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Ⅱ．過去の研究歴

1985.7～1988.9 中国白求恩医科大学で口腔微生物と免疫学の研究について

1988.9～1991.7. 中国白求恩医科大学の大学院で唾液腺癌用重中C-mycC原位癌遺伝子の表達について

1991.7～1995.2 头頸部癌用重の分子遺伝学の研究について

Ⅲ．過去の研究実績

Ⅰ. 29例粘液表皮癌、臨床病理学的分析、診断病理学雑誌、1:81－84, 1994.

Ⅱ. 1類提高PCR, 敏感性と特異性の方法、診断病理学雑誌、4:239－241, 1995.

Ⅲ. Lipopolysaccharide Stimulates osteoclast-like multinucleate cell formation CFU-GM-derived cell cultures. J. Meikai Univ. Sch. Dent 24: 413－421, 1995.

Ⅳ．本年度の研究業績

(1) 学会、研究会等においての口頭発表（学会名・内容）

Eighteenth Annual Meeting of the American Society for Bone and mineral Research. 1996

The cDNA of h. BMP mature region was cloned into Pkk223.3. SDS-PAGE revealed a new protein band about 14kD. it was dissolved in derafuring solvent and purified by using heparin saphanose.

(2) 学会誌等に発表した論文　　無 ・ (有)　（雑誌名・論文名）

Lipopolysaccharide Stimulates Osteoclast-like Multinucleate Cell Formation in CFU-GM-derived Cell Cultures

Junichi TATSUMI, Noriyoshi KURIHARA, Shuhua YANG, Masamichi SHIMOYAMA, Fumio ARAI and Katsumi IKEDA Department of Periodontology, Meikai University School of Dentistry, Sakado, Saitama, Japan 明海大学歯学雑誌, 24(2) 1995

Ⅴ．今後の研究計画及び希望

東北大学歯学部大学院口腔微生物学講座にて大学院生として研究に従事、研究終了後は中国に帰国し、中国の大学の発展に寄与したい。

研　究　報　告（日本語、又は英語で書いて下さい。 2,000字程度で記載して下さい。）

来日後,明海大学歯学部歯周病学講座 池田克巳教授ならびに教室員の配慮のおかげで私の研究活動は円滑に開始できた。また，実験に必要な機械・器具で教室内に不足していたものは，速やかに購入していただくことができた。一年目の私の研究テーマはヒト破骨細胞の培養方法の習得と破骨細胞形成に関わる菌体内毒素の作用機序を主に行った。また，中国にて研究をしていたヒトBMP-4についての新しい精製方法についての研究も同時に進行させた。ヒト破骨細胞形成におけるLPSの作用については以前より本講座栗原徳善講師ならびに辰巳順一講師らが中心となって研究を継続していたことから，私はこの実験系については途中から参加した。その結果，破骨細胞前駆細胞にLPSを作用させるとこの前駆細胞がIL-1αおよびβを産生しこれがオートクラインに働いて破骨細胞形成を促進する結果を見出した。本研究内容については，すでに論文発表されている (別刷り添付) 。さらに私は，上記先生らと共にヒトBMP-4の精製に精進した。大腸菌内に導入したBMP-4発現遺伝子は順調にBMP-4を含むタンパク質を産生したが，本タンパクの性質上その精製には困難を極めた。現在ダイマーの状態までは精製が可能となったが，精製効率，モノマーへの転換が現在の課題として残った。この段階での研究内容は，日本骨代謝学会，アメリカ骨代謝学会で演題発表したが，より優れた論文発表のためにも研究内容を完結したいと考えている。(この研究内容については別添資料の通りである。)本教室での研究活動は，私を中国から招いていただいた池田教授の御定年もあり，困難となってきた。私は現在，東北大学歯学部大学院を受験し入学の内諾を得ている。，今後私の研究は東北大学で継続し，初期の目的を達成したいと考えている。

Ⅶ. 指導教官の意見

まず、このたびは、本研究に対し貴協会から有益な研究助成金を賜り、本人はもとよりわたくしも心から深く感謝致しております。

ところで、本研究のメインである「BMP-4」の精製については、本人も上述のように、今後の課題を残す結果となっていますが、できれば現在入学の内諾を得ている東北大学でこれが継続できればと念じています。省みて、本研究で本人は直接の指導教員からの助言・示唆等にはよく応え、所期の成果を求め、たえず熱心に日夜努力を続けたことに小生「概ね良好」といえる評価を与えたい。別添に第１４回日本骨代謝学会(平成８年７月)で公表した内容等についてでありますが、この点よろしく願いあげます。

