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Full Length Research Paper

Effect of promoter strength and signal sequence on the periplasmic expression of human interferon-α2b in *Escherichia coli*

Ramakrishnan Nagasundara Ramanan[1], Wong Bee Tik[1], Hamid Rajabi Memari[1,2], Siti Nor Ani Azaman[1], Tau Chuan Ling[3], Beng Ti Tey[1,4], Mohd. Azmi Mohd. Lila[1], Mohd. Puad Abdullah[5], Raha Abdul Rahim[1,5] and Arbakariya B. Ariff[1,6]*

[1]Institute of Bioscience, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia.
[2]Shahid Chamran University, Golestan BLV. Ahvaz, Iran.
[3]Department of Process and Food Engineering, Faculty of Engineering, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.
[4]Department of Chemical and Environmental Engineering, Faculty Engineering, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.
[5]Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.
[6]Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.

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Two plasmids, pFLAG-ATS and pET 26b(+), were studied for the periplasmic expression of recombinant human interferon-α2b (IFN-α2b) in *Escherichia coli*. The pFLAG-ATS contains ompA signal sequence and *tac* promoter while pET 26b(+) contains pelB signal sequence and T7*lac* promoter. It was observed that periplasmic expression of IFN-α2b from pET 26b(+) was around 3000 times higher than pFLAG-ATS. Difference in the expression level was attributed to the difference in the promoters and the signal sequences. *In silico* analysis of mRNA secondary structures were analyzed using Vienna RNA package and MFOLD. The results suggested that the increase of expression would mainly due to the difference in the translation initiation associated with secondary structure of mRNA transcribed by both plasmids.

Key words: Interferon-α2b, Periplasm, Signal sequence, mRNA secondary structure, *Escherichia coli.*

INTRODUCTION

Escherichia coli is considered to be a primary workhorse for the protein expression particularly when post-translational modification like glycosylation is not necessary. The recombinant DNA technology has paved the way for the drastic improvement in heterologous protein expression. Different strategies like gene dosage (Kane, 1995), promoter strength (Boer et al., 1983), codon bias in the initiation region (Stenström et al., 2001), engineering 5'-untranslated initiation region (UTIR) (Cebe and Geiser, 2006; Zhang et al., 2006) were used to overcome the limitations during over expression of protein. Combination of plasmids and signal sequences are often studied for the suitability of higher expression (Denefle et al., 1989; Khushoo et al., 2005; Sletta et al., 2007), but in-depth analysis is lacking for the particular choice.

Human interferon-α2b (IFN-α2b) is a biopharmaceutical protein that has excellent antiviral, antimicrobial and anti-tumor actions. It is used to cure the diseases like hairy cell leukemia, chronic hepatitis C, chronic hepatitis B (Ramanan et al., 2008). Recombinant IFN-α2b is non-glycosylated and does not need any post-translational modification for its action. Industrially, IFN-α2b is currently produced in the form of inclusion bodies (IB)

*Corresponding author. E-mail: arbarif@biotech.upm.edu.my. Tel: +603-8946 7591. Fax: + 603-8946 7593.

Abbreviation: ompA, outer membrane protein A; **pelB,** pectate lyase B; **PCR,** Polymerase chain reaction; **ATCC,** American type culture collection; **RNA,** Ribonucleic acid; **rRNA,** ribosomal RNA; **RBS,** Ribosomal binding site

