In vivo evidence for a new concept of bacterial translation initiation

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Summary

The canonical initiation mode starts with the small ribosomal subunit, which with help of initiation factors and the initiator tRNA finds the start signal for protein synthesis on an mRNA. However, there are many observations in the literature that cannot easily be reconciled with this initiation mode. We found that a resolution of these difficulties can be provided with an alternative initiation mode, the so-called 70S-scanning mode. In this thesis we provide *in vivo* evidences supporting it. Towards this end we identify features that critically participate in this process.

1. According to general wisdom the initiation factor IF1 binds to 30S subunits helping IF2 and IF3 to select the ribosomal binding site on the mRNA. The factor is believed to leave the 30S subunit upon association with the large ribosomal subunit. Therefore, it should never be present on 70S ribosomes. We determined the ribosome location of IF1 using cytosol-profiles on sucrose gradients and identified IF1 via Western blotting. It was strikingly found that IF1 is present specifically on 70S and polysomes rather than on 30S subunits. IF3 is thought to be an anti-association factor and thus should not be able to bind to 70S ribosomes. We found it on 30S subunits as expected, but 1/3 of the amount was present on 70S and significant amounts even on disomes. Obviously, the functional horizon of these initiation factors is wider than thought before.
2. IF1 is an essential factor. To study its function *in vivo* we constructed a strain, where the chromosomal IF1 was knocked out and the IF1 gene present on a plasmid after an inducible AraB promoter (in the presence of arabinose IF1 is expressed, in the presence of glucose it is not). The effects of insufficient IF1 amounts can be summarized as follows: (i) A slowed growth rate with doubled generation time. (ii) Serious defects in 50S assembly leading to accumulation of 50S-precursors, accumulation of 30S subunit and strikingly poor polysome pattern. Obviously, the block in 50S formation reduces the formation of 70S and thus explains the indispensability of IF1 for bacterial cell survival. The specific defects of the 50S assembly are consistent with an assumption of involvement of IF1 in scanning ribosomes leading to stoichiometric synthesis of ribosomal proteins.
3. Our assumption was that IF1 is essential for the 70S scanning initiation. To test this we constructed a bicistronic mRNA expressing Renilla and Firefly luciferase,

respectively. The hypothesis predicts that the first cistron might be preferentially initiated in the canonical mode, whereas for the second expression the IF1-dependent scanning mechanism would be more important. Precisely this was observed: The expression of the second cistron was 4.5 times reduced, when the cells were starved for IF1. Remarkably, the expression of the first cistron was practically not affected at all by reducing IF1 amounts. Western blotting showed that this expression-reduction was accompanied by reducing the IF1 amounts for about 50%. This observation distinctly presents the evidence of involvement of IF1 in sliding 70S ribosomes, leading to translation initiation of second cistron.

4. Eventually we demonstrated that the 70S-scanning type of initiation also exists for the expression of monocistronic mRNAs. With a strong secondary structure in the 5'-UTR abolishing the scanning mode we observed that the expression of the reference protein GFP was not affected by various levels of IF1, whereas without secondary structure allowing both initiation modes the expression was strongly IF1 dependent.

In summary we provided strong evidence that IF1 is a 70S specific factor and participates crucially in the 70S-scanning type of initiation. Since the 70S-scanning mode is importantly involved in the expression of both mono- and polycistronic mRNAs it might be even the prevailing initiation mode in the bacterial cell.

Zusammenfassung

Das Standardmodell der Initiation beginnt mit der kleinen Untereinheit, die mit Hilfe der Initiationsfaktoren und der Initiator fMet-tRNA das Startsignal für die Proteinsynthese auf einer mRNA findet. Jedoch sind einige Beobachtungen nicht einfach mit dem Standardmodell in Einklang zu bringen. Wir prüfen in der vorliegenden Arbeit eine Hypothese, nach der neben dem Standardmodell eine zweite Initiationsart existiert, das sogenannte 70S-Scanning Modell, das die Ungereimtheiten befriedengend erklären kann. Die erzielten Ergebnisse der *in vivo* Untersuchungen unterstützen das Modell:

