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Full Length Research Paper

High level expression of human basic fibroblast growth factor in *Escherichia coli:* Evaluating the effect of the GC content and rare codons within the first 13 codons

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High-level expression of recombinant human basic fibroblast growth factor in *Escherichia coli* presents research opportunities such as analysis of hbFGF expression after translation initiation region (TIR) mutagenesis. In our study, *hbfgf*-cDNA was expressed in three stains of *E. coli* comprising OrigamiB (DE3), BL21 (DE3) and modified strain carrying copies for rare codon tRNAs (BL21 (DE3)-codonplus-RP). During the course of these experiments, we investigated the role of rare codon replacement and of GC content reduction in N-terminal, just downstream of the ATG start codon. As standardized procedure, two forward primers were designed for modification of N-terminal of *hbfgf*-cDNA. N-terminally modified genes were PCR amplified and cloned into the expression vector, pET-22b. Meanwhile, wild-type gene remarkably expressed in all the strains especially in codon plus strain, rare codon substituted hbFGF gene construct surprisingly displayed undetectable levels of protein production; modified gene construct with reduction in GC content of the first 13 codons contributes to 2.5 folds increased expression level. In addition, recombinant hbFGF were purified and the biological activity of the recombinant growth factor was demonstrated by its ability to stimulate proliferation of NIH/3T3 cells. Purified rhbFGF exhibited proliferative activity comparable to commercial rhbFGF.

Key words: Translation initiation region mutagenesis, modified gene, codon optimization, GC content reduction.

INTRODUCTION

Escherichia coli is an attractive heterologous expression host for recombinant proteins, because of its availability of versatile vector systems, host strains and exceeding high expression levels (Makrides, 1996; Sahdev et al., 2008; Terpe, 2006). In most heterologous expression studies, high level expression is not routinely achieved. Thus, it is important to reach the highest possible expression levels. One parameter that has to be considered for efficient protein expression is the phenomenon called codon bias. Codon bias is the observation that organisms do not use all codons for one amino acid at the same frequency and codon preferences vary between different organisms. Based on the observation that most low usage codons are found within the first 25 codons in *E. coli*, it has been proposed that such codons have a regulatory role in expression process (Chen and Inouye, 1994; Chen and Inouye, 1990; Goldman et al., 1995; Rosenberg et al., 1993). However, another study from *E. coli* suggests that codon bias is unlikely to be an important

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Abbreviations: **TIR**, Translation initiation region; **IPTG**, isopropyl β-D-thiogalactopyranoside; **PMSF**, phenylmethyl-sulfonyl fluorides; **DAB**, diaminobenzidine; **PCR**, polymerase chain reaction; **DMEM**, Dulbecco's modified eagle medium; **MTT**, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole; **SDS**-**PAGE**, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Table 1. The 5′ sequence of wild-type gene, its mutagenic forward primers and reverse primer.

Parameters	Primers
Wild type gene sequence at N-terminal	5′ATG CCC GCC TTG CCC GAG GAT GGT GGT TCG GGC GCC ATC CCG CCC GGC CAC TTC 3’
Forward mutagenic primer for codon optimization	5' TACATATGCCG GCC TTG CCG GAG GAT GGT GGT TCT GGC GCC ATC CCG CCC GGC CAC TTC 3'
Forward mutagenic primer for GC content reduction	5′ TACATATG CCA GCT TTA CCA GAA GAT GGT GGT TCT GGT GCT ATT CCG CCC GGC CAC TTC 3′
Reverse primer	5'TACTATTAGATCTTGGCCATTAAAATCAGC 3'

mode of modulating gene expression (Kurland, 1991). For *E. coli*, a correlation between codon usage and expression levels has been presented, indicating that existence of optimal codons in a gene may contribute to high expression levels (Calderone et al., 1996; Gustafsson et al., 2004; Ikemura, 1981; Makoff et al., 1989; Sorensen and Mortensen, 2005).