Construction of Recombinant Mature Region of Human Bone Morphogenetic Protein-4 in *Escherichia coli* by use of the Polymerase Chain Reaction

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Key Words : human osteosalcoma cells, BMP-4, recombinant

Escherichia coli における RT-PCR をもちいたヒト BMP-4 成熟領域のクローニングについて

明海大学歯学部歯周病学講座

楊　淑華、辰巳順一、栗原徳善、曲 建香、池田克己

抄録：本研究においてわれわれはまず、ヒト骨原性細胞株(OS-732)より得られたヒト Bone morphogenetic protein-4 (BMP-4)を含む mRNA を採取し、プライマーを作成し RT-PCR 法を用いて成熟活性領域を含む BMP-4 の cDNA を作製した。つぎに BMP-4 成熟活性領域 cDNA をプラスミドベクターpKK223.3 の 48 クローンに挿入し、その中の 8 クローンにリコンビナント cDNA の発現が restriction enzyme analysis によって確認できた。以上の結果から、われわれは原核細胞(Escherichia coli)からヒト BMP-4 の成熟活性領域を含むタンパクを産生させる可能性をみいだした。

ABSTRACT

In this study, we amplified the complementary DNA of the mature region of human bone morphogenetic protein-4 (hBMP-4) from a human osteogenetic cell line (OS-732), using the reverse transcription-polymerase chain reaction (RT-PCR) method with two specific primers flanking the mature region . The cDNA of hBMP-4 mature region was cloned into the expression plasmid vector-pKK223.3. Forty-eight clones were selected, and eight of them were identified to be recombinants contains cDNA of the mature region by restriction enzyme analysis. Human bone morphogenetic protein-4 mature region recombinants were thus established. This study may lead to availability of mature region peptide of recombinant BMP for study bioactive hBMP from *Escherichia coli*.

Introduction

Bone has a remarkable potential for a continuous balance between its resorption and formation. Bone growth and establishment depend upon bone formation. Urist *et al.* demonstrated that extracts from demineralized bone could induce bone formation if implanted into ectopic sites in rodents (1). The factor responsible for bone formation was named bone morphogenetic protein (BMP). The bone-inducing activity of demineralized bone matrix suggested the possibility of using it to treat large bony defects caused by trauma, surgical resection, or periodontal disease and led to a search for macromolecules possessing bone-inducing activity. The purification of BMPs from bone matrix is rather cumbersome and difficult (2), but high-grade purified BMP was obtained in 1988 when Wozney *et al.* cloned and expressed human full-length BMP cDNA in a eukaroytic cell vector (3).

Several laboratories have cloned and expressed BMP in mammalian cells (4,5). The reports have indicated, however, that considerable expense, time and effort are required to purify the recombinant protein due to a relatively low efficiency of production of BMP in eukaroytic cells(6). Thus, one of the difficulties of studying the biological activities of BMP in basic research and therapeutic application is the deficiency of pure BMP.

To research the possibility of engineering *Escherichia coli* to produce biologically active BMP, we decided to clone the cDNA of the mature region of hBMP-4 alone instead of its full-length cDNA and to apply a denaturation-renaturation protocol to reconstitute the dimer *in vitro* as a novel approach to the production of bioactive BMP from *E.coli.* As a first step toward this goal, we report here in the method and strategy for cloning the cDNA of the mature region of hBMP-4 into a *E.coli* vector by PCR. This study will aid in meeting the basic requirement for researching the biological activity of this protein from *Escherichia coli.*

MATERIALS AND METHODS

Culture of human osteogenetic sarcoma cells and RNA preparation

Cells of a human osteogenetic sarcoma cell line (OS-732) were cultured in RPMI 1640 medium (GIBCO, Grand Island, NY, USA) containing 10% heat-inactivated fetal calf serum (FCS ; GIBCO) and 10 mM HEPES, and incubated at 37°C. After a monolayer had formed, the cells were washed *in situ* with cold PBS and lysed in lysis buffer (0.5% Nonidet P-40, 0.14M NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, 1 mM dithiothreitol, and 100 units/ml placental RNAse inhibitor). After digestion with 50μg/ml proteinase K and extraction with phenol/chloroform, the RNA was precipitated by ethanol. The RNA concentration and purity were calculated by absorption at 260 and 280 nm. One μl RNA was subjected to reverse transcription.

cDNA synthesis

cDNA from total RNA of human osteogenetic sarcoma cell was synthesized by AMV reverse transcriptase with oligo(dT)12-18 as a primer. Briefly, 1 μg of total RNA from human osteogenetic sarcoma was added to the reverse transcription reaction buffer to make a final volume of 20 μl (50 mM Tris-HCl, pH 8.3; 50 mM KCl, 10 mM concentration of each of DTP, 10 pM of oligo(dT) 12-18, 20 units of AMV reverse transcriptase), and the mixture was incubated at 37°C for 45 minutes. A 5-μl volume of the reaction mixture was subjected to the PCR.

