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Cloning and high level nonfusion expression of recombinant human basic fibroblast growth factor in *Escherichia coli*[1]

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KEY WORDS basic fibroblast growth factor; molecular cloning; gene expression

ABSTRACT

AIM: To obtain high-level expression of nonfusion recombinant human basic fibroblast growth factor (rhbFGF). **METHODS:** hbFGF cDNA was prepared from the total RNA of embryonic brain tissue. As a template, the obtained gene was used to clone nonfusion rhbFGF. New primers were employed to alter the translation initiation region (TIR) and reduce the G+C content through nucleotide change. Using pET-3C as vector, the cloned rhbFGF was expressed in BL21 (DE3). **RESULTS:** rhbFGF was expressed in *E coli* up to 30 % of the total cellular protein. Cation exchange and heparin affinity chromatography were employed to purify the target protein from the supernatant of bacteria lysate. The bioactivity of the purified rhbFGF was identical with the standard bFGF. **CONCLUSION:** Modification of TIR is an effective means to increase nonfusion expression rate of recombinant proteins, such as rhbFGF, in *E coli*.

INTRODUCTION

Human basic fibroblast growth factor (hbFGF) is a potent mitogen for many mesenchyma or neuroectoderm-originated cells. As a single-chain protein, hbFGF has 146 amino acids and pI of 9.6, and M_r is about 17 200. The hbFGF cDNA is a 155-amino acid variant. Natural bFGF was isolated initially from pituitary extracts. Exogenously, bFGF can heal wound, cure bone damage, graft vascula, and regenerate lens, *etc*. In addition, as neuronotrophic factor, bFGF plays an important role in nerve system. In animal study, bFGF can prevent the brain from injury of ischemia and reperfusion in rats[1].

It is almost impossible to obtain sufficient bFGF from animal tissues due to its extremely low quantity and high expense. Therefore, genetic technology has

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been employed to produce bFGF at low cost.

In the last decade, insertion of a short exogenous gene in upstream of bFGF gene to produce recombinant bFGF is a popular practice. Used this fusion technique, the host rhbFGF peptide can be stabilized and expressed effectively. However, fusion bFGF is limited to only external use in clinic due to its potential immunoreaction. Therefore, nonfusion rhbFGF is preferred. Unfortunately, no nonfusion bFGF strain with expression rate of above 10 % of total cellular protein is available.

mRNA translation efficiency is critical in foreign gene expression in *E coli*. Translation initiation region (TIR) is defined as the beginning 70 codon-proximal nucleotide containing important information for expression initiation[2]. Secondary structure of mRNA controls gene expression. It has been well established that the recognition of critical sites within TIR by ribosomal RNA can be seriously stalled in an excessively stable secondary structure, and consequently the initiation of translation is impeded[3].

Evidences have shown that the reduction of G+C content will decrease the stability of secondary structure[4]. On the other hand, according to the codon preference of host bacteria *E coli*, the usage of preferred codons will enhance the translation initiation[5].

Intergration of the aforementioned factors modifies the TIR. In this experiment, an appropriate expression system including pET-3c and BL21 (DE3) was selected, the primer of changed sites is designed to construct the TIR.

MATERIALS AND METHODS

Restriction enzymes *Bgl* II, *Nde* I, and *Bam*H I were purchased from Takara Company (Japan). *Taq* DNA polymerase was from Promega Company (USA). PCR purification kit, gel extraction kit, and Miniprep kit for plasmid extraction were obtained from Qiangen Company (USA).

Vector and host *E coli* strain, BL21 (DE3), and expression vector, pET-3c, were provided by Bioengineering Institute of Jinan University. Primer was synthesized by Shanghai Bioasia Company (China).

RNA extracts and RT-PCR By using acid guanidium isothiocyanate-phenol-chloroform extraction methods, the total RNA was prepared from 100 mg human embryonic brain. Oligonucleotide primers were synthesized according to bFGF coding sequence. The designed upstream primer, bFGF-F, is 5' ATG CAG GCC GGG AGC ATC3' according to GENBANK while the down upstream primer, bFGF-R, is 5' TCA GCT CTT AGC AGA CAT 3'. bFGF-R was used for reverse transcription. PCR was conducted in a sequential procedure: (1) 94 ℃ for 4 min; (2) 32 cycles at 94 ℃ for 30 s, 60 ℃ for 30 s; 72 ℃ for 30 s; (3) 72 ℃ for 10 min.