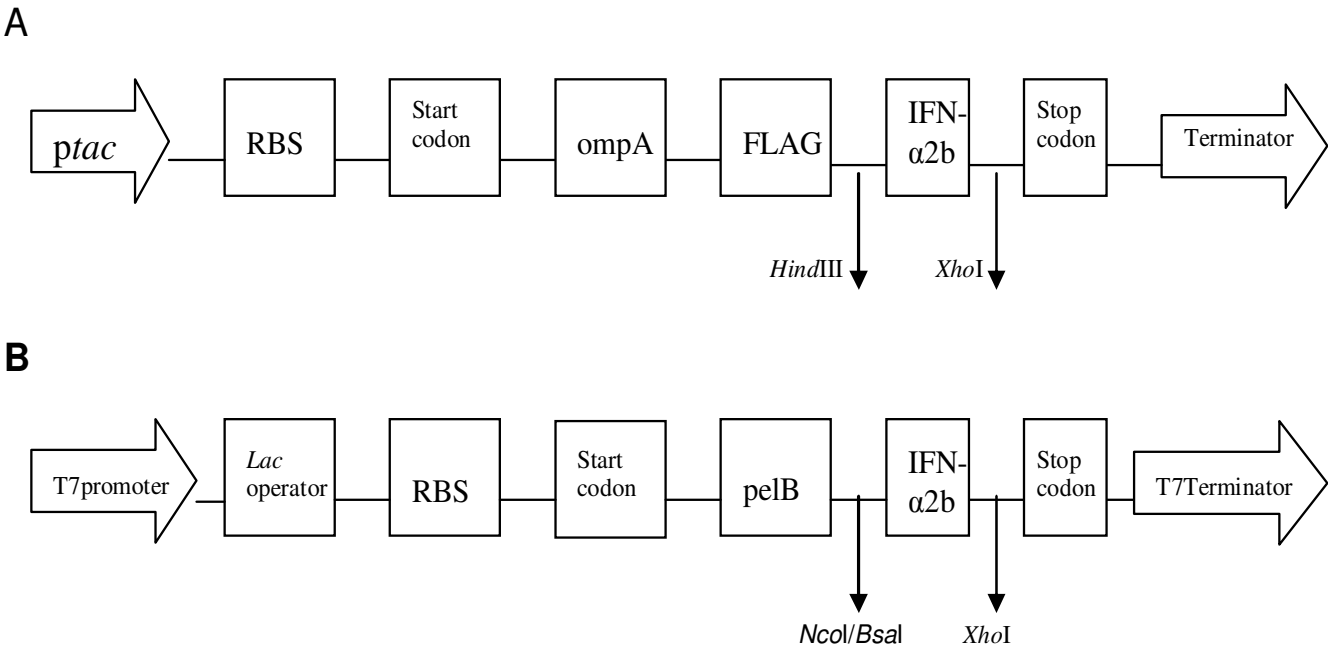


Figure 1. Construct of pFLAG-IFN (A) and pET-IFN (B).

(Graumann and Premstaller, 2006). Different types of IFN, such as IFN-α2 (Barbero et al., 1986), IFN-α2c (Voss et al., 1994) and IFN-γ (Medina-Rivero et al., 2007) have capability to express in periplasmic space of *E. coli*. Recently, Sletta et al. (2007) showed that even in the high cell density culture and with the optimized codon sequence of IFN-α2b, soluble periplasmic expression was not detected.

The objective of the present study was to compare the expression of IFN-α2b cloned in two different plasmids (pFLAG-ATS and pET-26b(+)). This knowledge may be applied in the strategy for the selection of suitable plasmid for the protein expression.

MATERIALS AND METHODS

Materials

Plasmid pFLAG-ATS (Catalog No. E5769) and kanamycin (Catalog No. K4378) was purchased from Sigma-Aldrich, USA. Plasmid pET-26b(+) (Catalog No. 69862), Rosetta-gami 2 (RG 2) competent cells (Catalog No. 71350), RG 2(DE3) competent cells (Catalog No. 71351), overnight express instant terrific broth (ITB) (Catalog No. 71491), anti-α-IFN mouse monoclonal antibody (anti-IFN) (Catalog No. 407290), goat Anti-Mouse total immunoglobulin (Ig) peroxidase conjugate (anti-mouse) (Catalog No. DC08L), ampicillin (Catalog No. 171254), chloramphenicol (Catalog No. 220551), glycerol (Catalog No. 356352) and 3,3 Diaminobenzidine (DAB) tetra hydrochloride (Catalog no. 281751) were obtained from Merck, USA. 30% hydrogen peroxide (Product no. 2186) was obtained from J.T.Baker, USA. Prestained broad range protein marker (Catalog No. P-7708S) and all the restriction enzymes were obtained from New England Biolabs, UK. ELISA kit (Catalog No. RPN5960) was purchased from GE Healthcare, UK. IFN-α2b standard (Catalog No. ORP-16029) was obtained from Affinity Bioreagents, USA. Polyvinylidene fluoride (PVDF) membrane (Immbilon-P^{SQ} membrane, Catalog No. ISEQ 00010) was obtained from Millipore, USA. All other chemicals were purchased from either Sigma-Aldrich or Merck.