Primer Synthesis

A pair of primers flanking the mature region of hBMP-4 was designed and synthesized according to the cDNA sequence of human BMP-4 (GenBank, Los Alamos National Laboratory, Los Alamos, NM.). An additional sequence ATAG for quarantining the restriction enzyme recognition ; the restriction enzyme *EcoRI* recognition sequence GAATTC ; and a starting codon, ATG, were added to the upstream of the 5'-primer codon sequence (Fig. 1), 5'-primer sequence (P1) : 5'-ATAGGAATTCATGCCTAAGCATCACTCACAGC-3'. An additional sequence TAAT, Hind III recognition sequence AAGCTT, and anti-stop codon TCA were added upstream

of the 3'-primer. Whose sequence was : 5'-TAATAAGCTTTCAGCAGCGGCACCCACATCC CTCT-3'.

Polymerase Chain Reaction (PCR)

The cDNA of hBMP-4 mature region from osteogenetic sarcoma cells was amplified mediated by the primers. In a 100-μl reaction, 5 μl of total cDNA was amplified in 10 mM Tris-HCl (pH 8.3), 50 mM KCl , 1.5 mM $MgCl_2$, 0.001% gelatin, 200 mM concentration of each dNTP, 50 pM concentration of each primer, and 2.5 units of Taq DNA polymerase (Primega, Madison,WI,USA). The mixture was overlaid with 50 μl of mineral oil and subjected to 30 cycles of amplification in a thermal cycler (Program Temp Control System PC-700 ; ASTEC Co., Fukuoka, Japan), according to the following cycle parameters : denaturation for 60 sec. at 93℃, reanealing for 30 sec. at 54℃ and extension for 30 sec. at 72℃. A denaturation step of 60 sec at 93℃ and an extension for 5 min. at 72℃ were added at the initial and final cycle, respectively. The PCR products were precipitated by ethanol and digested with restriction enzyme.

← 図 1 挿入

Preparation of plasmid

Expression plasmid pKK 223.3 was chosen as the vector (Fig. 2). pKK223.3 DNA isolation was carried out by the standard alkaline extraction method (7).

← 図 2 挿入

Digestion of DNA with EcoRI and Hind III restriction enzyme

Twenty micrograms of plasmid DNA and PCR-products were digested respectively for 2 hr at 37℃ with 40 units of restriction endonuclease Hind III in a 100-μl reaction volume consisting of 100 mM NaCl, 10 mM Tris-HCl (pH 8.5), 5 mM $MgCl_2$, and 1 mM 2-mercaptoethanol. About 5μl of the digestion mixture was electrophoresed on a 1% agarose gel to confirm the complete digestion of the plasmid DNA. The digested DNA was extracted with phenol/chloroform, and then the aqueous phase was extracted twice with chloroform and precipitated with ethanol. The pellet was washed with 70% ethanol and was dissolved in 20 μl of TE buffer. The EcoRI reaction to cleave the DNA was performed for 3 hr at 37℃ in a 100-μl reaction volume comprising 100 mM NaCl, 50 mM Tris-HCl (pH 7.5), and 10 mM DTT. The

DNA fragments were precipitated with ethanol and purified by electrophoresis on a low-melt temperature agarose gel.

Gel purification of DNA fragment

The digested plasmid DNA and PCR-product DNA were eletrophoresed on 1% low-melt temperature agarose gel at 4℃. The gel was stained with ethidium bromide (Sigma, St.Louis, MO, USA). The desired DNA was identified and excised in the smallest possible volume of agarose. The agarose containing the band was diluted 5 fold with Tris -HCl buffer and liquified by heating to 65℃ for 5 min. The DNA was isolated by phenol-chloroform extraction and then concentrated by ethanol precipitation.

Ligation and transformation

DNA ligation was performed in a 20 μl reaction volume consisting of 1 unit of T_4 DNA ligase, 0.5 μg cDNA of BMP-4, and 1 μg plasmid DNA added to the ligation buffer to make final concentrations of 50 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$,20 mM DTT, and 1 mM ATP , and the mixture was incubated at 16℃ for 4 hr. The ligation mixture was used to transfect *E.coli* JM105 [supE endA sbcB15 hsdR4 rpsL thi⊿ (lac-proAB)] by the standard $CaCl_2$ procedure. The transfected bacteria were spread on ampicillin-containing LB plates and incubated at 37℃ for 16 hr. The ampicillin-resistant colonies were selected and analyzed with restriction enzymes.