1. Nach allgemeiner Ansicht bindet der Initiationsfaktor IF1 an die kleine Untereinheit und unterstützt die Faktoren IF2 und IF3, die ribosomale Bindungsstelle für den Start der Proteinsynthese auf einer mRNA zu finden. Die Annahme ist, dass IF1 die 30S Untereinheit nach Assoziation der großen verläßt. Daraus folgt, daß dieser Faktor nicht auf 70S Ribosomen anzutreffen sein soll. Wir bestimmten die IF1 Lokalisation auf ribosomalen Partikeln mittels Cytosol-Profilen auf Sucrose-Gradienten und anschließender IF1 Identifikation mittels Western-Blot. Zu unserer Überraschung fanden wir IF1 ausschließlich auf 70S und zu einem geringen Anteil auf Disomen, aber nicht auf der kleinen Untereinheit. IF3 als Antiassoziationsfaktor wird für einen spezifischen 30S Faktor gehalten, der nicht auf 70S Ribosomen anzutreffen sein soll. Unsere Western-Analyse zeigte, dass wie erwartet ein großer Teil des IF3 wie erwartet an die 30S Untereinheit bindet, aber etwa ein Drittel der Menge an 70S Ribosomen bindet. Es folgt daraus, dass der funktionelle Horizont beider Faktoren offenbar weiter reicht als allgemein angenommen.
2. IF1 ist ein essentieller Faktor. Um dessen Funktionen *in vivo* zu testen, haben wir einen *E. coli* Stamm konstruiert, dem das chromosomale IF1-Gen fehlt und der Zelle auf einem Plasmid angeboten wird, dessen AraB Promoter die IF1 Synthese an- und ausschalten kann (in Gegenwart von Arabinose wird IF1 synthetisiert, während Glucose die Synthese abschaltet). Die Effekte einer IF1 Verarmung können folgendermaßen zusammengefaßt werden: (i) Eine 50% Reduktion der IF1 Menge halbiert die Wachstumsrate. (ii) Die Bildung der großen 50S Untereinheit ist schwer geschädigt, 50S Vorstufen werden angehäuft, 30S Untereinheiten akkumulieren mit dem Ergebnis, dass 70S Ribosomen sowie Polysomen deutlich vermindert sind. Die spezifischen Defekte des 50S Aufbaus können damit erklärt

werden, daß IF1 an einem 70S-Scanning Initiations-modus beteiligt ist, was zu einer stöchiometrischen Synthese der ribosomalen Proteine führt.

3. Unsere Annahme, dass IF1 wichtig für eine 70S-Scanning Initiation ist, wurde folgendermaßen getestet: wir konstruierten eine bi-cistronische mRNA, mit der die Renilla und die Feuerfliegen Luziferase exprimiert werden kann. Die produzierte Menge beider Luziferasen können in einem Ansatz ohne Überlappung getestet werden. Unsere Hypothese sagt voraus, dass das erste Cistron vornehmlich nach dem kanonischen 30S Modell initiiert wird, während bei der Expression des zweiten Cistrons der IF1 abhängige Scanning-Modus deutlicher beteiligt ist. Genau das wurde beobachtet: Eine IF1 Reduktion um 50% reduzierte die Translation des zweiten Cistrons um das 4,5 fache, während interessanterweise die Translationsleistung am ersten Cistron durch reduzierten IF1 Gehalt gar nicht beeinträchtigt wurde. Dieser Befund ist eine deutliche Unterstützung des Scanningmodells und belegt zum ersten Mal, dass IF1 wahrscheinlich ein spezifischer 70S Initiationsfaktor ist und eine geringe Rolle – wenn überhaupt – bei der kanonischen 30S Initiation spielt.
4. Schließlich haben wir gezeigt, dass der 70S-Scanning Typ auch bei der Translation von mono-cistronischen mRNAs beteiligt ist. Bei einer mRNA mit einer starken Sekundärstruktur an der 5'-UTR, die 70S-Scanning verhindert aber eine 30S abhängige Initiation erlaubt, ist die Expression von GFP unabhängig von der IF1 Menge in der Zelle, während ohne Sekundärstruktur die GFP Synthese stark von der IF1 Menge abhängig war.