The general agreement from the published data on heterologous gene expression and codon usage is that optimizing codon sequences will increase expression levels in various organisms such as *E. coli* (Hale and Thompson, 1998; Hanning and Makrides, 1998; Zhang et al., 1999), yeast (Brocca et al., 1998; Cormack et al., 1997) and mammalian cells (Kim et al., 1997; Mirzabekov et al., 1999; Nagata et al., 1999; Uchijima et al., 1998). Studies of expression regulation in *E. coli* reveal that rare codons are most effective in reducing expression levels when they are situated in the 5′-region of a gene (Chen and Inouye, 1994; Goldman et al., 1995; Johansson et al., 1999), but it is not evident whether optimizing the 5′-region alone is sufficient to reach high expression levels. In most genes, an exchange of low usage or rare codons with synonymous more optimal codons is neutral or even increase yield of expression (Makoff et al., 1989; Rangwala et al., 2003; Zahn, 1996). For some genes, however, it has been observed that such an exchange surprisingly lead to a reduction in expression level (Griswold et al., 2003; Jiang et al., 1996; Wu et al., 2004).

A second parameter for efficient expression is the regulation of ribosome assembly at the translation initiation region (Kozak, 1999). Initiation sites are most efficient if they conform to an unstable sequence motif. There are evidences to reveal that reduction in GC content will decrease the stability of secondary structure and enhance the translation initiation (Ishida and Oshima, 2002).

In this paper, hbFGF expression in *E. coli* was investigated by comparing different strategies to enhance gene expression. We compared expression levels quantitatively and analyzed the yield of hbFGF of wild type and two N-terminally modified genes, where the 3 rare codons were substituted with optimal codons and where GC content of 13 first codons were reduced from 69 to 46% according to codon usage of host.

MATERIALS AND METHODS

Cell, vectors and reagents

Plasmids were amplified in *E. coli* Top10f strain (Invitrogen, USA). pET-22b vector was obtained from Novagen (USA) and pGEM-T Easy vector was constructed by Promega Company (USA). Luria Bertani media for growth of *E. coli*, antibiotics, Xgal while isopropyl β-D-thiogalactopyranoside (IPTG) were purchased from Merck (USA). Restriction enzymes and T4 DNA ligase were purchased from TAKARA (Japan). PCR (polymerase chain reaction) purification kit, gel extraction kit and Miniprep kit for plasmid extraction were obtained from Roche Company (Germany).

Primer designing, PCR amplification

We created two versions of hbFGF-cDNA using oligonucleotide primers. Table 1 represents the designed upstream and downstream primers. Primers were synthesized by MWG Company (Germany). The template used for all amplifications was pET-1006 construct which contains mature cytoplasmic isoform of hbFGF that was inserted into pET-22b vector (Mirzahoseini et al., 2007). *Taq* polymerase (Fermentas, USA) and recommended protocol was applied during gradient PCR. The PCR products were analyzed and quantified by agarose gel electrophoresis. Subsequently, the amplified DNA fragments about 480 bp with 3´OH adenine nucleotide overhangs and coding sequences for hbFGF were purified from gel and were cloned into pGEM-T Easy vector.

Construction of expression vectors

The N-terminally modified hb*fgf*-cDNA fragments were digested with *Nde*I and *Bgl*II enzymes and were isolated from pGEM-T Easy vector. Two variants of hb*fgf* genes with *Nde*I and *Bgl*II overhangs were directionally coloned between *Nde*I and *Bam*HI sites of pET-22b vector. In this way, two expression constructs comprising pET-1008 and pET-1009 were made. Nucleotide sequences of the modified hb*fgf*-cDNA fragments were determined by MWG Company (Germany). Plasmid pET-1008 contains *hbfgf* genes with low GC content at N-terminal, while pET-1009 plasmid consists of *hbfgf* gene with replaced 3 rare codons with optimal codons at N-terminal.

Expression and purification

For the expression of hbFGF, expression constructs were transformed into chemically competent (Bussow et al., 2005) three strains of *E. coli* comprising, BL21 (DE3), BL21-CodonPlus (DE3)-RP and OrigamiB (DE3). The cultures were plated on LB agar

plates containing 50 µg/ml of ampicillin for plasmid selection and were incubated approximately 16 h at 37°C. In order to achieve high yields of rhbFGF, different inducing time points, temperatures and addition concentration of IPTG were varied and evaluated in the expression procedure.