Assays of recombinant by restriction enzyme maps

Three kinds of restriction endonucleases, EcoR I, Hind III, and Sty I, were selected to analyze the recombinants according to recombinant plasmid restriction enzyme map (Fig. 2). The plasmid DNA was prepared from the cloned bacteria by rapid lysis for minipreparation of DNA by the alkaline extraction method. The method of EcoRI and Hind III digestion was performed as above, and Sty I mediated cleavage of the DNA was done in 100 mM NaCl, 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 1 mM DTT. Samples of plasmid DNA were digested respectively with Hind III, EcoRI + Hind III, or Sty I. After digestion by the restriction enzymes, the DNA was electrophoresed on an ethidium bromide-agarose gel.

RESULT

cDNA of human BMP-4 mature region amplified by PCR

For amplifying the cDNA of the hBMP-4 mature region from human osteosarcoma cells, we designed and synthesized a pair of primers that flanked the mature region (Fig 1). To ensure cloning efficiency, restriction enzyme EcoRI and Hind III recognition sequences were incorporated into the primers. Because the sequence at terminal region is difficult to be recognized by restriction enzymes, four basic pairs 5'-ATAG-3' and 5'-TAAT-3' were added upstream of the restriction enzyme recognition sequence. A starting codon and stop anti-codon were designed in the primers for future research on for the expression of hBMP-4. The PCR was performed on cDNA synthesized from the RNA of human osteogenetic sarcoma. The PCR product with the desired length of 348 bp, corresponding to the C-terminal 116 amino acids of the hBMP-4 mature region, was obtained (Fig. 3, line B). To confirm the nature of the PCR product, we treated the product with Sty I, as there is a Sty I recognition sequence covering base pairs 131 to 136 of the hBMP-4 mature region . Two fragments with length of about 130 bp and 210 bp were obtained, after the PCR product had been cleaved with the Sty I restriction enzyme (Fig. 3, Line C). The result of restriction enzyme analysis demonstrated that the 384-bp PCR product is the specific cDNA of the hBMP-4 mature region.

← 図3挿入

Cloning cDNA of hBMP-4 mature region into plasmid vector

The plasmid pKK 223.2 was selected as the vector, and the cloning strategy was shown on Fig. 2. After the cDNA of the hBMP-4 mature region and pKK 223.3 were cleaved by EcoRI and Hind III, the cDNA was ligated into pKK 223.3. Three kinds of restriction enzyme maps were prepared to analyze the recombinants. The DNA cleavage by Hind III was used to detect the length of recombinants. Hind III and EcoRI cooperative digestion detected the cDNA of the hBMP-4 mature region insert. The restriction enzyme Sty I recognition sequence present in pKK 223.3 (1249) and hBMP-4 mature region (131) was used to identify the specific cDNA of hBMP mature resion to be inserted cDNA. Eight of 48 clones were identified to be recombined with cDNA of the hBMP-4 mature region by restriction enzyme analysis (Fig 4).

← 図4挿入

DISCUSSION

Progress in using BMP to treat bone defect disease and in our understanding of bone metabolism has been limited by lack of large amounts of purified, biologically active protein. BMPs could be purified from demineralized bone (8), but only 40 μg of BMP could be obtained from 40 kg of bovine bone power, indicating this approach to be rather cumbersome. The purification of recombinant protein BMP from eukaroytic cells has been hampered by inefficient expression in eukaroytic cells. The expression rate of BMP, which protein is related to the TGF superfamily, was rather low compared with that of other proteins, unrelated to the TGF-β family proteins, produced by use of the same eukarotic vector (9). The inefficiency of expression is probably due to the fact that biosynthesis of TGF-related proteins involves post-translational modification, such as dimerization of polypeptides and processing of large precursors into their mature form.

The method of expressing protein from a cloned gene in *E. coli* has proven invaluable in the purification and functioned analysis of eukaroytic proteins (10,11). A high level of protein expressed in *E. coli* often results in cytoplasmic granules that can separated from crude cell lysates by centrifugation (12,13). But so far there have been no reports of BMP with bone-inducing activity, produced by prokaryotic cells. For this reason we cloned the cDNA of the hBMP-4 mature region into the prokaryotic *E.coli*.