Zusammengefasst, haben wir sehr starke Hinweise, dass IF1 ein spezifischer 70S-Scanning Initiationsfaktor ist, der eine bedeutende Rolle bei diesem neuen Typ der bakteriellen Initiation spielt. Da die 70S-Scanning Initiation sowohl bei der Initiation von poly- als auch mono-cistronischen mRNAs eine Rolle spielt, könnte dieser Initiationstyp sogar der in der bakteriellen Zelle vorherrschende sein.

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Abbreviations

AA	Acrylamide
aa-tRNA	Aminoacyl-tRNA
AcPhe-tRNAPhe	N-Acetyl-Phe-tRNAPhe
Å	Angstrom
ATP	Adenosine tri-phosphate
BAA	Bis-acrylamide
BCIP	5-Bromo-4-Chloro-3-indolyl phosphate
BPB	Bromophenol blue
cfu	colony forming unit
dsDNA	Double strand DNA
EF-G	Elongation factor G
EF-Tu	Elongation factor thermo unstable
eRF1	Eukaryotic release factor 1
GDP	Guanosine di-phosphate
GFP	Green Fluorescent protein
Gly	Glycine
GTP	Guanosine tri-phosphate
HCl	Hydrochloric acid
H_2SO_4	Sulphuric acid

$H_tM_uN_vSH_wSpd_xSpm_y$	H Hepes	t mM
	M $Mg(Ac)_2$	u mM
	N NH_4Ac	v mM
	SH ß-mercapto-ethanol	w mM
	Spd Spermidine	x mM
	Spm Spermine	y mM

HPLC	High Performance Liquid Chromatography
IF	Initiation factor
kb	kilo bases
kDa	kilo Dalton
kJ	kilo Joules
kV	kilo Volts
Leu	Leucine

mA	milli Ampere
µCi	micro Curie
MDa	mega Dalton
$Mg(Ac)_2$	magnesium acetate
MQ	milli Q water
M.W.	molecular weight
NaAc	sodium acetate
NBT	nitroblue tetrazolium
NH_4Ac	ammonium acetate
nt	nucleotide(s)
NTP	Nucleoside tri-phosphate
Ω	Ohm
PAGE	Poly acrylamide gel electrophoresis
Phe	Phenylalanine
Poly(U)	Long poly-uridine mRNA
PPi	Inorganic Pyrophosphate
PTF	Peptidyl transferase centre
RF	Release factor
rpm	revolutions per minute
rRNA	ribosomal RNA
RRF	Ribosome recycling factor
RTS	rapid translation system
SD	Shine Dalgarno sequence
SDS	Sodium dodecyl sulphate
30S	small ribosomal subunit
50S	large ribosomal subunit
70S	Prokaryotic ribosome
80S	Eukaryotic ribosome
V	Volts
v/v	volume/volume
w/v	weight/volume
pBER-GFPcyc3	Berlin GFP
pET-GFPcyc3	canonical cyc3-GFP
pBER-int-GFPcyc3	Berlin intermission GFP

1 Introduction

The ribosome is a macromolecular ribonucleoprotein complex which catalyses peptide bond formation – in a process known as translation. Information is transported from the genome *via* mediator molecules called messenger RNAs (mRNAs) to the ribosome, and it translates the sequence of the codons on the mRNA into the corresponding sequence of amino acids, using adaptor molecules – transfer RNAs (tRNAs).

Protein synthesis can be divided into four main steps: initiation, elongation, termination and ribosome recycling. Each ribosome consists of a small and large subunit, which are primed on the translation initiation region (TIR) of the mRNA during the initiation phase of translation {Laursen, 2005}. Initiation is the rate-limiting step {Kozak, 1999}, where the ribosomal subunits are associated on the mRNA together with the initiator tRNA assisted by the initiation factors {Gualerzi, 1990}. Elongation is the process where the ribosome slides over the mRNA, one codon at a time, translating the genetic information and adding one amino acid to the growing peptide chain after each codon being read. When the ribosome reaches one of the three stop codons, the nascent peptide chain is released upon interaction with the release factors {Nakamura, 1996} thus causing termination. During recycling phase the termination factors and the tRNAs are released from the ribosome, and it is thought that this is followed by dissociation of the two subunits from the mRNA {Janosi, 1996; Janosi, 1998}. A single ribosome can incorporate 10 to 20 amino acids per second {Bremer, 1996} with an accuracy of about one misincorporation per 3000 amino acids incorporated {Bouadloun, 1983}.