Characteristics of plasmids

The two plasmids (pFLAG-ATS and pET-26b(+)) contain ColE1 origin of replication and can produce approximately 40 copy numbers. The main reason of choosing these plasmids is to get more soluble product, to avoid the segregation instability and to reduce metabolic burden. The main difference between these two plasmids is the promoter for the transcription and the signal sequence for the periplasmic expression. pFLAG-ATS contains *tac* promoter and ompA signal sequence where as pET-26b(+) contains T7*lac* promoter and pelB signal sequence. In addition to this difference, pFLAG-ATS contains FLAG fusion tag which is used for the affinity purification. The presence of this tag would stabilize the fusion protein by protecting against proteases and thereby enables high expression level (Einhauer and Jungbauer, 2001).

Preparation of constructs

pFLAG-IFN was prepared by subcloning the coding sequence of IFN-α2b from the source plasmid pALCA1SIFN (ATCC 53369) to the target plasmid pFLAG-ATS using specific forward primer 5'AAATATAAGCTTATGTGTGATCTGCCTCAAACCCAC3' and reverse primer 5'ATTCTCGAGTCATTATTCCTTACTTCTTAAT CA3' between *Hind*III and *Xho*I restriction sites by PCR-based subcloning strategy (Figure 1a). The recombinant plasmid DNA was then transformed into RG 2 competent cell. pET-IFN was prepared by amplifying the coding sequence of IFN-α2b from the same source plasmid mentioned above using specific forward primer 5'GAATGGTCTCTCATGTGTGATCTGCCTCAA3' and reverse

primer 5'GCAGTCCTCGAGTTATTCCTTACTTCTTAAAC3'. The amplified gene was double-digested using *Bsa*I and *Xho*I restriction enzymes. The overhang prepared was subcloned in pET 26b(+) between *Nco*I and *Xho*I restriction sites (Figure 1b). The recombinant plasmid DNA was then transformed into RG 2(DE3). Both the resulting clones were verified by colony PCR amplification and sequencing. RG 2 and RG 2(DE3) are similar expression hosts, containing the plasmid that carries the genes for seven codons, rarely used in *E. coli*. This is useful in increasing the expression level of heterologous proteins by overcoming codon bias (Schumann and Ferreira, 2004; Sørensen and Mortensen, 2005). Additionally, RG 2(DE3) contains a lamda prophage which has the gene for T7 RNA polymerase.

Culture condition

ITB was used as a culture medium at a concentration of 60 g/L. Sterile glycerol (10 mL) was added to 1 L of sterile culture medium along with the respective antibiotics for the particular host (34 mg/L of chloramphenicol and 50 mg/L of ampicillin for the host containing pFlag-IFN where as 34 mg/L of chloramphenicol and 30 mg/L of kanamycin for the host containing pET-IFN). The production of IFN-α2b was carried out in 500 mL Erlenmeyer flask containing 100 mL medium inoculated with 1% of respective stock culture. The flask was incubated at 37°C in a rotary shaker (Certomat® BS-1 B. Braun, Germany), agitated at 225 rpm.

Protein extraction

The cells were harvested after 24 h of cultivation and centrifuged at 2860 x g (rotor model 1619, Universal 32R centrifuge, Hettich AG, Switzerland) for 15 min in 25°C. The cell pellets resuspended in Tris buffer (20 mM Tris-HCl, pH 8.0). were treated with osmotic shock technique to release periplasmic protein and then by glass bead shaking technique to release soluble cytoplasmic protein (Ramanan et al., 2009).