Recently, the study of TGF-β-related protein biosynthesis has shown that the polypeptides of the TGF-β superfamily are synthesized as part of large secretory precursors. The precursor polypeptide chains consist of a signal sequence, a proregion of several hundred amino acids, and a highly conserved C-terminal region of approximately 100 amino acids. These protein in C-terminal domain of the precursor are generally cleaved to form the mature bioactive dimers (14). The association between the mature region and the proregion of TGF-β masks the biological activity of the mature dimers, resulting in the formation of an inactive latent form (15). BMP-4 alone can induce bone formation without addition of related growth factors (16). Human BMP-4 is a 33 kDa protein that is a glycosylated, disulfide-linked homodimer of the mature region. The mature region of hBMP-4 comprises the COOH-terminal 116 amino acids of a 408-amino acid precursor. Because accurate proteolytic processing to form the mature region from

the precursor is not feasible in *E.coli,* we chose to clone the cDNA of the mature region of hBMP-4 instead of its full-length cDNA into *E.coli* as a novel approach to obtain biologically active BMP from *E. coli.*

BMP in osteosarcoma have been identified by immunhistochemical studies (17). BMP immunostaining was restricted predominantly to the cytoplasm of malignant cells with a primitive mesenchymal appearance. Particularly, the extraction of a protein fraction with bone morphogenetic activity from human osteosalcoma implies that BMP might be secreted by osteosarcoma cells (18). Thus we selected a human osteosarcoma cell line (OS-732) as a source of hBMP-4 mRNA. For cloning the precise fragment of the hBMP-4 mature region into *E. coli*, we took advantage of the speed and efficiency of the polymerase chain reaction. The PCR is the method not only for directly amplifying minute quantities of genomic DNA from a complex background, but also for amplifying specific cDNA molecules by reverse transcription of complex mixtures of mRNA. The specific fragment amplified is determined by the primers used to anneal to a part of the target sequence. We designed a pair of primers flanking the mature region of hBMP-4. Cloning a blunt-ended of PCR product is often less efficient than that of the other blunt-end DNA fragment (19). The inefficient cloning might result from the addition to the end of PCR product of an A nucleotide by the intrinsic terminal transferase activity of Taq polymerase. To avoid this problem , we introduced the restriction sites of EcoRI and Hind III at the 5' end of the oligonucleotide. Four additional nucleotides were added to ensure the efficient cleavage of the PCR product by restriction enzymes. After the mature region of hBMP was amplified by the PCR method, the specific product could be identified by restriction enzyme analysis. The human BMP-4 recombinant was thus constructed by use of reverse transcriptase-polymerase chain reaction technology.

The disulfide-linked dimer of BMP, which is related to the TGF-β superfamily, is essential for the biological activity of bone formation as loss of induction activity occurs upon reduction of the active dimer (20). But dimerization can not take place in the reducing environment of *E. coli*. However, many proteins expressed in *E.coli* have been reconstructed *in vitro* by reduction and reoxidation method to show biological activity (21). McNally *et al.* demonstrated that *E.coli* could express and assemble of individual component to polymer without

the requirement of a specific eukarotic cell mechanism (22). Currently we are attempting to apply denaturation-renaturation to reconstruct dimer *in vitro* to produce the bioactive BMP.

The authentic bioactive BMP is a glycosylated protein, but the carbohydrate moiety of BMP is not necessary for the induction of bone formation, as euidenced by the fact that deglycosylated BMP could induce bone formation *in vivo* (23). Presumably, BMP without glycosylation from *E.coli* would have effective biological activity. Bioactive, non-glycosylated, disulfid-linked BMP may thus be expected to be obtained from BMP produced by *E. coli.*

As an early stage of our study, we have completed the strategy for cloning the BMP-4 mature region and are now examing this recombinant for protein expression and for biological activity achieved by the *in vitro* denaturation-renaturation approach.

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Figure Regends

Fig.1 Amino acid sequence of human bone morphogenetic protein-4 and the pair of oligonucleotide primers used

Schematic diagram of domain structure of hBMP-4.

Signal peptide, pro-region, and mature region are shown. Two oligonucleotide primers for PCR were synthesized.

Fig.2 Strategy for cloning of hBMP-4 mature region cDNA into pkk223.3

hBMP mature region cDNA was amplified by PCR after reverse transcription of OS-732 cell mRNA. Two gene-specific primers containing the restriction sites were used. After cutting the PCR product and the vector with EcoRI and Hind III restriction enzymes, ligation of the molecules was carried out to obtain the recombinant vector pkk223.3-BMP.

