Biased expression, under the control of single promoter, of human interferon α-2b and *Escherichia coli* methionine amino peptidase genes in *E. coli*, irrespective of their distance from the promoter

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Abstract: Human interferon α-2b and *Escherichia coli* methionine amino peptidase genes were cloned independently as well as bicistronically in expression plasmid pET-21a (+). Production of human interferon α-2b was comparable to that of *E. coli* methionine amino peptidase when these genes were expressed independently in *E. coli* BL21-CodonPlus (DE3)-RIL. However, human interferon α-2b was produced in a much less amount whereas there was no difference in the production of methionine amino peptidase when the encoding genes were expressed bicistronically. It is important to note that human interferon α-2b was the first gene in order, after the promoter and *E. coli* methionine amino peptidase was the next with a linker sequence of 27 nucleotides between them.

Keywords: Human, interferon α-2b, *Escherichia coli*, methionine amino peptidase, bicistronic expression.

INTRODUCTION

Several expression systems including bacterial, yeast, insect and mammalian are available for producing recombinant proteins in high levels. Among the bacterial expression systems, *Escherichia coli* is the most popular organism for the production of recombinant proteins in high levels. However, not every protein can be produced in higher amount in this organism. This may be attributed to the structural features of the gene encoding that protein or major differences in codon usage. Certain *E. coli* strains have been developed in order to minimize the codon bias and high levels of proteins have been produced using these strains despite of the major difference in codon usage. However, sometimes co-expression of two genes is required for protein: protein interaction studies. There are two types of strategies for heterologous co-expression of two genes: the dual-vector and the bicistronic systems. The dual-vector system, as reflected by its name, employs two compatible plasmids for expression of individual genes in the same host. On the other hand, the bicistronic vector copies the bacterial operon composition that contains more than one gene under the control of a single promoter. In principle, the expression of the second gene could be similar to that of the first gene, because each translation sequence has independent ribosomal binding site. However, in practice quite lower expression of the second gene has been observed (Rucker *et al*., 1997). Multiple copies of a gene have also been expressed under a single promoter and ribosomal binding site for the production of recombinant protein in high amount (Gallagher *et al*., 1992).

We systematically evaluated bicistronic vector system for the production of human interferon α-2b and *E. coli* methionine amino peptidase. Interferons are small proteins produced in the human body and perform functions related to the immune system (Reyes-Vázquez *et al*., 2012). The use of recombinant technologies has allowed the production of interferons. Proteins in bacterial expression systems are usually produced with a starting formylmethionine, which may be cleaved, after deformylation, (Flinta *et al.,* 1986) by methionine amino peptidase that has been characterized from various organisms (Ben-Bassat *et al*., 1987; Chang *et al*., 1992; Kendall and Bradshaw, 1992; Arfin *et al*., 1995). This enzyme has higher affinity for substrates that contain a physically small residue located next to the initiator methionine. Studies on mutant forms of methionyl-tRNA synthetase in *E. coli* indicated that the starting methionine could be efficiently cleaved from proteins that have glycine, alanine, serine, or threonine residues adjacent to the starting methionine (Hirel *et al*., 1989). Proteins having valine or cysteine as penultimate residue could only be partly processed by the methionine amino peptidase of the host (Hwang *et al*., 1999). In order to get the completely processed human interferon α-2b it is required that the methionine residue of the recombinant protein is removed to generate the mature human interferon α-2b with Cys residue at the N-terminal. We report here cloning of genes encoding human interferon α-2b and *E. coli* methionine amino peptidase independently as well as bicistronically in pET-21a (+) vector and their expression profiles.

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MATERIALS AND METHODS

Materials

E. coli strains DH5α and BL21-CodonPlus (DE3)-RIL (Novagen) were employed for cloning and expression of the genes, respectively. Plasmids pTZ57R/T (Fermentas) and pET-21a (+) (Novagen) were employed for cloning and expression, respectively. DNA extraction kit, Gene JET Plasmid Miniprep kit and InsTA PCR cloning kit were purchased from Fermentas. QI Aquick Gel Extraction kit was obtained from QIAGEN. DNA cutting enzymes and DNA polymerase were obtained from Fermentas. All other reagents and chemicals were purchased either from Sigma-Aldrich or Acros Organics.