Calculation

The fold increase in the expression of IFN-α2b was calculated as follows:

$$\text{Fold increase in expression} = \frac{\text{Higher expression} - \text{Lower expression}}{\text{Lower expression}} \quad (1)$$

Analytical procedures

Western blot analysis was performed using 20% SDS-PAGE, run at 130 V using mini Protean 3 apparatus (Bio-Rad, USA) in Tris-glycine buffer and transferred in PVDF membrane for 45 min at 15 V. The transferred membrane was incubated with 2% bovine serum albumin (BSA) in phosphate-buffered saline with 0.05% Tween 20 (PBST) for 1 h followed by 0.5% of primary antibody (anti- IFN) and 1% BSA in PBST for 1 h and then by 0.1% of secondary antibody (anti-mouse) and 1% BSA in PBST for 30 min. The washing was done thrice with PBST in between each step. The blot was developed using DAB and hydrogen peroxide. Quantification of IFN-α2b was performed using ELISA as described previously (Ramanan et al., 2008).

Analysis of mRNA secondary structure

The possible mRNA secondary structures were accessed by submitting the particular RNA sequence in Vienna RNA package online software (http://www.tbi.univie.ac.at/~ivo/RNA/). The free energies of the stack and the loop were accessed using MFOLD (http:// mfold.bioinfo.rpi. edu/) (Mathews et al., 1999). The minimum free energy (mfe) for each sub loop was calculated by summing up both free energies of the stack and the loop.

RESULTS

Expression of IFN-α2b

The expression of IFN-α2b was carried out using RG 2 and RG 2(DE3) which carries pFLAG-IFN and pET-IFN plasmids, respectively. Western blot analysis was carried out for the periplasmic and cytoplasmic fractions of pET-IFN. RG 2(DE3) containing pET-26b(+) (without IFN-α2b gene) was grown similar to RG 2(DE3) containing pET-IFN and its periplasmic and cytoplasmic fractions were used as negative control. IFN-α2b standard was used as positive control. While the presence of IFN-α2b in RG 2(DE3) was detected in western blot analysis (Figure 2), it could not be identified as clearly in RG 2 due to the existence of very low amount of IFN-α2b.

The amount of IFN-α2b in different locations of the cell was quantified using ELISA and the comparison in expression is shown in Table 1. The total soluble expression level in pFLAG-IFN and pET-IFN was around 0.4 ng/mL and 447 ng/mL, respectively.

mRNA secondary structure prediction and its minimum free (mfe) energy calculation

The possible secondary structures of mRNA of both plasmids (from transcription starting site) were calculated randomly between 80 and 151 nucleotides (nt). Out of eight structures analyzed (by changing number of nt), four structures gave similar patterns and one of the patterns for both plasmids is shown in Figures 3 and 4 and the corresponding sequences are shown in Figure 5. Both the Shine Dalgarno (SD) sequence and the start codon were closed inside the loop for pFLAG-IFN. On the other hand, the start codon was fully exposed for pET-IFN. The mfe plot is shown in Figure 6 and the calculated free energy of each subloop is also depicted inside the Figure.

DISCUSSION

Effect of promoter strength

Promoter strength is an important factor for the amount of transcripts produced and thereby increasing the protein expression. The plasmids pFLAG-IFN and pET-IFN have *tac*I and T7*lac* promoter, respectively. The efficiency of *tac*I promoter is the highest among their parental promoters (*lac* UV5 and *trp*) and its similar hybrid promoter