Fig.3 Agarose gel (2.5%) electrophoresis of PCR products and PCR products digested by Sty I

Line A: DNA marker[PQEM-7Zf(+)]

Line B: PCR products

Line C: PCR products cleaved by StyI

Fig.4 Restriction enzyme pattern of recombinant pKK223.3-BMP

Line A: pkk223.3-BMP digested with HindIII [4900bp fragment]

Line B: pkk223.3-BMP digested with StyI [1300bp and 3500bp fragments]

Line C: pkk223.3-BMP digested with EcoRI +HindIII[340bp and 4500bp fragment

Line E: pkk223.3-BMP plasmid DNA

Line D: DNA marker [DNA / Hind III]

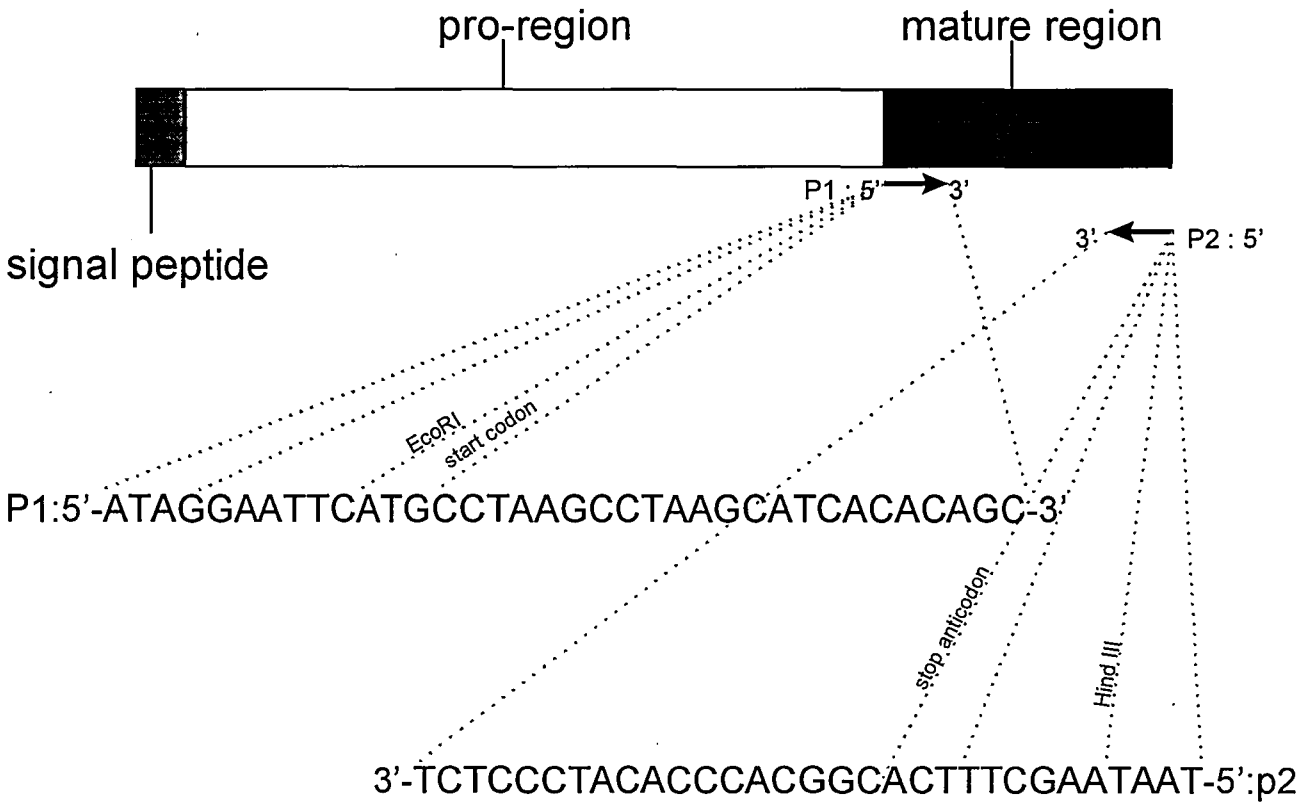
pro-region
mature region
signal peptide
P1 : 5'
3'
3'
P2 : 5'
EcoRI
start codon
P1:5'-ATAGGAATTCATGCCTAAGCCTAAGCATCACACAGC-3'
stop anticodon
Hind III
3'-TCTCCCTACACCCACGGCACTTTCGAATAAT-5':p2