Modified TIR primer design The upstream primer, bFGF-MF, is 5' ATGC TAG CAT ATG GCT GCT GGT AGT ATT ACA ACC CTG CCG GCG CTG CCG GAA GAT GGT GGT AGT GGT GCA TTC CCG CCG GGC CAC TTC AAG 3'. The downstream primer, bFGF-MR, is 5' TGCA AGA TCT TTA GCT CTT AGC AGA CAT TGG AAG 3'.

Sequence analysis Nucleotide sequence of the modified bFGF was sequenced by Takara Company.

Construction of expression vector including bFGF cDNA With RT-PCR bFGF as template and bFGF-MF and bFGF-MR as primers, PCR production was performed as follows: (1) 94 ℃ for 30 min; (2) 32 cycles at 94 ℃ for 30 s, 58 ℃ for 30 s, 72 ℃ for 30 s; (3) 72 ℃ for 10 min. The obtained product was cut by *Bgl* II and *Nde* I, and cloned into pET-3C to obtain pET-hbFGF (Fig 1-3).

Induction and expression of rhbFGF The obtained vector was transformed into BL21 (DE3). Cells were grown in LB medium with addition of IPTG at mid-log time of cultivation. The level of expression was determined using SDS-PAGE followed by densitometor scanning (Fig 4).

Purification of recombinant bFGF BL21 (DE3) cells were harvested by centrifugation and ultrasonically ruptured. The supernatant of the liquid mixture of the cell debris was fed to the appropriate chromatography columns (Fig 4, 5).

Bioassay of mitogenicity of recombinant hbFGF By using MTT method, the mitogenic effect of hbFGF

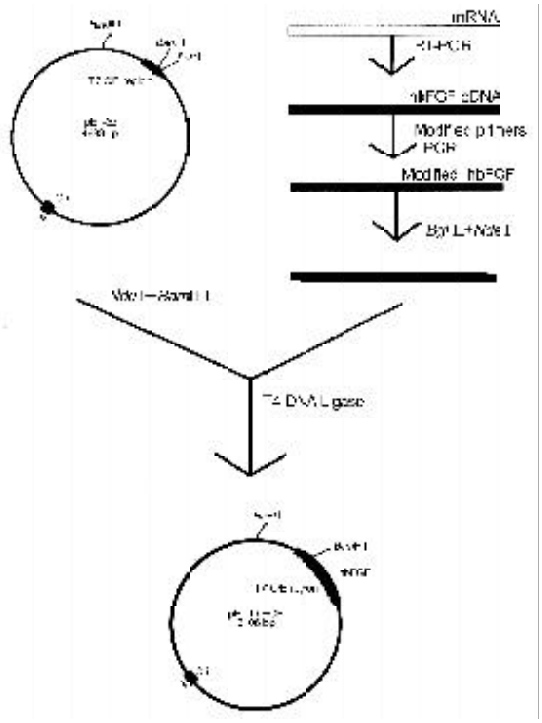

Fig 1. The process of recombinant pET-hbFGF.

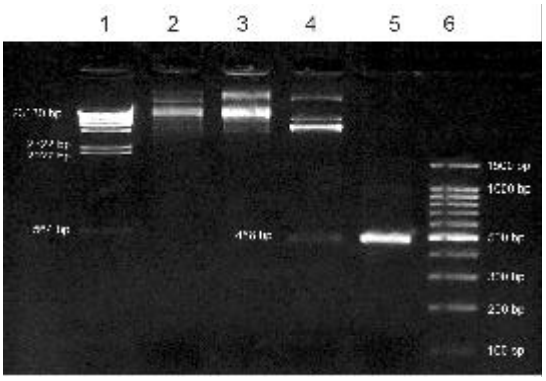

Fig 2. Analysis of pET-hbFGF by restriction enzymatic digestion. Lane Marker: Lamda DNA/*Hind* III (Takara Company). Lane 2: pET-3C plasmid. Lane 3: pET-hbFGF plasmid. Lane 4: pET-hbFGF/*BamH*I+*Hde*I. Lane 5: RT-PCR production of hbFGF. Lane 6 Marker: 100 bp DNA ladder (Takara Company).

on 3T3 fibroblasts was quantified in a 96-well plate.

