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Design, Construction and Expression of a Synthetic β-Interferon (IFN-β) Gene in *E. coli*

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Abstract: In this study, a synthetic gene encoding 166 residues of interferon-β was constructed. For an efficient expression of IFN- β in *E. coli*, the synthetic gene was designed according to the *E. coli* codon usage. Cysteine 17 was replaced with serine to avoid multimer formation and random intramolecular disulfide bridges. The sequence of the designed gene was analyzed with Web Cutter and RNA secondary structure softwares to make sure about the cloning and expression of the recombinant gene in the proposed cloning and expression vectors. Twelve overlapping oligonucleotides ranging from 55 to 83 in length were designed. A step-wise assembly polymerase chain reaction was used for the construction of the synthetic gene through three steps PCR programs using overlapping adjacent primers as well as PCR products of a previous steps. The final PCR product was further amplified and cloned into T/A cloning vector. The constructed gene was sub-cloned into pQE30, pKK322 and pGEMEX-1 expression vectors and the expression was analyzed with Western-blotting.

Key word: Synthetic gene, interferon β, step-wise assembly PCR, codon usage

INTRODUCTION

Human interferon β is a 166 amino acids glycoprotein that can be produced by most cells in the body in response to viral infection or exposure to other biological factors. It binds to a multimeric cell surface receptor which results in a cascade of intracellular events leading to expression of IFN-β inducible genes. The induction of these genes in turn leads to diverse effect that can be classified as antiviral, antiproliferative and Immunomodulatory activities. These activities form the basis for clinical benefits that have been observed with interferon therapy of viral hepatitis, glyoma, melanoma and recently multiple sclerosis (MS) (Noronha *et al*., 1993; Ossage *et al*., 2001). Two types of recombinant IFN-β produced in eukaryotic cell (glycosylated) and *E. coli* (nonglycosylated) are biologically active and have been used for therapeutic purposes (Antonetti *et al*., 2002). Recombinant IFN-β has been expressed in *E. coli* under the control of *trp, lpp* (Ithoh *et al*., 1984), λP_L and λP_R (Sergey *et al*., 1990) promoters using native and synthetic genes. In this study, the production of INF-β in different *E. coli* expression system has been considered through construction of synthetic gene.

MATERIALS AND METHODS

Bacterial strains and plasmids: Strains of TG1, XL1Blue, DH5α, M15 and JM109 of *E. coli* K12 were used for cloning and expression of the rIFN-β. Plasmids pQE-30 (Qiagen), containing T5 promoter and *lac* operator, pKK223-3 containing *tac* promoter and pGEMEX-1 containing T7 promoter and the gene 10 from T7 phage (Promega) were used for the expression of synthetic β-Interferon (IFN-β) gene.

Construction of synthetic gene: Oligonucleotides ranging from 55-83 nucleotides in length were designed and synthesized (MWG Company, Germany). The oligonucleotides used in the construction and PCR amplification of IFN-β gene are listed in Table 1. In the first round of step-wise assembly polymerase chain reaction (Lin *et al*., 2002), six reactions were carried out, each containing adjacent pairs of oligonucleotides at a final concentration of 0.8 μM in 50 μl reaction. In the second round, equal volumes of the products from the reaction one and two, three and four, five and six were combined in separate 50 μl reactions. Assembly reactions were repeated successively with the products generated by adjacent pairs of oligonucleotides until only a single reaction was carried out.

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Table 1: The oligonucleotides for construction and cloning of the synthetic gene