Cloning of human interferon α-2b gene

Human interferon α-2b gene, consisting of 501 nucleotides, was amplified by polymerase chain reaction (PCR) using a set of forward (5'-CTCTCATATGTGTGATCTGCCTCAAACCCACAGC) and reverse (5'-TGAAAAGCTTTAAATCGTGTCATGGTC) primers and human genomic DNA. Underlined sequences in the forward and reverse primers are *Nde*I and *Hin*dIII recognition sites, respectively. The gene fragment amplified by PCR was ligated in pTZ57R/T vector using InsTAclone^{TM} kit (Fermentas) and the resulting plasmid was named as pTZ-Ifn. In order to clone human interferon α-2b gene in the expression vector, plasmid pTZ-Ifn was cut with *Nde*I and *Hin*dIII and the liberated DNA was ligated in pET-21a (+). The resulting plasmid was named as pET-Ifn.

Cloning of E. coli methionine amino peptidase gene

E. coli methionine amino peptidase gene, consisting of 795 nucleotides, was amplified by PCR utilizing a set of forward (5'-CATATGGCTATCTCAATCAAGACCCC) and reverse (5'-TTATTCGTCGTGCGAGATTATCGCC) primers and genomic DNA of *E. coli*. The gene fragment amplified by PCR was cloned in pTZ57R/T and the resulting plasmid was named as pTZ-Map. For cloning in expression vector, plasmid pTZ-Map was cut with *Nde*I and *Eco*RI and the liberated gene was ligated in pET-21a (+). The resulting plasmid was assigned the name pET-Map.

Cloning of human interferon α-2b and E. coli methionine amino peptidase genes bicistronically

In order to clone both the genes in a single expression vector, new primers were designed. A set of forward (5'-AATCTAGACTGCAGTAAGAAGGAGATATAACTATGTGTGATCTGCCTCA) and reverse 5'-CATATGCGATGCATCTTTCATTCCTTACTTCTTAAACTTTCTTGCAAGT) primers were used to amplify human interferon α-2b gene by PCR. Forward primer contained *Xba*I (single under lined), *Pst*I (double under lined) and ribosome binding (shown in bold) sites before the start of human interferon α-2b gene. Reverse primer contained *Nde*I (single under lined) and *Nsi*I (double under lined) sites after the stop codon (shown in bold). The gene, after PCR amplification, was ligated in pTZ57R/T vector and the resulting plasmid was named as pTZ-Ifn2. For cloning in the expression vector, plasmid pTZ-Ifn2 was cut by using *Xba*I and *Bam*HI and the liberated DNA fragment was ligated in pET-21a (+) that was cut with *Xba*I and *Bam*HI. The resulting plasmid was named as pET-Ifn2.

Similarly, a set of forward (5'-AATCTAGACTGCAGTAAGAAGGAGATATAACTATGGCTATCTCAATCAAGACC) and reverse (5'-CAT ATGCGATGCATCTTTTATTCGTCGTGCGAGATTATCGCC) primers was used for the amplification of *E. coli* methionine amino peptidase gene by PCR. The restriction enzyme and ribosomal binding sites were identical to the human interferon α-2b in both the primers. PCR generated gene fragment was cloned in pTZ57R/T and the resulting plasmid was named pTZ-Map2.

For bicistronic cloning, plasmid pTZ-Map2 was restricted with *Pst*I and *Nde*I. The liberated methionine amino peptidase gene fragment (Map3) was ligated in pET-Ifn2, which was cut with *Nsi*I and *Nde*I (pET-Inf2L). The cohesive ends of DNA obtained after cleavage with *Pst*I and *Nsi*I are complementary to each other and can be joined together. The resulting sequence, obtained after the joining of *Pst*I and *Nsi*I cohesive ends, cannot be cut either with *Pst*I or *Nsi*I. The resultant derivative plasmid was named pET-Ifn-Map having human interferon α-2b as the first gene and *E. coli* methionine amino peptidase as the second gene. Both the genes had independent ribosome binding sites and were separated by 27 nucleotides. The schematic representation of the strategy adopted for cloning both the genes bicistronically is shown in fig. 1.