To perform expression of rhbFGF, 100 µl of prepared seed cultures of transformants were inoculated in 5 ml of LB medium containing ampicillin (50µg/ml); then were shaken in a rotatory shaker at 37°C and 200 rpm. When the absorbance value at 600 nm reached 0.2 - 0.6 (1 - 4 h), the cultures were supplemented with 0.5, 0.8, 1, 1.5 and 2 mM IPTG to induce the expression of the rhb*fgf* gene regulated by the *T7* promoter. After induction, the cultures were further shaken at 20, 30, and 37°C and 200 rpm until OD_{600} reached 0.2.

Subsequently, cells were harvested by centrifugation (6000 rpm, 4°C, 10min). The cell palettes were re-suspended in 200 µl of 50 mM phosphate buffer (pH 7.2) containing 0.1 mM phenylmethyl-sulfonyl fluorides (PMSF), 0.5 mM $MgCl_2$, 1 mM EDTA and 250µg/ml lysozyme. The suspension was kept on ice for 30 min and then cells were sonicated (3 times, 30 s each time) using an ultrasonic processor (Misonix, Framingdale, NY). Over-expression of the recombinant protein was analyzed in the cell lysate. For large scale expression, the best transformant clone was cultured in 1 L LB medium. Purification under native conditions via cation exchange chromatography with CM Sepharose (Pharmacia, Sweden) was performed to assess biological activity of purified hbFGF.

A CM Sepharose column (25 ml, Pharmacia Biotech, Sweden) was equilibrated with 250 ml phosphate buffer (pH 7). The super-natant was harvested by centrifugation (10000 rpm, 10 min and 4°C) and was loaded onto the CM Sepharose column at the rate of 1 ml/min. The column was washed extensively with the same buffer at the rate of 4 ml/min. The bound protein was eluted with salt gradient (0.2 - 2 M NaCl) while the flow rate was maintained at the rate of 4 ml/min. Fractions were collected across the major peaks during the chromatography process.

Protein assay and detection

The protein concentrations in the samples were determined with Bradford method (Bradford, 1976) using bovine serum albumin as the concentration standard. Cell lysates and purified hbFGF were analyzed with 15% polyacrylamide separation gels as described by Laemali (1970). Expression levels in cell lysates and yields of purified protein were quantified by densitometry scan of 1-D gels using Quantity-One 1D-gel analysis software (Bio-Rad, USA). For Western blotting assay, proteins in the gel were transferred to a 0.2 µm pore size nitrocellulose membrane (schlecher and Schuell, USA) using a semi-dry electroblotting apparatus (Bio-Rad, USA) at 12 V for 45 min. The membrane was blocked and then incubated with Rabbit anti-hbFGF antibody (Sigma, USA). After being washed, the membrane incubated with goat anti-rabbit IgG conjugated to HRP (Sigma, USA). The immune-reaction was visualized using 0.5 mg/ml 3,3′-diaminobenzidine (DAB) and 0.01% H_2O_2. For precise measurement of hbFGF, an ELISA kit (R and D, USA) was employed which includes a standard hbFGF for plotting a standard curve.

Activity assay of rhbFGF

Purified recombinant hbFGF and commercial hbFGF (Invitrogen, USA) were assayed for mitogenic activity on mouse embryo fibroblast; NIH 3T3 cells (NCBI C162, National Cell Bank of Iran). Cells were seeded into 96-well microtiter plates in Dulbecco's modified eagle medium (DMEM) (Invitrogen, USA) with 10% fetal calf serum (10000 cells in 100 µl per well) and incubated for 3 days at 37°C. The spent medium was removed and replaced with 100 µl fresh DMEM per well containing 0.3% FBC and various dilutions of recombinant and commercial hbFGF (5, 50, 100 and 200 ng/ml). After 24, 48 and 72 h incubation with hbFGF, 20 µL dissolved 3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide, a tetrazole (MTT) (Sigma, USA) in PBS was added to each well and incubated for a further 4 h at 37°C. The culture medium including MTT solution in the well was removed and 150 µl of dimethyl sulfoxide (DMSO) was added to each well and mixed to dissolve the crystals. The plates were read at 492 nm. By obtaining the absorbance values, cell proliferation was determined. Activity of recombinant hbFGF was determined with reference to commercial hbFGF. Each dilution of hbFGF was assayed in triplicate.

