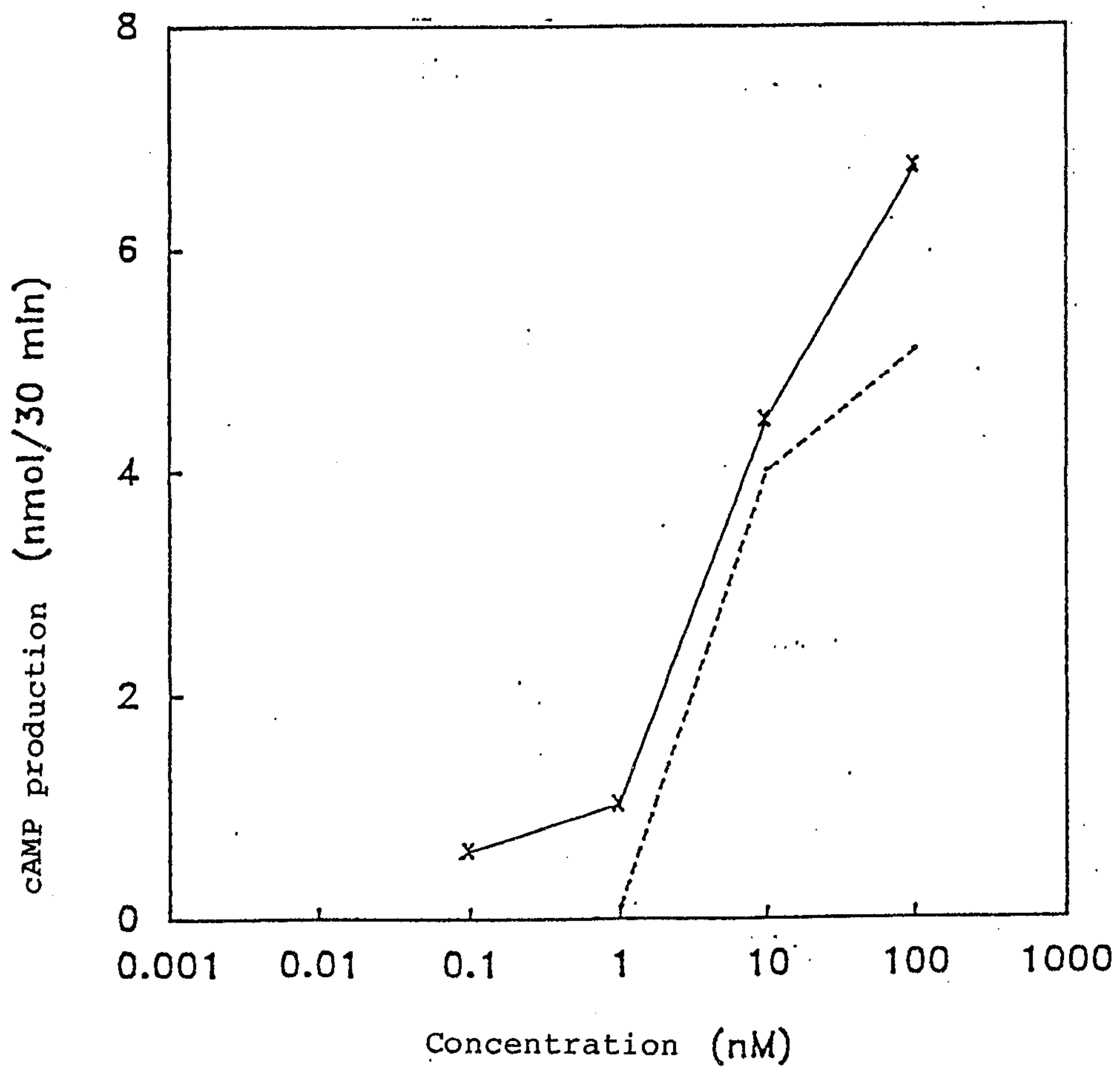


Fig. 10



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Fig. 11



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