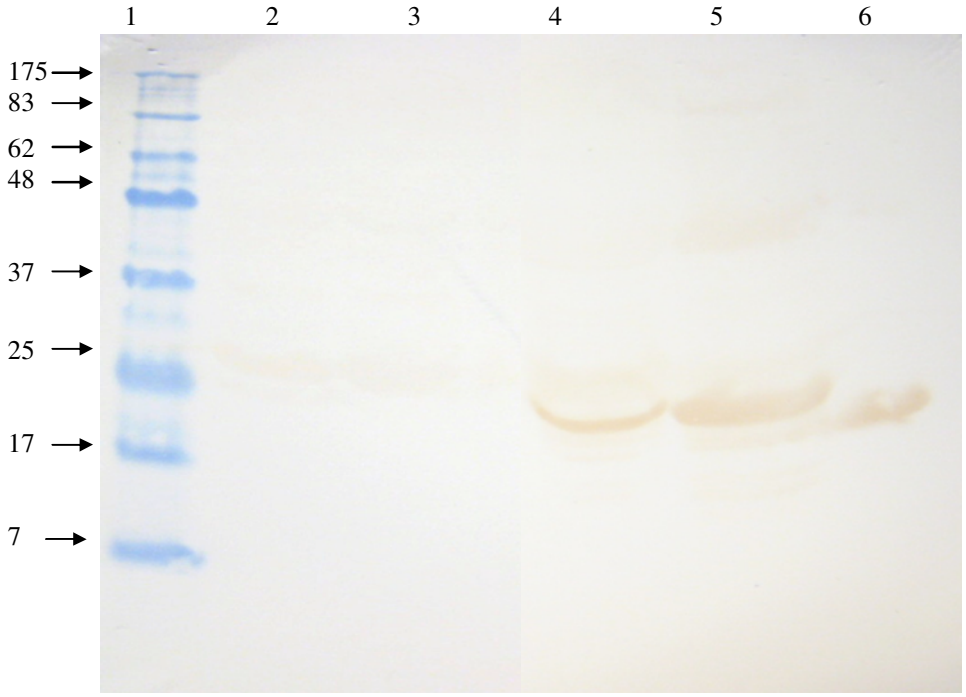


Figure 2. Western blot for pET-IFN. Lane 1, Prestained protein marker (7-175 kDa broad range protein marker, P-7708S from New England laboratories); Lane 2, soluble cytoplasmic fraction of pET 26b(+) in RG2(DE3); Lane 3, soluble periplasmic fraction of pET 26b(+) in RG2(DE3); Lane 4, soluble cytoplasmic fraction of pET-IFN in RG2(DE3); Lane 5, soluble periplasmic fraction of pET-IFN in RG2(DE3); Lane 6, IFN-α2b standard

Table 1. Comparison of IFN-α2b expression between pET-IFN and pFLAG-IFN.

Expression	pET-IFN (ng/mL)	pFLAG-IFN (ng/mL)	Fold increase in expression
Periplasmic	299.7	0.1	2997
Cytoplasmic	147.1	0.3	490
Total Soluble protein	446.8	0.4	1117

The data represents the average values of two shake flask cultures

(*tac*II) (Boer et al., 1983). The efficiency of the T7*lac* promoter would be higher than the *tac*I promoter as their transcription rate is 5 times higher than the normal *E. coli* (Sørensen and Mortensen, 2005). Com-parative studies done by Deuschle et al. (1986) revealed that all the T7 promoters considered for their analysis were higher in their strength than the *tac*I promoter and up to 5 fold differences was noted. This shows that the difference in promoter strength might increase the expression in few folds but definitely not in orders.

On the other hand, study done by Khushoo et al. (2005) revealed that the tac (pTACAsp) promoter yield slightly higher soluble production (~2 UI/mL) than T7lac (p29AsP) promoter. However, their study was focused on soluble but not total product expressed. Often, the T7 promoters would lead to the formation of IB (Baneyx, 1999; Xu et al., 2006).

Effect of signal sequence on mRNA secondary structure

The increase in expression level due to the presence of signal sequence has been reported previously (Khushoo et al., 2005; Sletta et al., 2007). Sletta et al. (2007) suggested that the addition of signal sequence would increase both the transcript and translation levels. The role of secondary structure in translation initiation and its effect in higher expression have been studied by several researchers (Steitz and Jakes, 1975; Lee et al., 1987; Looman et al., 1987; De Smit and van Duin, 1990a; De Smit and Van Duin, 1990b; Gross et al., 1990; Wikstrom et al., 1992; Helke et al., 1993; De Smit and Van Duin, 1994; Tsai et al., 1995; Le Calvez et al., 1996; Satchidanandam and Shivashankar, 1997; Cebe and Geiser, 2006; Zhang et al., 2006) and some of them were