RESULTS

Results of RT-PCR As indicated in the agarose gel, bFGF produced by RT-PCR was about 468 bp (Lane 5 of Fig 2).

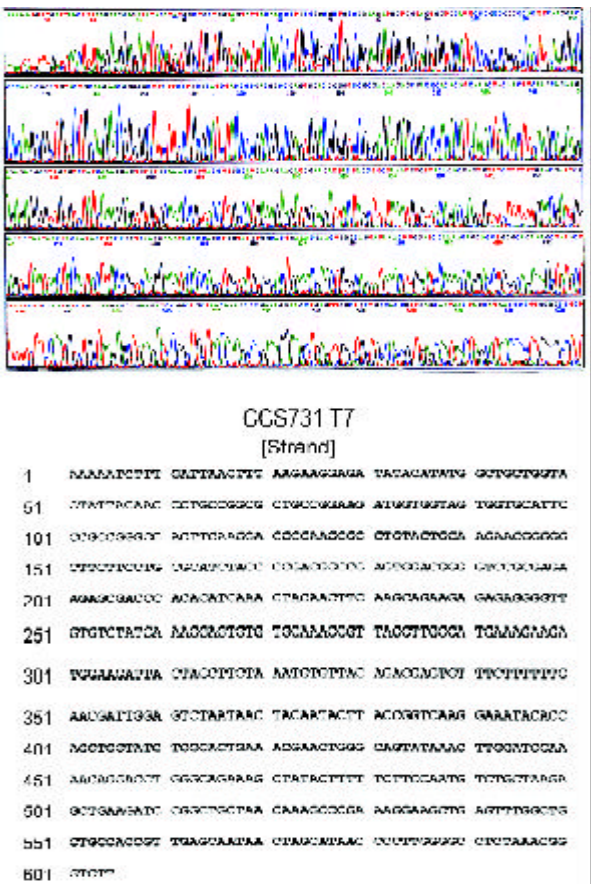

Fig 3. The sequence result of pET-hbFGF.

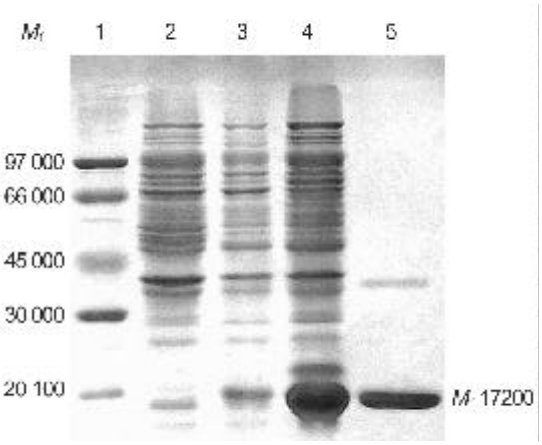

Fig 4. SDS-PAGE analysis of the bacteria lysate. Lane 1: low molecular protein marker (Pharmacia Company). Lane 2: control, BL21 (DE3)/pET-hbFGF. Lane 3: BL21 (DE3)/pET-hbFGF induced by IPTG in test tube culture. Lane 4: BL21 (DE3)/pET-hbFGF induced by IPTG in fermentation culture. Lane 5: purified hbFGF after two-steps purification.

Induction, expression, and identification of rhbFGF Based on SDS-PAGE assay followed by den-

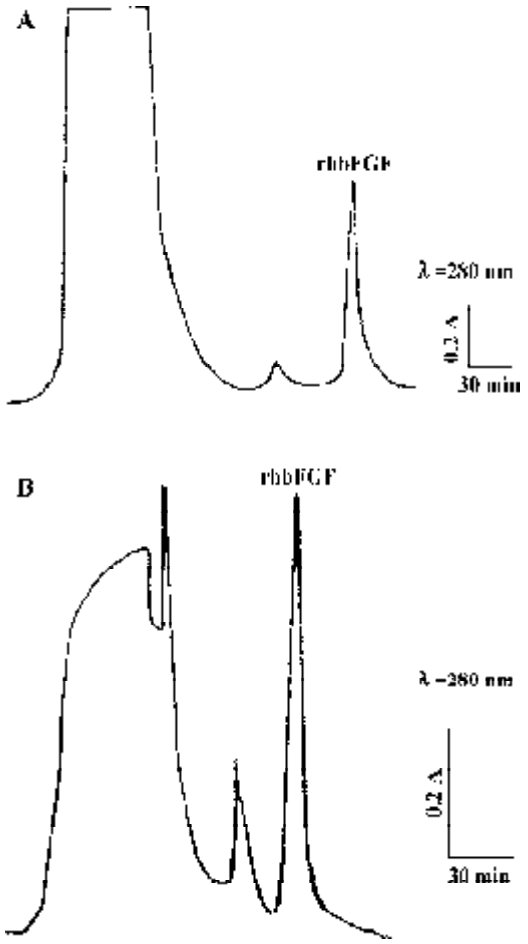

Fig 5. rhbFGF purified by cation exchange column (A), then purified by heparin affinity column (B).