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圖1

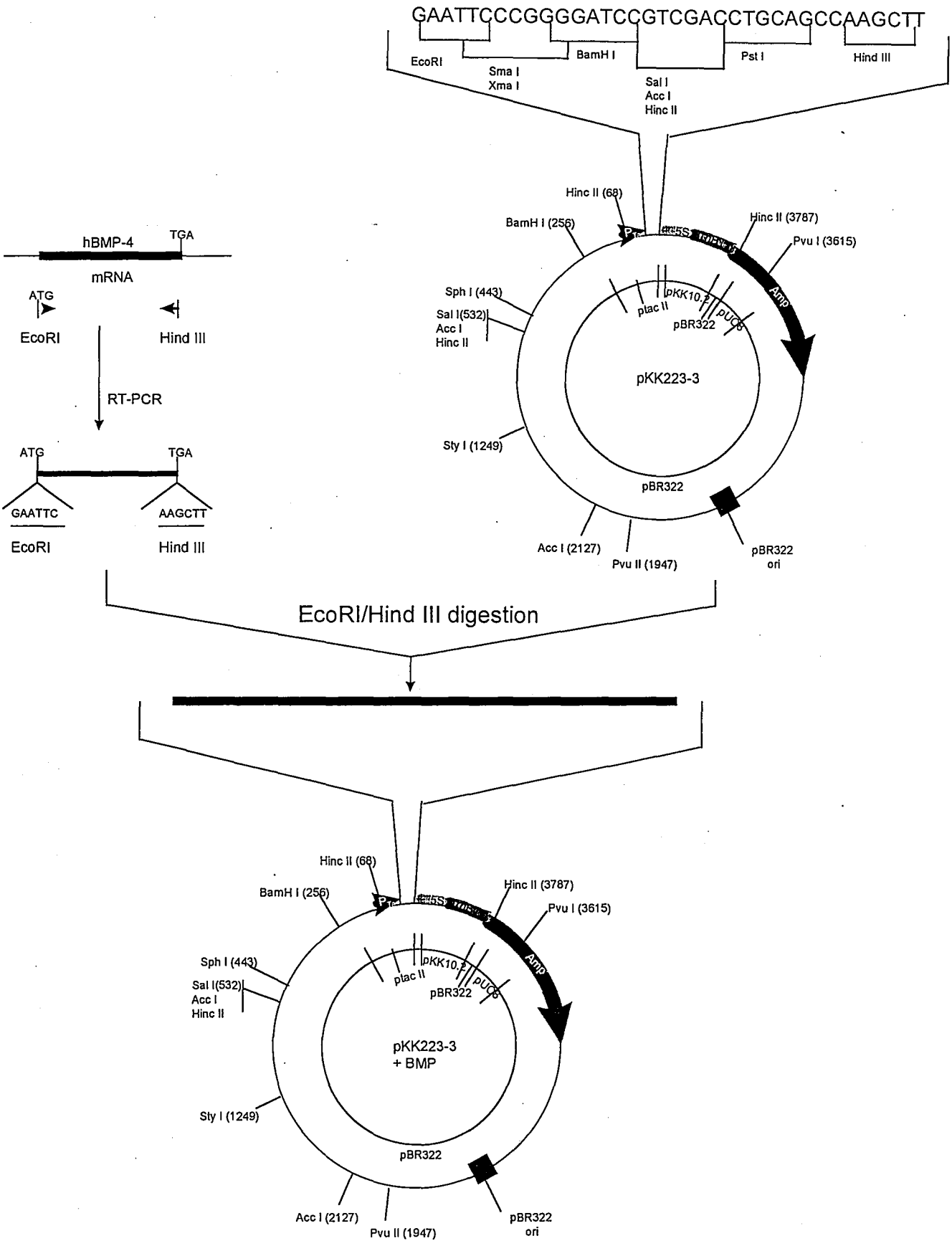
GAATTCCCGGGGATCCGTCGACCTGCAGCCAAGCTT
EcoRI
Sma I
Xma I
BamH I
Sal I
Acc I
Hinc II
Pst I
Hind III
hBMP-4
TGA
mRNA
ATG
EcoRI
Hind III
RT-PCR
ATG
TGA
GAATTC
AAGCTT
EcoRI
Hind III
Hinc II (68)
BamH I (256)
Hinc II (3787)
Pvu I (3615)
Sph I (443)
Sal I(532)
Acc I
Hinc II
ptac II
pKK10.2
pUC8
pBR322
Amp
pKK223-3
Sty I (1249)
pBR322
Acc I (2127)
Pvu II (1947)
pBR322
ori
EcoRI/Hind III digestion
Hinc II (68)
BamH I (256)
Hinc II (3787)
Pvu I (3615)
Sph I (443)
Sal I(532)
Acc I
Hinc II
ptac II
pKK10.2
pUC8
pBR322
Amp
pKK223-3
+ BMP
Sty I (1249)
pBR322
Acc I (2127)
Pvu II (1947)
pBR322
ori