Expression of human interferon α-2b and E. coli methionine amino peptidase genes in E. coli

In order to express the genes, *E. coli* BL21-CodonPlus (DE3)-RIL cells were transformed using pET-Ifn, pET-Map and pET-Ifn-Map, independently. The cells, containing the respective plasmid, were grown to early log phase and gene expression was induced with 1mM lactose at 37°C in Luria Bertani medium containing ampicillin at a final concentration of 100µg/mL. The cells were harvested by centrifugation at 5,000x *g* for 10min at 4°C, resuspended in 50mM Tris-HCl (pH 8.0), and disrupted by sonication for 10min at 4°C before analysis by gel electrophoresis.

RESULTS

Cloning of interferon α-2b and methionine amino peptidase genes

Polymerase chain reaction for the amplification of human interferon α-2b gene, using the set of primers given in the materials and methods section, resulted in the amplification of 0.5 kb DNA fragment exactly matching the size of human interferon α-2b gene (fig. 2A). Similarly, polymerase chain reaction for the amplification of *E. coli* methionine amino peptidase gene, using the set of primers given in the materials and methods section as priming strands and *E. coli* genomic DNA as a template, resulted in the amplification of 0.8 kb DNA fragment (fig. 2B) exactly matching the size of *E. coli* methionine amino peptidase gene.