QE-30 INTB 1	5`CGGAATTCATTAAAGAGGAGAAATTAACTATGA GCTACAACCTG CTGGGCTTCCTGCAACG	For construction of synthetic gene
QE-30 INTB 2	5` TCAGTTGCCACAGTAACTTCTGGCTCTGAAAA TTACTGCTACG TTGCAGGAAGCCCAGCAG	For construction of synthetic gene
QE-30 INTB 3	5` AGAAGTTACTGTGGCAACTGAATGGCCG CCTGGAATATTGCTTA AAAGACCGC ATG	For construction of synthetic gene
QE-30 INTB4	5` CTGGAACTGCTGCAGCTGTTTAATCTCTT CGGGATATCAAAGTT CATGCGGTCTTTTAAGC	For construction of synthetic gene
QE-30 INTB5	5` AAACAGCTGCAGCAGTTCCAGAAGGA AGATGCCGCGCTGACC ATTTATGAAATGCTGCAG	For construction of synthetic gene
QE-30 INT B6	5` CCGGTGCTCGAGCTATCTTGGCGAAAAATC GCAAAGATGTTCTG CAGCATTTCATAAATGG	For construction of synthetic gene
QE-30 INTB7	5` CCAAGATAGCTCGAGCACCGGCTGGAAC GAGACGATTGTTGAA AACCTCCTGGCGAATGTG	For construction of synthetic gene
QE-30 INTB8	5` TTTCTTCCAGCACGGTTTTCAGATGGT TGATCTGATGATACAC ATTCGCCAGGAGG	For construction of synthetic gene
QE-30 INTB9	5`TGAAAACCGTGCTGGAAGAAAAACTGGAGAA AGAAGATTTTACCCGCGGTAAACTCATGAGCAG	For construction of synthetic gene
QE-30 INTB10	5` ATGCAGAATGCGGCCATAATAACGTTTC AGGTGCAGACTGCT CATGAGTTTACCG	For construction of synthetic gene
QE-30 INTB11	5`TATTATGGCCGCATTCTGCATTACCTGAAGGC GAAAGAGTATAGCCACTGTGCCTGGACCATCGTGCGTGT	For construction of synthetic gene
QE-30 INTB12	5`CCCAAGCTTA GTTACGCAGATAACC CGTCAGACGGTTAATAAAGTAAAAGTTGCGA AGAATTTCCACACGCACGA T GGTC CA G	For construction of synthetic gene
KKINTB1	GGGAATTCTATGAGCTACAACCTGCTG	For cloning of the synthetic gene in pKK223-3

PCR reactions were supplemented with 0.2 μl of 25 mM dNTP and 2 units of high fidelity DNA polymerase *PFU* (Roche) in each round. Conditions for first round assembly reactions were: 10 cycles at 94°C for 15 sec, 52°C for 30 sec, 72°C for 15 sec, 20 cycles at 94°C for 15 sec, 52°C for 30 sec, 72°C for 20 sec and one cycle at 72°C for 10 min. Full-length products were further amplified using Taq DNA polymerase (Roche) and the QE-30INTB1 and QE-30INTB12 primers. The PCR program was carried out with the following temperature profile: 94°C for 30 sec, 52°C for 30 sec and 72°C for 30 sec, (30 cycles) and finally 72°C for 5 min. The PCR products were then cloned into T/A cloning vector and verified by restriction endonuclease mapping of their plasmids. Dideoxy Termination sequencing with the ABI automated sequencer was used to confirm the authenticity of the constructed gene. All molecular methods were in accordance to the standard methods (Sambrook *et al*., 2001).

Antibody production: Polyclonal antiserum against rhIFN-β (betaseron, Shering Germany) was raised in New Zeland white rabbit. The specificity of the polyclonal antibody was confirmed by the Western blotting test. The first inoculum was prepared by emulsification of 100 μg of protein in Freund's complete adjuvant (Gibco); all subsequent inoculations were prepared in Freund's incomplete adjuvant. Rabbit was injected subcutaneously in 6-8 sites on the animal's back (0.1-0.2 ml/site). Boosting doses of emulsified antigen were carried out every 4 weeks.

Expression of recombinant protein: In order to study the expression of synthetic IFN-β gene, TG1, XL1Blue, M15 strains of *E. coli* K12 were transformed with pQE30-IFNβ, DH5α was transfored with pKK223- IFNβ and JM109 with pGEMEX1-IFNβ. The recombinant bacteria were grown in LB medium with ampicillin with the final concentration of 100 μg ml^{-1} and in the case of M15 with ampicillin and kanamicin with the final concentrations of 100 and 25 μg ml^{-1}, respectively at 37°C, to a cell density of OD600: 0.5-0.8. Induction was carried out by adding IPTG with the final concentration of 0.5-1 mM. After induction, bacteria were further incubated at 37°C. Bacterial cells from 0.5 ml of the cell culture were mixed with 100 μl of sample solvent at room temperature and boiled for 5 min and were then used to study protein expression. The expression of rIFN-β was monitored by Western Blotting at different time intervals (2, 3 and 4 h post-induction) according to standard methods (Sambrook *et al*., 2001). The proteins were probed with polyclonal antiserum prepared against rIFN-β and with the Rabbit poly anti human IFN-β (Koma Biothec Inc). The protein-antibody complex were treated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin (Tebsan-Iran) and visualized using a solution of 4-chloro-1-naphthol, with hydrogen peroxide as an enzyme substrate.