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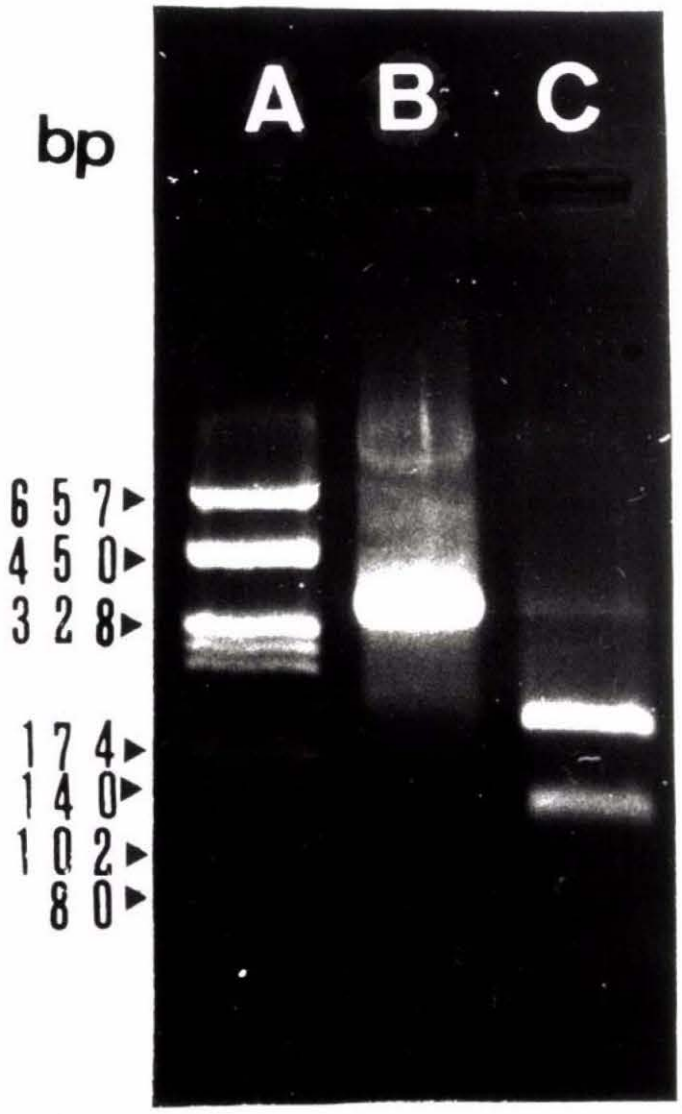
圖 2



横論文

図3

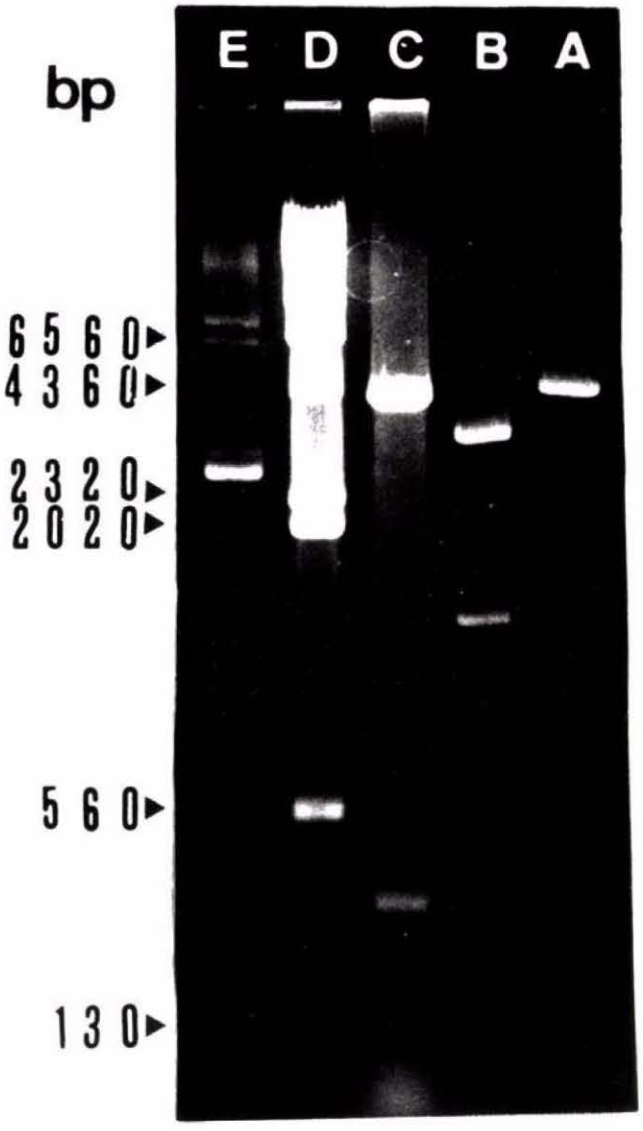


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図4