RESULTS

Construction of pET-1008 and pET-1009

In our system, the upstream sequence relating to AUG of hbFGF in pET-22b contains *T7* promoter and SD sequence. The elaborately designed *T7* promoter and SD sequence were not altered. The 5' end of the hbFGF coding sequence was focused while particular nucleotides in the first 13 codons of the hb*fgf* 5' sequence were modified. Two variations of hb*fgf* genes were produced during PCR using different forward primers and same reverse primer. The amplified *hbfgf* genes contained different changes in N-terminal of *hbfgf* cDNA: one had reduction in GC content (69 to 46%); another one contained 3 rare codons replaced with optimal codons (Table 1). The DNA fragments which corresponded to 450 bp were detected on 1.5% agarose gel.

PCR products were cloned in to pGEM-T easy vector (T-vector) and transformed into Top10f cells for amplification. Transformants colonies were chosen and their plasmids were extracted. Recombinant hb*fgf* genes were isolated from T-vector by *Nde*I and *Bgl*II digestion and ligated into *Nde*I and *Bam*HI sites of pET-22b vector. Correct insertion was confirmed by *Hind*III and *Bgl*II digestion. Subsequently, detection of DNA fragments about 683 bp on 1.5% agarose gel was verified in the correct insertion of hb*fgf* genes into pET-22b vector. The use of hbFGF PCR analysis and DNA sequencing also ensured the correct insertion and modification of hbFGF cDNA. The PCR results showed that there was an insert fragment of about 480 bp.

Expression and detection of hbFGF in *E. coli*

pET-1006, pET-1008 and pET-1009 were transformed into three *E. coli* strains [OrigamiB(DE3), BL21(DE3) and BL21-CodonPlus(DE3)-RP] for expression. By analysis of the pre-expression and optimized expression process, the optimal culturing conditions were achieved by using an optimum temperature of 37°C and an optimal induction time points of about 3h for the strains and IPTG addition concentration of 1 mM (data not shown). Under these conditions, high level expression transformant of *E. coli* strains was manifested by sodium dodecyl sulfate

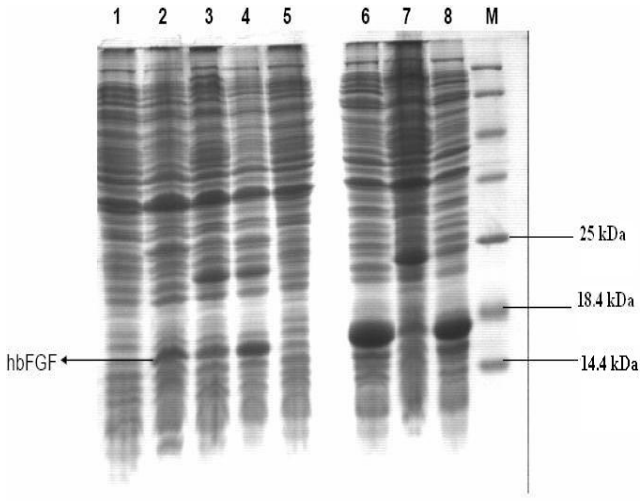

Figure 1. 15% SDS-PAGE. Lane 1: Uninduced OrigamiB (DE3) cell containing pET-1006; lane 2: induced OrigamiB (DE3) cell containing pET-1006; lane 3: induced BL21(DE3) cell containing pET-1006; lane 4: induced BL21Codon Plus(DE3)-RP cell containing pET-1006; lane 5: uninduced OrigamiB(DE3) cell containing pET-1008; lane 6: induced BL21(DE3) cell containing pET-1008; lane 7: induced BL21CodonPlus(DE3)-RP cell containing pET-1008; M: protein molecular weight marker.