RESULTS

Construction of synthetic gene: A synthetic gene with preferred codon usage of *E. coli* was designed for an efficient expression of recombinant β-interferon in *E. coli*.

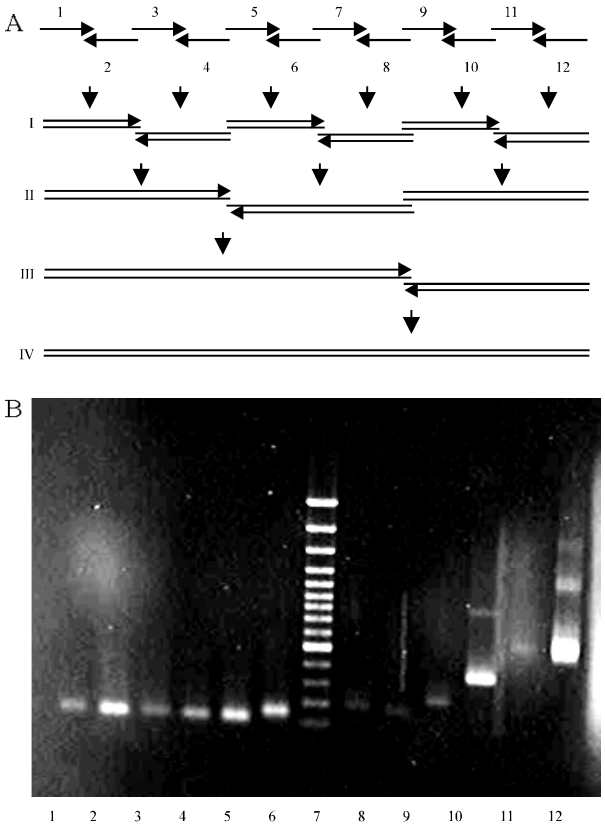

Fig. 1: Step-wise assembly PCR for production of synthetic IFN-β gene, A: The strategy for construction of the synthetic gene. B: Agarose gel electrophoresis of the products of the step-wise assembly PCR. Lane 1-6 are the PCR products of round 1, line 7 is DNA Molecular weight marker 100 bp ladder, line 8, 9, 10 are the PCR products from the PCRs 1, 2 and 3, 4 and 5, 6 (round 2), Line11 is PCR product 8, 9 (round 3) and line 12 is the PCR product of line 10, 11

Cysteine 17 was replaced with serine to avoid multimer formation and random intramolecular disulfide bridges as well (Runkel *et al*., 1998). The designed gene was analyzed with Web Cutter (www.firstmarket.com/cutter/cut2.html) and RNA structure soft wares (www.genebee.msu.su/services/rna2_reduced.html) to make sure about cloning and expression of recombinant protein in the proposed cloning and expression vectors. Analysis of RNA structure revealed that the start codon and ribosome binding site of the mRNA were not in the stem, so theoretically it was suitable for expression. To construct the synthetic gene 12 overlapping (15-20 nucleotides) oligonucleotides ranging from 55-83 nucleotides in length were designed (Table 1). The step-wise assembly polymerase chain

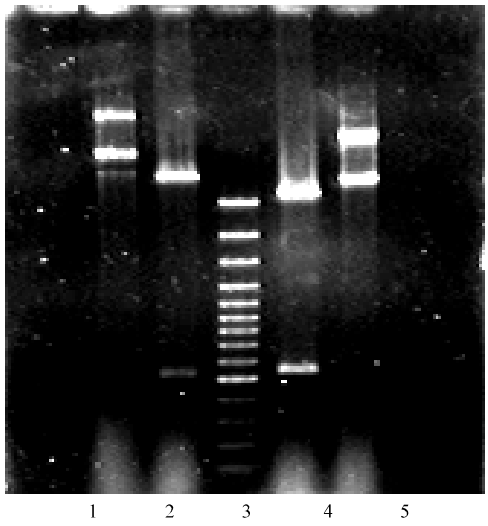

Fig. 2: Restriction Enzyme analysis of pYG83 and pYG84:, Lane 1 uncut pYG83, lane 2 pYG83 digested with *Eco*RI/*Hin*dIII, lane 3 100 bp DNA Molecular weight lane 4 uncut pYG84 and lane 5 pYG4 digested with *Eco*RI/*Hin*dIII