1.1 The machinery of translation initiation

1.1.1 The ribosome and its subunits

The bacterial ribosome has a mass of approximately 2.6-2.8 mega daltons (MDa), a relative sedimentation rate of 70S and a diameter of 200-250 Å. There can be 3,000-70,000 ribosomes per cell, the maximal number at mid log phase of *E. coli* {Bremer, 1996}. Under certain functional states the 70S ribosome falls apart into two unequal subunits: a large 50S subunit and a small 30S subunit.

The small ribosomal subunit has a relative sedimentation rate of 30S and a mass of 0.8 MDa, whereas the large ribosomal subunit has a relative sedimentation rate of

50S and a mass of 1.5 MDa. Each subunit is a ribonucleoprotein particle with one third of the mass consisting of protein and the other two thirds of ribosomal RNA (rRNA) {Ramakrishnan, 2001}: 33 proteins and two rRNA molecules – 5S (120 nts), and 23S (~2,900 nts) rRNA in the large subunit, and 21 proteins and a single 16S (~ 1,500 nts) rRNA in the small subunit (**Figure 1.1.1-1**).

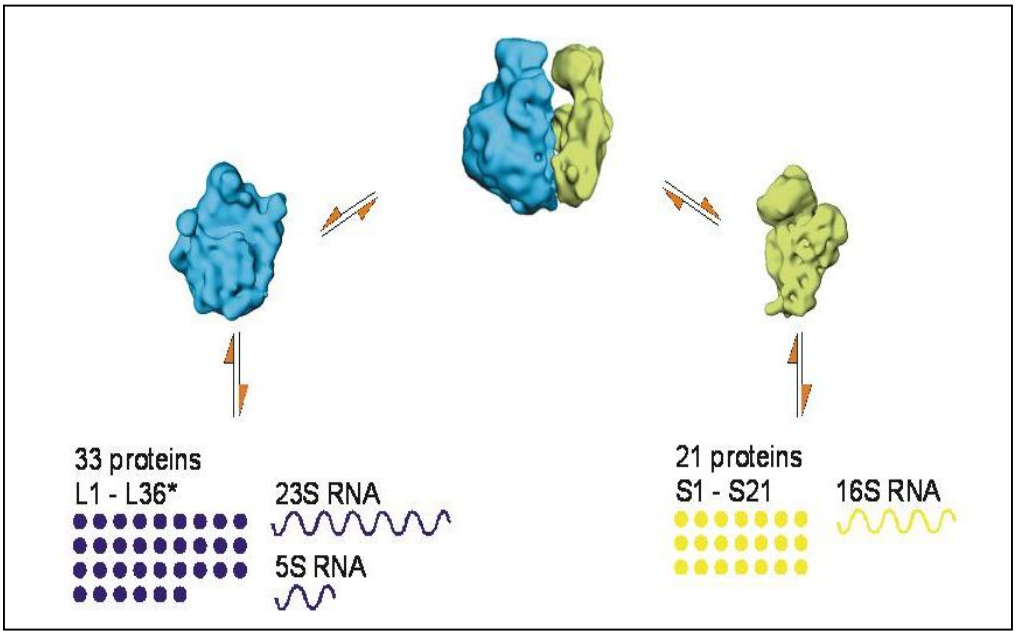


Figure 1.1.1-1: Composition of the* E. coli *ribosome

Thus, the ribosome is a large polyanion. The tertiary structure of the 16S and 23S rRNA is mainly stabilized by three types of interactions: Mg^{2+} bridges, RNA-RNA interactions and RNA-protein interactions. The magnesium ions form neutralizing bridges between two or more phosphate groups in secondary structure distant in sequence {Laursen, 2005}.

Both subunits differ in functions as well. On the 30S subunit the codon-anticodon interaction between the mRNA and tRNA occurs, namely the process of decoding. The large 50S subunit performs the central catalytic function of protein synthesis *i.e.* the active center forms peptide bond between the nascent polypeptide chain and the incoming aminoacylated tRNA and it also provides the path for emerging proteins.