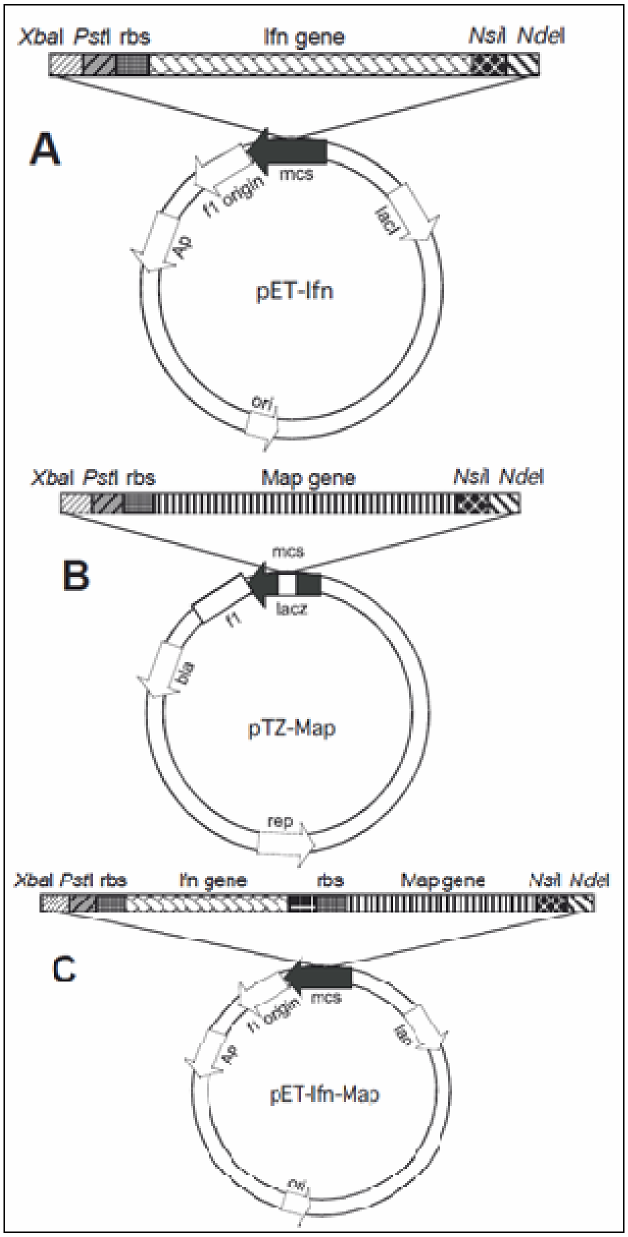

Fig. 1: Construction of plasmids for bicistronic cloning and expression of human interferon α-2b and *E. coli* methionine amino peptidase genes. A) pET-Ifn2 plasmid containing human interferon α-2b gene with *Xba*I, *Pst*I and ribosomal binding sites at the N-terminus, and *Nsi*I and *Nde*I sites at the C-terminus. Digestion of pET-Inf2 with *Nsi*I and *Nde*I produces TGCA and TA overhangs (pET-Inf2L). B) pTZ-Map containing *E. coli* methionine amino peptidse gene with *Xba*I, *Pst*I and ribosomal binding sites at the N-terminal end, and *Nsi*I and *Nde*I site at the C-terminal end. Digestion of pTZ-Map2 with *Pst*I and *Nde*I liberates the Map gene fragment having ACGT and AT overhangs (Map3). C) The ligation of pET-MapL with Map3 produces pET-Ifn-Map containing human interferon α-2b and *E. coli* methionine amino peptidse genes in a single vector with independent ribosomal binding site.

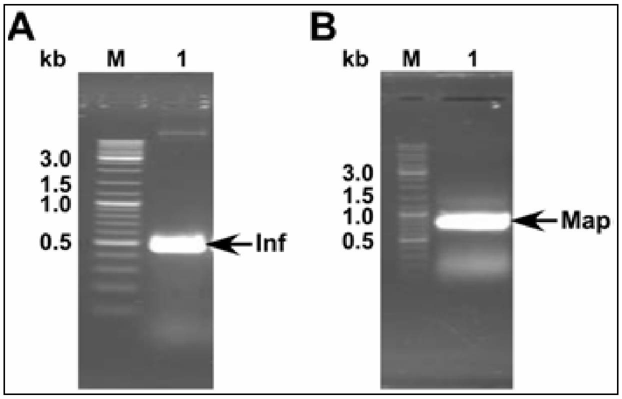

Fig. 2: Analysis of PCR amplified genes by ethidium bromide stained agarose gel electrophoresis. A) PCR amplified human interferon α-2b gene (lane 1). B) PCR amplified *E. coli* methionine amino peptidase gene (lane 1).

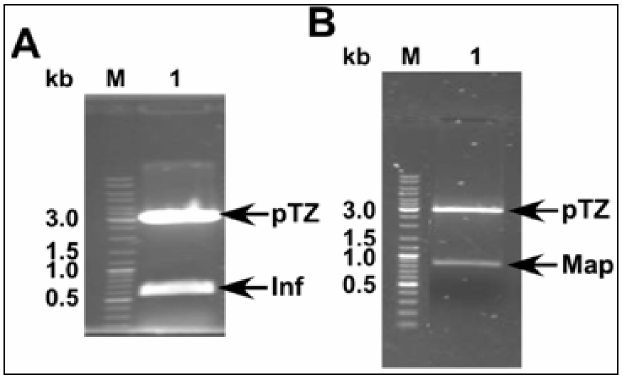

Fig. 3: Ethidium bromide stained agarose gel demonstrating cloning of the PCR amplified gene in pTZ57R vector. A) Cloning of human interferon α-2b gene. Lane 1, recombinant plasmid digested with *Nde*I and *Hin*dIII. B) Cloning of *E. coli* methionine amino peptidase gene. Lane 1, recombinant plasmid digested with *Nde*I and *Eco*R1.

PCR amplified human interferon α-2b gene fragment was purified from the agarose gel and inserted in pTZ57R/T. When the resulting plasmid pTZ-Ifn was used to transform *E. coli* DH5α competent cells several blue and white colonies appeared on the selection plates. White colonies were further screened for the presence of human interferon α-2b gene by colony PCR. Plasmid DNA was extracted from one of the positive clones and digested with *Nde*I and *Hin*dIII which resulted in the liberation of a 0.5 kb DNA fragment from the vector (fig. 3A). DNA sequence of the inserted fragment was determined and the results confirmed that the cloned fragment encodes human interferon α-2b. In order to clone in expression vector, pET-21a (+), the vector was cut with the same two restriction enzymes (*Nde*I and *Hin*dIII) and resolved on agarose gel. Expression vector pET-21a (+) and human interferon α-2b gene were purified from the gel and ligated. When the resulting plasmid pET-Ifn was used to transform *E. coli* DH5α competent cells, a few colonies appeared on the selection plate. Positive clone was selected by colony PCR and restriction enzyme digestion analysis (fig. 4A).