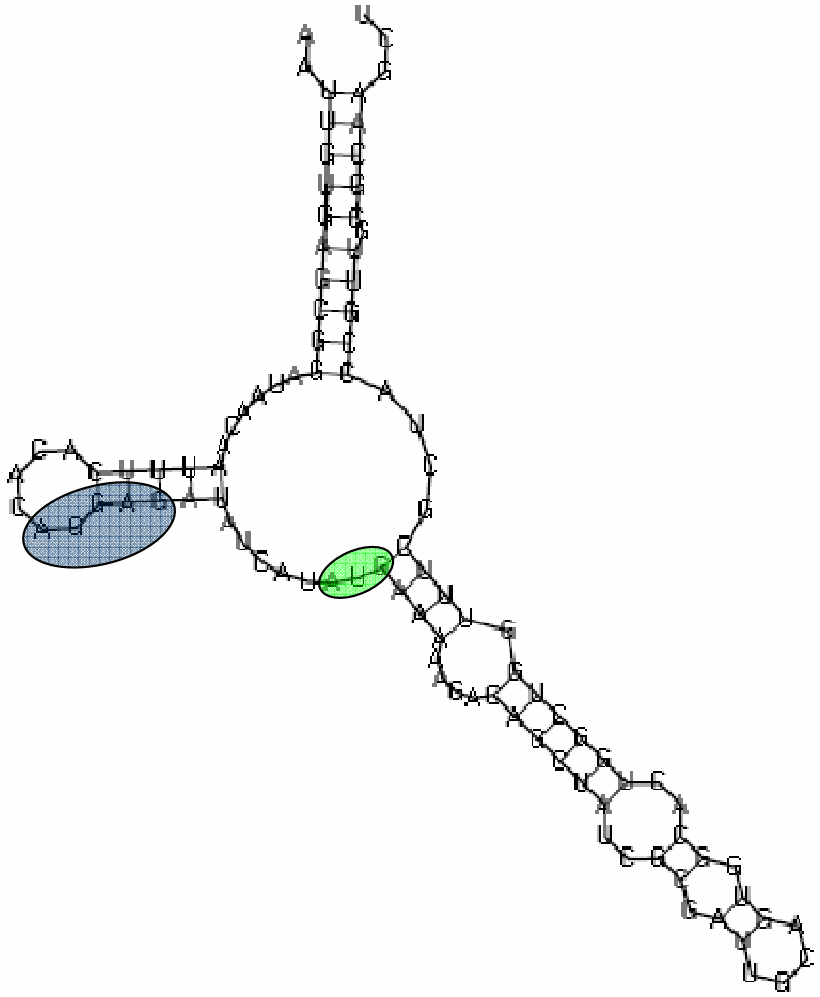


Figure 3. Secondary structure diagram for pFLAG-IFN taken from Vienna RNA package. The blue and green color shading shows the region of SD sequence and start codon.

specific to IFN (Lee et al., 1987; Gross et al., 1990). It should be noted that difference in the secondary structure of mRNA can affect the efficiency of translation as much as 1000 fold (Looman et al., 1987; Helke et al., 1993).

Exposure of SD sequence and correct start codon was necessary for efficient translation (Looman et al., 1987; Wikstrom et al., 1992; Tsai et al., 1995). As the initiation starts by the recognition and base pairing of SD sequence by 16S rRNA (Steitz and Jakes, 1975) and the translation starts by the recognition of start codon, initially it was suggested that recognition could be done in single stranded structure (Looman et al., 1987). A series of research conducted by De Smit and Van Duin (1990a, 1990b; 1994) revealed that the translation enhancement was directly coupled with the free energies present within these region.

A reduction of free energy by 1.4kcal/mol would reduce the expression by 10 fold, above certain reference point (De Smit and Van Duin, 1990b). This reduction may be due to the need of same amount of energy to destabilize the mRNA secondary structure so that it can bind with ribosome. In most cases, the minimum free energy should be higher than -6 kcal/mol for efficient translation (De Smit and Van Duin, 1994; Satchidanandam and Shivashankar, 1997; Zhang et al., 2006). Indeed, the free energy should be in such a way that it should give protection to the nucleases and at the same time high enough to bind with ribosome (De Smit and Van Duin, 1990b).