The ribosome has three binding sites for tRNA, shared between the two subunits, namely the A site, P site and the E site. The aminoacyl (A) site has high affinity for aminoacyl-tRNA (aa-tRNA); the peptidyl (P) site has high affinity for peptidyl-tRNA; and the exit (E) site has high affinity for deacylated tRNA {Ramakrishnan, 2002}.

1.1.1.1 The small ribosomal subunit

The small ribosomal subunit is composed of 21 proteins (designated from S1-S21) and an rRNA of 1,542 nucleotides (in *E. coli*) sedimenting at 16S. The 30S subunit is divided into three domains (head, body, and platform).

This subunit performs decoding, the process in which accurate aminoacyl-tRNA (aa-tRNA) is selected during translation. It sets the translational fidelity {Ogle, 2002; Youngman, 2006}. The decoding centre consists almost entirely of rRNA containing the upper part of helix 44 and the 3´ and 5´ ends of 16S rRNA {Schluenzen, 2000}. Nucleotides G530 from the shoulder domain, and A1492 and A1493 in helix 44 come together to span the minor groove of the codon–anticodon duplex at the first two codon positions. This results in a closed conformation of the 30S subunit, compared to open structure, when the A site is unoccupied {Wimberly, 2000}. Cognate anti stem loop (ASL) induces a closure of the 30S subunit around the A site, which is enhanced in the additional presence of paromomycin. During decoding it is essential that high fidelity is maintained. The error rate of tRNA selection in the decoding process is 10^{-3}-10^{-4} {Kurland, 1992}.

In the process of high fidelity of translation, ribosome recognizes the geometry of codon-anticodon base pairing in a way that would discriminate against near-cognate tRNAs. In short, cognate tRNA are the ones that follow complementarity of the all three nucleotide of the codon and the anticodon region, near cognate follows strictly the middle and first one with third one wobbling but the non cognate has complete mismatch and no complementarity between codon and anticodon nucleotides. The minor groove of the first and second base pairs between the codon and anticodon is closely monitored by a set of interactions that are induced by the binding of cognate tRNA. These interactions would be disrupted by mismatches, so that the induced structural changes would no longer be energetically favourable. The third or “wobble” position has less stringent constraints, and therefore can allow a broader range of base-pairing geometries, consistent with the requirements of the genetic code {Ogle, 2001}. This checking of stereo-chemical correctness of codon-anticodon interaction plays the most important role in the selection of correct amino acid. In the kinetic proofreading mechanism, the binding energy for the near cognate amino acid is lower than the cognate one. Therefore the probability for triggering conformational change required for the accommodation of aa-tRNA into the A site is lower for near cognate

one. This in turns will favor their fall off. Also their re-binding is unlikely to occur in the presence of ternary complexes that have 2-3 orders of magnitude higher affinity to A site. Kinetic proofreading mechanism requires EF-Tu-dependent GTP hydrolysis. It was shown that incorporation of the near cognate amino acid resulted in 10-fold increase in GTP consumption as compared to cognate one, making it bit unfavorable for selection whereas non cognate amino acids which could not stimulate GTP consumption at all are rejected at the first step on base pairing {Ogle, 2005}. Hence significant contribution to accuracy of translation (1000 fold) lies in stereo-chemical monitoring whereas kinetic proofreading mechanism plays only a minor role.

1.1.1.2 The large ribosomal subunit

The large ribosomal subunit is composed of 34 proteins (designated L1-L34) and two rRNAs containing about 120 and 2,900 nucleotides sedimenting at 5S and 23S rRNAs, respectively. Within the 23S rRNA six secondary structure domains are defined {Noller, 1981}, while the 5S rRNA is considered as the seventh domain of the large subunit {Steitz, 2003}. The 50S subunit is composed of a rounded base with three protuberances named the L1 protuberance, the central protuberance and the L7/L12 stalk {Wilson, 2003}. The protuberances are highly mobile, which is functionally important.