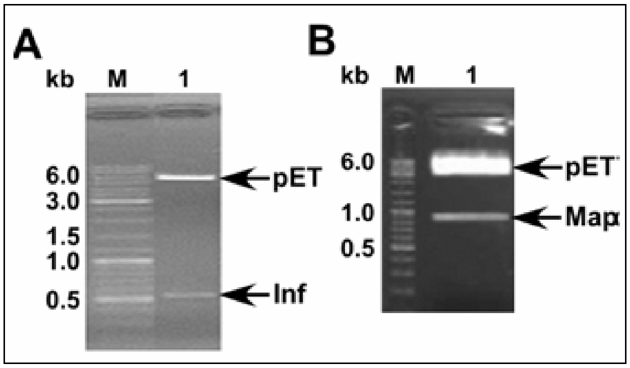

Fig. 4: Analysis of cloning of the two genes in pET-21a (+) vector by ethidium bromide stained agarose gel. A) Cloning of human interferon α-2b gene. Lane 1, recombinant plasmid digested with *Nde*I and *Hin*dIII. B) Cloning of *E. coli* methionine amino peptidase gene. Lane 1, recombinant plasmid digested with *Nde*I and *Eco*R1.

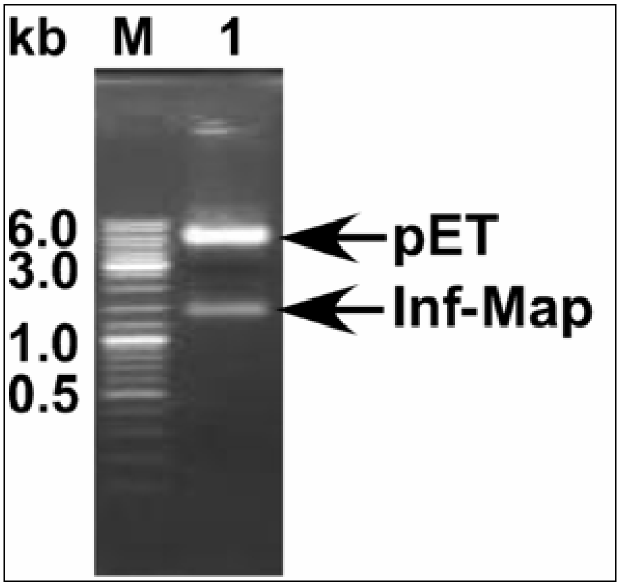

Fig. 5: Ethidium bromide stained agarose gel demonstrating bicistronic cloning of the two genes in pET-21a (+) vector. Lane 1, recombinant plasmid pET-Inf-Map digested with *Xba*I and *Nde*I.

Similarly when *E. coli* DH5α cells were transformed using pTZ-Map, several blue and white colonies appeared on the selection plate. After screening of the white colonies by colony PCR, plasmid DNA was isolated from one of the positive clones and digested with *Nde*I and *Eco*R1 which resulted in the liberation of 0.8 kb DNA fragment (fig. 3B). For cloning the gene into expression vector, pET-21a (+) was cut with *Nde*I and *Eco*R1 and ligated with amino peptidase gene fragment liberated from pTZ-Map. When the resulting plasmid pET-Map was used to transform *E. coli* DH5α cells, several colonies appeared on the selection plate. Positive clone was selected by colony PCR and confirmed by restriction enzyme digestion analysis (fig. 4B).