reaction (material and methods) was led to production of a ~530 bp full length gene product (Fig. 1). The full length gene was then further amplified using QE-30INTB1 and QE-30INTB12 primers with Taq DNA polymerase and cloned into T/A cloning vector to produce plasmid pYG81. This construct is suitable for cloning in pQE30 expression vector because containing the ribosome binding site.

The pYG81 was used as template for amplifying the synthetic gene suitable for cloning in pKK223-3 expression vector using KKINTB1 and M13 Reverse primers. The PCR product was then cloned in T/A cloning vector and the authenticity of the synthetic genes were confirmed by restriction enzyme analysis and sequencing. The resultant plasmid was named pYG82.

Expression of IFN -β synthetic gene: To construct the expression plasmid, the *Eco*R1/*Hin*dIII DNA fragments (~530 and ~512 bp) containing IFN-β gene isolated from pYG81 and pYG82, was inserted into *Eco*R1/*Hin*dIII sites of pQE30 and pKK223-3 expression vectors downstream of the T5 and *tac* promoters to produce recombinant plasmids pYG83 and pYG84 respectively. The recombinant plasmids were then transferred into DH5α strain of *E. coli* and verified by estimating the length of the cloned fragment after digestion with *Eco*R1/*Hin*dIII enzymes (Fig. 2). The expression of rIFN-β was investigated using SDS-PAGE and Western blot analysis using polyclonal antiserum and polyclonal antibody against IFN-β but no recombinant IFN-β was detected in TG1, XL1Blue, DH5α, M15 strains of *E. coli* harboring pYG83 and pYG84 .

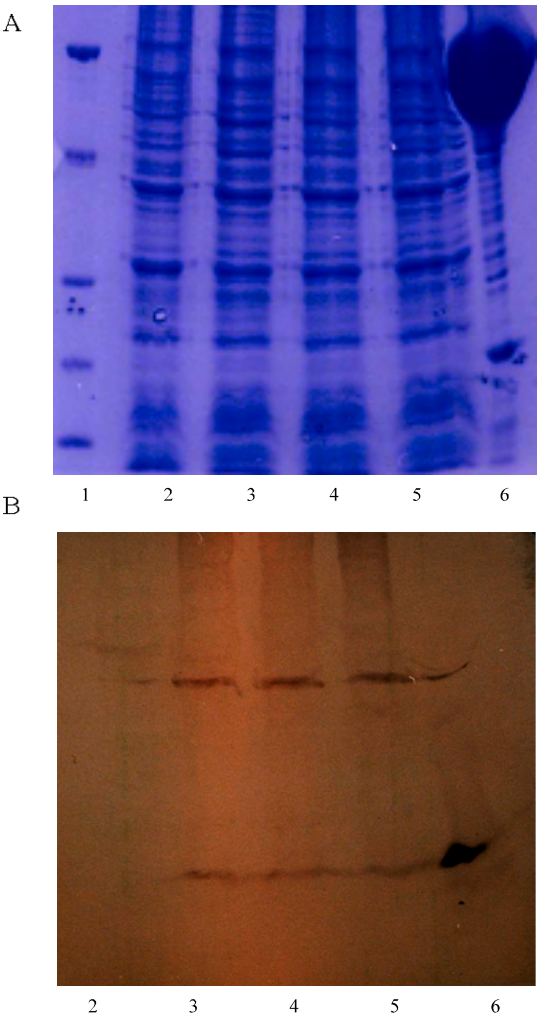

Fig. 3: SDS-PAGE (A) and Western Blot (B) analysis of fusion IFNβ::T7Gene 10, Lane 1: Protein molecular weight marker (14.4, 21.5, 26.6, 39.2, 66.2, 97.4 KD, respectively), lane 2: JM109 strain of *E. coli* harboring pGEMEX, lane 3-5: JM109 strain of *E. coli* harboring pYG85, lane 6: IFN-β (positive control)