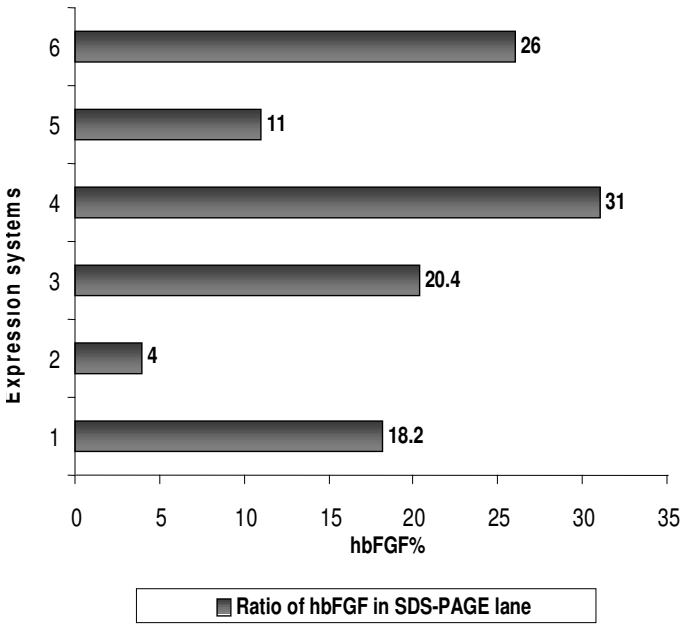

Figure 2. Density scanning of the electrophoretic bands as shown in SDS-PAGE lane. The ratio of hbFGF in the cell lysates. 1: OrigamiB (DE3) containing pET-1006; 2: BL21 (DE3) containing pET-1006; 3: BL21CodonPlus (DE3)-RP containing pET-1006; 4: OrigamiB (DE3) containing pET-1008; 5: BL21 (DE3) containing pET-1008; 6: BL21CodonPlus (DE3)-RP containing pET-1008.

polyacrylamide gel electrophoresis (SDS-PAGE) and densitometry scan of gels. Results presented in Figures 1

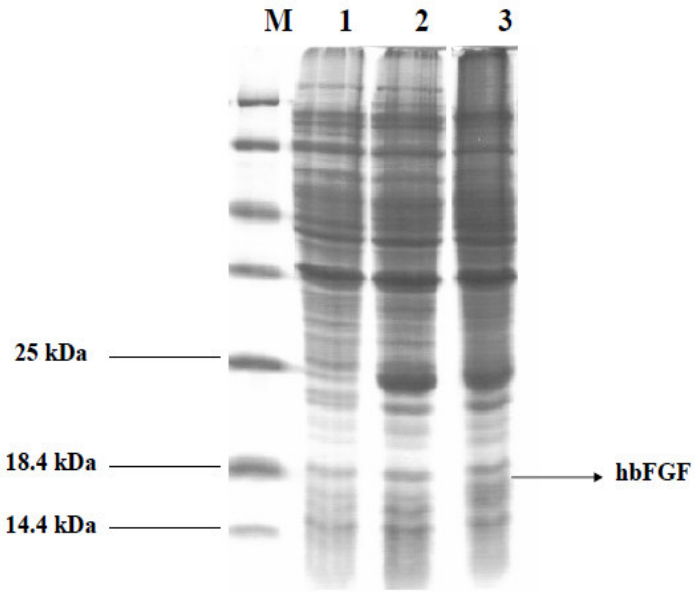

Figure 3. 15% SDS-PAGE. M: Protein molecular weight marker; lane 1: induced OrigamiB(DE3) cell containing pET-1009; lane 2: induced BL21(DE3) cell containing pET-1009; lane 3: induced BL21CodonPlus(DE3)-RP cell containing pET-1009.

and 2 showed that transformant cells containing pET-1008 plasmid expressed highest yield of rhbFGF in OrigamiB (DE3) strain while low levels of hbFGF were obtained in BL21 (DE3). Meanwhile no band for hbFGF was observed in SDS-PAGE, which was the lysate of pET-1009 transformants (Figure 3), wild-type *hbfgf* gene was properly expressed especially in the codon bias-adjusted strain. Based on the amino acid sequence, the calculated molecular weight of rhbFGF is 18 kDa which is consistent with the result of SDS-PAGE measurement.

Western blotting analysis demonstrated that produced recombinant hbFGF by pET-1006 and pET-1008 constructs could bind with the rabbit anti-human bFGF polyclonal antibody (Figure 4). Table 2 presents the exact amount of synthesized rhbFGF in 1000 milliliter of cell culture through two different transformants containing pET-1008 and pET-1006 plasmid.

Biological activity of purified rhbFGF

The protein was purified from the supernatant of 1 L cell lysate (OrigamiB (DE3) containing pET-1008) by CM-Sepharose ion exchange chromatography (Figure 5). The purity of rhbFGF was more than 85%. Bradford protein assay and gel densitometry showed that about 180 mg rhbFGF was obtained from 1 L cultivation broth, the final recovery of the recombinant protein was 78.54%.