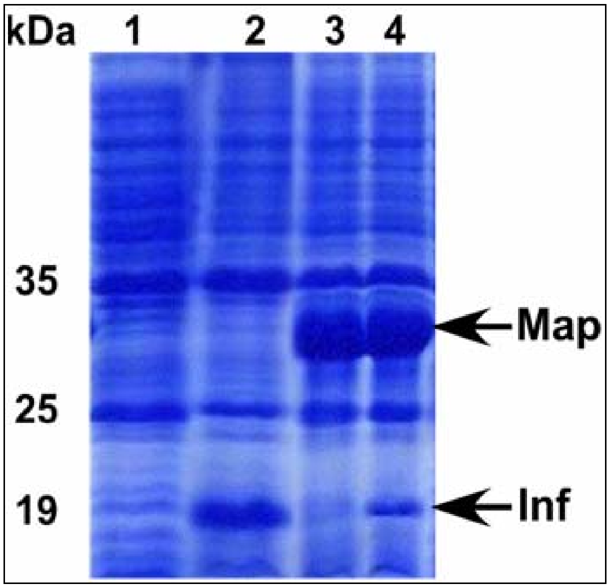

Fig. 6: Coommassie Brilliant Blue stained SDS-PAGE showing the production of recombinant human interferon α-2b (Ifn) and *E. coli* methionine amino peptidase (Map). Lane 1, host cells containing pET-21a (+); lane 2, host cells containing pET-Inf construct; lane 3, host cells containing pET-Map construct; lane 4, host cells harboring pET-Inf-Map construct.

Bicistronic cloning

Strategy adopted for cloning both the genes bicistronically is described in the material and methods section (fig. 1). When the bicistronic plasmid, pET-Ifn-Map, was used to transform *E. coli* DH5α cells, several colonies appeared on the selection plate. Transformants were screened by colony PCR. When plasmid DNA from one of the positive clones was cut with *Xba*I and *Nde*I, a 1.4 kb DNA fragment liberated from pET-Ifn-Map indicating the cloning of the two genes (fig. 5). Furthermore, DNA sequencing confirmed the proper cloning of the two genes in a single vector.

Production of interferon α-2b and methionine amino peptidase in E. coli

For expression of the two genes, *E. coli* BL21-CodonPlus (DE3)-RIL cells were transformed using recombinant plasmids pET-Ifn and pET-Map, independently. When the host cells carrying these plasmids were induced overnight with lactose, interferon α-2b and methionine amino peptidase were produced in a comparable amount (fig. 6).

However, when *E. coli* BL21-CodonPlus (DE3)-RIL cells were transformed using recombinant plasmid pET-Ifn-Map for co-expression of both the genes, there was no difference in the production level of methionine amino peptidase whereas, to our surprise, human interferon α-2b was produced in a much less amount (fig. 6) although human interferon α-2b gene was the first in order, after the promoter, and *E. coli* methionine amino peptidase was the next with a linker sequence of 27 nucleotides between them.

DISCUSSION

Several studies have reported the bicistronic expression of two genes. In principle, the expression of the second gene should be similar to that of the first gene, because each translation sequence has independent ribosomal binding site. However, in practice quite lower expression of the second gene has been observed (Rucker *et al*., 1997; Aslam *et al*., 2013). In some cases incorporation of the promoter sequence before the second gene enhances the expression of the second gene (Kim *et al*., 2004). When we systematically evaluated the bicistronic expression of human interferon α-2b and *E. coli* methionine amino peptidase genes we found that human interferon α-2b, first in order after the promoter, was produced in a quite fewer amount. This is in contrast to the previous work on the bicistronic expression where lower expression of the second gene has been reported (Rucker *et al*., 1997; Aslam *et al*., 2013). The formation of mRNA secondary structures can be ruled out because when there was no competition both the proteins were produced in a comparable amounts and when expressed bicistronically the native protein was produced in a much higher amount in spite of the fact that it was far from the promoter. Based on our results of bicistronic expression we propose here that it is not the order of the genes that determines the amount of recombinant proteins in *E. coli* BL21-CodonPlus (DE3)-RIL cells, rather it is the preference of native over non-native by the host cells. This knowledge should be taken into account when two non-identical genes are being expressed bicistronically.

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