sitometry scanning, the amount of synthesized hbFGF reached 30 % of the total cellular protein.

Purification of rhbFGF protein Using cation exchange and heparin affinity chromatography, from the supernatant of the bacteria lysate, the rhbFGF was purified. The purity of the obtained rhbFGF was confirmed as high as 95 % by SDS-PAGE. Bioassay of mitogenicity suggested that the bioactivity of the obtained rhbFGF was identical with that of the standard bFGF (not shown).

DISCUSSION

Mature bFGF is a single chain polypeptide without glycosylation, and is suitable to be expressed in *E coli*. In this study, BL21 (DE3) & pET-3C systems are employed to express hbFGF. pET-3C vector contains a promoter, which can be exclusively recognized by T7 RNA polymerase. BL21 (DE3) is a genetically engineered *E coli* strain containing the T7 RNA polymerase gene with LacUV5 promotor. With pET-hbFGF transformed into BL21 (DE3) vector, IPTG was utilized to induce *E coli* to express T7 RNA polymerase. The hbFGF gene was then transcribed through interaction with T7 promotor.

Translation efficiency of foreign genes in *E coli* is strongly influenced by the secondary structure of TIR. The range of TIR has not been strictly defined. Ganoza *et al* suggested that the 70-mer-long AUG-proximal nucleotides were important to initiate synthesis of protein[2].

In this study, the G+C content of the first 20 amino acids was estimated. The result shows that up to 78 % of the gene is comprised of G+C. This suggests that the stable TIR structure dramatically decreases the initiation efficiency of the translation. Therefore, without the change of amino acid sequence, modification of TIR is necessary.

In our system, the upstream sequence related to AUG of hbFGF in pET-3c contains T7 promotor and SD sequence. The elaborately designed T7 promotor and SD sequence are not to be altered. Furthermore, TIR in different genes has distinctive location. The 5' end of the hbFGF coding sequence was thus focused. Particular nucleotides in the first 20 codons of the hbFGF 5' sequence were modified.

Moreover, the codon preference in *E coli* must be considered. The excessive amount of the rare codons in bFGF TIR results in reduction of half-life of mRNA, premature termination of the translation, wrong frame shift during the translation, *etc*[5]. Based on computer-aided analysis, the start codon AUG is found in U type structure. hbFGF mRNA is thus difficult to be translated. Therefore, an extra long primer was designed to cover 60 bp of the 5' sequence of hbFGF. More preferred codons were employed to minimize the G+C content.

TIR modification is an effective tool to enhance the expression level of nonfusion hbFGF. However, inclusion body identified in *E coli* lysate implies that rhbFGF can be obtained from both supernatant and in-

clusion body. To increase rhbFGF concentration in supernatant, further computer-aided experiments are necessary to evaluate different TIR models.

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非融合重组人碱性成纤维细胞生长因子在大肠杆菌中的克隆及高效表达[1]

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关键词　碱性成纤维细胞生长因子；分子克隆；基因表达

目的：获得大肠杆菌中高效表达的非融合重组人碱性成纤维细胞生长因子(rhbFGF)．方法：采用 RT-PCR 技术，以人胎儿脑组织的总 RNA 克隆出 hbFGF 基因，再以此为模板，设计引物，对 hbFGF 的 TIR(翻译起始区)部分碱基进行改造和降低 G+C 含量，最后将该新基因克隆于质粒载体 pET-3C，转化于大肠杆菌中表达．结果：非融合 rhbFGF 在大肠杆菌中高效表达，占总蛋白量的 30 %以上．采用离子交换和亲和层析方法纯化后，生物活性与标准蛋白一致．结论：非融合 rhbFGF 及调整 TIR 区域的碱基序列能有效提高重组蛋白的表达效率．

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