The biological activity of rhbFGF was determined by MTT dye test. The results showed that both recombinant and commercial hbFGF markedly stimulated the prolife-ration of cultured 3T3 cells. The effects were concentration-

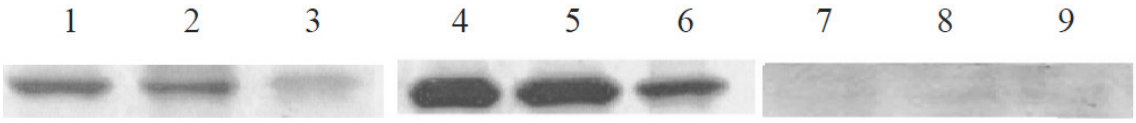

Figure 4. Western blotting analysis. Lane 1: Induced BL21CodonPlus(DE3)-RP cell containing pET-1006; lane 2: induced BL21(DE3) cell containing pET-1006; lane 3: induced OrigamiB(DE3) cell containing pET-1006; lane 4: induced OrigamiB(DE3) cell containing pET-1008; lane 5: induced BL21CodonPlus(DE3)-RP cell containing pET-1008; lane 6: induced BL21(DE3) cell containing pET-1008; lane 7: induced BL21CodonPlus(DE3)-RP cell containing pET-1009; lane 8: induced BL21(DE3) cell containing pET-1009; lane 9: induced OrigamiB(DE3) cell containing pET-1009.

Table 2. ELISA assay. The exact amount of synthesized rhbFGF in 1000 milliliter of cell culture through 6 different transformants.

Construct	Dilution rate	OD (450)nm	hbFGF(mg/l)
OrigamiB(DE3) containing pET-1006	1/10000000	0.392	110
BL21(DE3) containing pET-1006	1/10000000	0.371	84
BL21CodonPlus(DE3)-RP containing pET-1006	1/10000000	0.463	130
OrigamiB(DE3) containing pET-1008	1/10000000	1.23	280
BL21(DE3) containing pET-1008	1/10000000	0.341	70
BL21CodonPlus(DE3)-RP containing pET-1008	1/10000000	0.982	220
Uninduced BL21(DE3) containing pET-1008negative control	1/100000	~ 0.0	
BL21(DE3) without plasmid negative control	1/100000	~ 0.0	

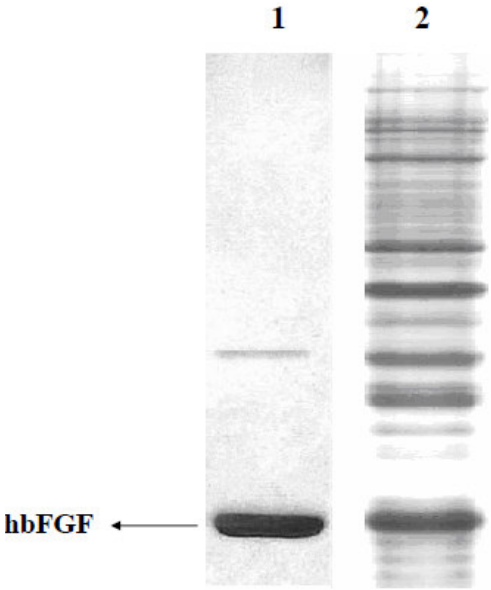

Figure 5. Lane 1: fraction passed CM Sepharose gel and lane 2: supernatant of 1 L cell lysate (OrigamiB (DE3) containing pET-1008).

dependent, in the range of 5 to 200 ng/ml, in similar manners for both recombinant and commercial hbFGF. The time-course of these effects on survival was almost the same between recombinant and commercial protein (Figure 6).

DISCUSSION

We have analyzed the heterologous expression characteristic in *E. coli* of wild type and two variations of hbFGF. The obtained results highlight critical concern in choosing strategies for expression of human genes in *E. coli.* Firstly, existence of rare codons at N-terminal of a gene does not necessarily correlate with expression levels and also does not refuse *E. coli* as a host for expression. Our results opposed previous findings that suggest that the existence of rare codons in a gene negatively affect expression levels in *E. coli* (Brinkmann et al., 1989; Kleber-Janke and Becker, 2000). Numerous instances of increased protein expression via codon optimization are found and making this methodology a common strategy for protein expression (Casimiro et al., 1997; Gold et al., 1981; Hamdan et al., 2002; Kleber-Janke and Becker, 2000; Li et al., 2003; Merril et al., 1981; Pan et al., 1999; Prapunwattana et al., 1996; Queen and Rosenberg, 1981).