Some studies have delineated that the stem loop present near/after the start codon alone would cause reduction of expression (Tsai et al., 1995; Le Calvez et al., 1996; Satchidanandam and Shivashankar, 1997) and its participation in the stem loop would block the expression totally (Wikstrom et al., 1992; Satchidanandam and Shivashankar, 1997; Zhang et al., 2006). The bioinformatics analysis of pFLAG-IFN revealed that the three bases of SD sequence were in pair and Guanine of the start codon

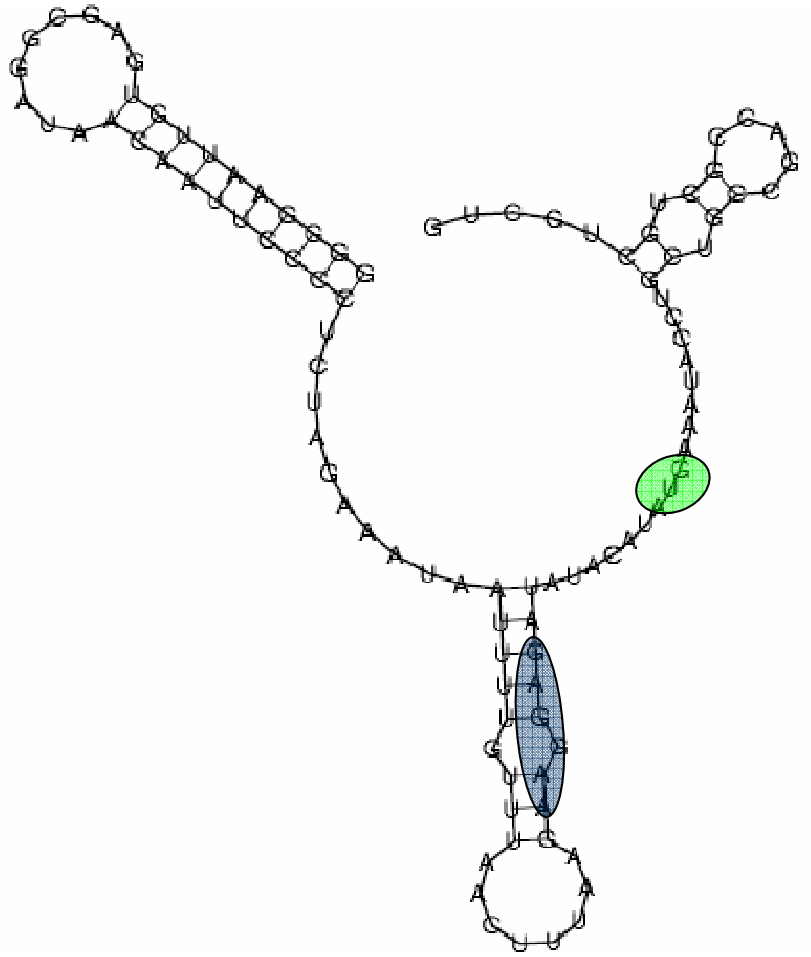


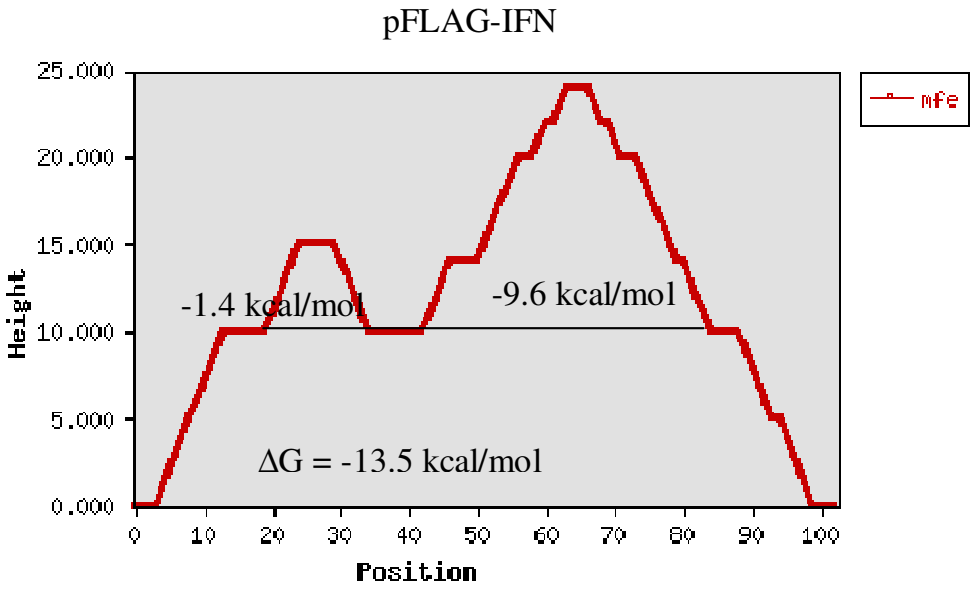
Figure 4. Secondary structure diagram for pET-IFN taken from Vienna RNA package. The blue and green color shading shows the region of SD sequence and start codon.

```
1                 10                  20                  30                  40
AAUUGUGAGC  GGAUAACAAU  UUCACACAGG  AGAUAUCAUA
                  50                  60                  70                  80
UGAAAAAGAC  AGCUAUCGCG  AUUG CAGUGG  CACUGGCUGG
                  90                  100
UUUCGCUACC  GUUGCGCAAG  CU
```

```
 1                 10                  20                  30                  40
GGGGAAUUGU  GAGCGGAUAA  CAAUUCCCCU  CUAGAAAUAA
                  50                  60                  70                  80
UUUUGUUUAA  CUUUAAGAAG  GAGAUAUACA  UAUGAAAUAC
                  90                  100
CUGCUGCCGA  CCGCUGCUGC  UG
```