Expression of synthetic gene as fusion protein: Based on the above results we assumed that fusion of the IFN-β to a protein which is already expressed may help its expression. The *Eco*R1/*Hin*dIII DNA fragments (~530 bp) containing IFN-β gene isolated from pYG81, was inserted into *Eco*R1/*Hin*dIII sites of pGEMEX expression vector to produce plasmid pYG85. In the resulting new recombinant pYG85 plasmid, the IFN-β gene was cloned in-frame downstream of the gene 10 of the T7 phage under the control of the T7 promoter, which is expressed as T7 gene 10 fusion in *E. coli* JM109 (DE3) host strain containing an IPTG inducible gene for T7 RNA polymerase. Expression of the fusion protein from plasmid pYG85 was evaluated by SDS-PAGE and western blot analysis, 4 h after induction with IPTG. SDS-PAGE did not show a visible band with the expected size, but a signal was observed in western blot analysis of bacterial lysate using antiserum and polyclonal antibody against rhIFN-β. As shown in Fig. 3 there is two band in each column .The upper bands as compared with protein marker are ~60 KD (fusion protein) and the lower band is ~20 KD as the size of the IFN-β.

DISCUSSION

A synthetic gene with preferred codon usage of *E. coli* was designed for an efficient expression of recombinant β-interferon in *E. coli*. The use of synthetic gene is suggested (Kane, 1995) since Interferon beta, like most other heterologous proteins, has several rare codons for Arginine and Leucine that correlates with low level of its corresponding tRNA and as a consequence, low level expression of the native gene in the heterologous host strain. By the use of synthetic gene, it will be possible to overcome this problem and also replace Cysteine 17 with Serine. As previously reported (Runkel *et al*., 1998), the presence of three Cysteins in Interferon beta gene, cause multimer formation and random intramolecular disulfide bridges during purification and refolding of the recombinant protein, which lead to the inefficient production of the IFN-β for therapeutic applications. The recombinant IFN-β has been expressed in *E. coli* under the control of the *trp*, *lpp* (Ithoh *et al*., 1984), λP_L and λP_R (Sergey *et al*., 1990) promoters using native and synthetic genes. The expression in those systems was usually low and in the case of synthetic gene no expression was obtained (Porter *et al*., 1986). In this study production of recombinant IFN-β in *E. coli* using a different synthetic gene under the control of T5 and *tac* promoters was considered. The analysis of the mRNA of the synthetic gene with RNA secondary structure prediction soft ware revealed that the ribosome binding site and ATG start codon was not in stem. As previously reported (Maarten and Dwin, 1994), the presence of ribosome binding site and ATG start codon in stem decrease the efficiency of translation. The lack of expression of the synthetic gene in recombinant bacteria harboring pYG83 and pYG84 may be as a result of toxicity of IFN-β. As previously reported (Warne and Thomes, 1986), IFN-β is highly toxic to *E. coli* and expression of IFN-β in early stages of growth is deleterious to host cell. In addition mRNA or protein might be unstable too. One way of overcoming these problems are the use of fusion protein (Germino and Bastia, 1984). The hybrid IFN-β was

expressed with T7 gene 10 as fusion protein under the control of T7 promoter. The expression of hybrid IFN-β may confirm the above assumptions. It seems that the mRNA of synthetic gene in pYG83 and pYG84 is unstable and when it was fused to the T7 gene 10, the mRNA became stable and expressed. Analysis of the expression of the fusion protein revealed two bands in Westren blot reacted with antibody against interferon beta. The upper band correspond with the hybrid protein and the lower band with the mature interferon beta as compared to the standard protein. This expression pattern could be related to the translation from two ribosome binding sites presented in the hybrid mRNA, one in the beginning of the mRNA and another one upstream of the interferon part of the hybrid mRNA. This structure can be used by translation machinery like polycistronic mRNA which is popular in prokaryotes (Synder and Champnenn, 2002). The future research would be the study of expression of the synthetic construct under the control of strong and tightly regulated promoters to evaluate the toxicity of the recombinant protein for the host.

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