Despite the reported advantage for codon optimization, it was found that the wild type hbFGF have already high expression in BL21 (DE3), OrigamiB (DE3) and especially in codon plus strain and substituting rare CCC/ CCC/TCG codons with optimal codon in the first 13

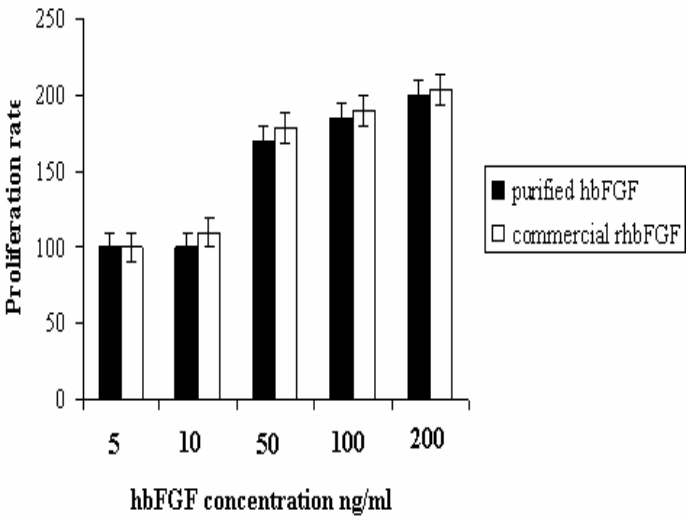

Figure 6. Stimulatory activities of purified rhbFGF on proliferation of NIH/3T3 cells compared to standard hbFGF. The mitogenic activity was estimated as the number of cells per well after incubation with 5, 10, 50, 100 and 200 ng/ml hbFGF.

codons of hb*fgf* gene is not efficient to increase expression level and also prevent hbFGF production. This effect has been observed in few other studies (Griswold et al., 2003; Nilsson and Mannervik, 2001) and could be due to the tendency of codon optimized mRNA to form stable 5' structures. In other respect, the modified gene with reduction in GC content led to 2.5 folds increase in expression level. Similarly, Ishida and Oshima (1994) have shown that the destruction of stable stem-loop by site directed mutagenesis at N-terminal of high GC content genes could enhance translation.

According to our investigations, the total free energy at the 5' end of the hb*fgf* gene in pET-1008 construct was increased from -23.40 to -15.20 kcal/mol while the total free energy at the 5' end of the hb*fgf* gene in pET-1009 construct was decreased to -24.40 kcal/mol. Obtained results imply that unstable RNA secondary structure at 5′ end eventually caused mRNA efficiency for translation. At least two possible explanations can be noted for obtained results. Firstly, the mutation in order to optimize codons may increase the structure stability of the RNA and, thereby, reduce the activity of the mRNA in initiation complex formation. Secondly, the mutation in order to reduce GC content could decrease the structure stability of the mRNA. In this case, the translation initiation site would become more accessible possibly resulting in increase in expression. Thus, reducing GC content and exposing the stability of secondary structure at translation initiation region could improve expression level.

These results highlight the important roles of 5' secondary structure for non-host protein expression in *E. coli.* Secondary structure of mRNA is a key factor in determining the efficiency of translation initiation in prokaryotes and translation efficiency is directly determined by the availability of unfolded translation initiation region (TIR) (De Smit and Van Duin, 1990). Eukaryotic genes do not have an active TIR in *E. coli*, so any alteration or nucleotide substitutions affect their expression in *E. coli* only by changes in secondary structure stability (De Smit and Van Duin, 1990; Sprengart et al., 1990). In another word, TIR redesign can lead to abnormal structure in translation initiation region and cause very low level of expression.

Whereas, expression trials of wild type, rare codon genes in various modified strains are the recommended first option, reduction in GC content (according to codon usage of host) at 5′ end of a gene is a considerable alternative preventing problems encountered by rare codon or specific RNA secondary structure formation.

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