Figure 5. Sequenence of pFLAG-IFN and pET-IFN numbered in 10s which was used to run the secondary structure prediction. The yellow, blue and green colors indicate the transcription start site/*Lac*I binding region, SD sequence and start codon, respectively.

A



B

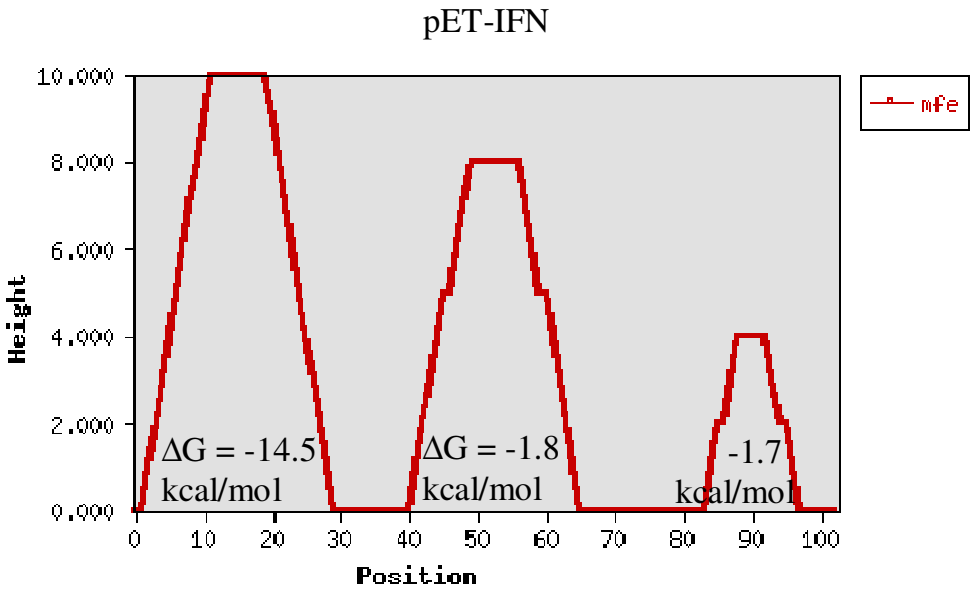


Figure 6. Minimum free energy plot for pFLAG-IFN (A) and pET-IFN (B) taken from Vienna RNA package. X axis shows number of bases used in the analysis. Y axis shows relative height of base pairs between the bases. The minimum free energy (mfe) of each loop is calculated as per the thermodynamic algorithm in MFOLD and pasted inside the loop.

was in the long stem loop (Figure 3) while the start codon of pET-IFN was fully exposed for active translation (Figure 4). The split up of minimum free energy (mfe) of pET-IFN (Figure 6b) also showed that the stem loop in the SD sequence and after the start codon was well above -6 kcal/mol, but this is different in the case of pFLAG-IFN (Figure 6a). Moreover, the upstream loop of pET-IFN would give protection against the nucleases as it was highly stable. The collective effect of all the above might have increased the expression by more than 1000

fold.

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