One of the special features of the 50S subunit is the tunnel for peptide exit. It runs from the peptidyl-transferase centre (PTC) at the foot of the central protuberance through the subunit down to the base of the cytoplasmic side of it with a length of about 100 Å and a width of 10 to 20 Å {Nissen, 2000}. Additionally, the 50S subunit has a factor-binding centre and all of the G-protein factors involved in protein synthesis interact with it during at least part of their duty cycles.

The central function of the large subunit is to perform peptidyl transfer and this performed by peptidyl transfer center (PTC). This is an exclusive function of the rRNA without the involvement of any protein component and therefore they are also termed as a ribozyme {Cech, 2000; Steitz, 2003; Wilson, 2003}. After peptide bond formation the deacylated tRNA from the P site has to move into the E site and the peptidyl-tRNA has to move from the A site to the P site. This process is called translocation, accompanied by the movement of mRNA by three nucleotides exposing the next codon in the A site {Noller, 2002}.

1.2 Translation

Each cycle is divided into three major steps, “Initiation”, “Elongation” and “Termination”.

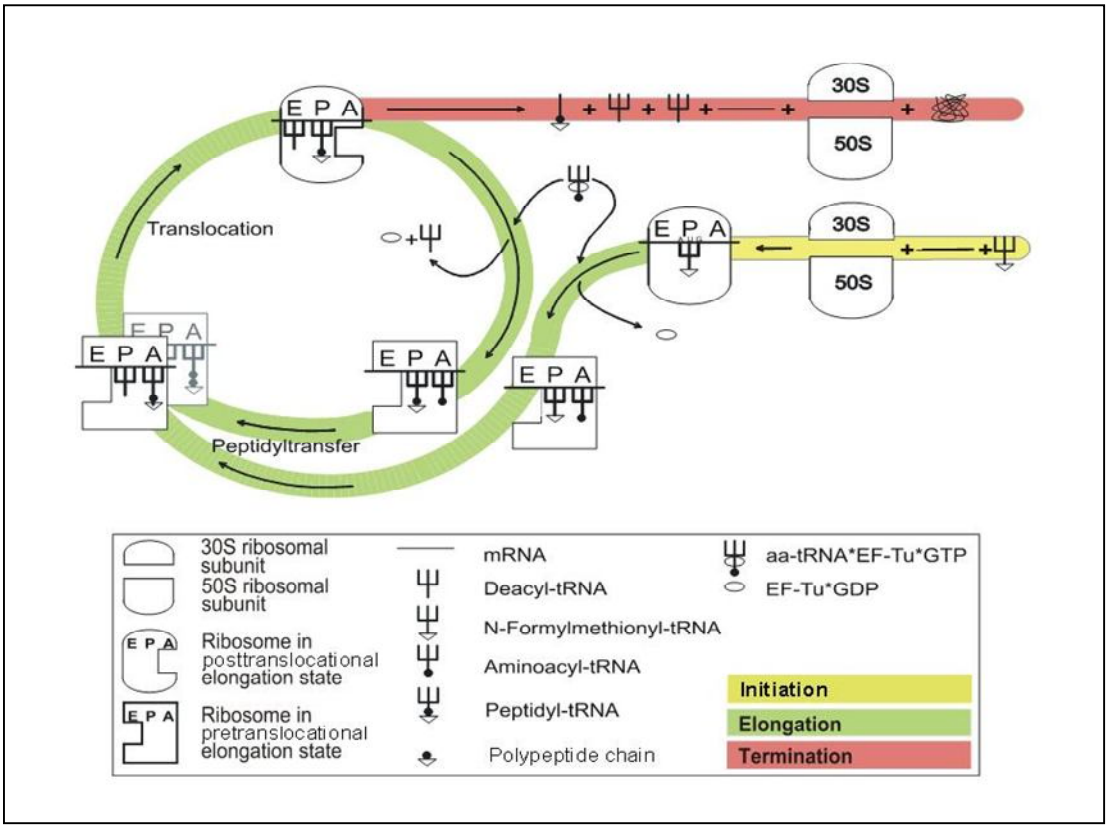


Figure 1.2-1 Summarizing all three steps of a bacterial translation cycle

1.2.1 Translation Initiation

Translation initiation involves recognition of the start codon by the initiator tRNA in the ribosomal P site. In bacteria, this process is thought to be kinetically controlled by three initiation factors (IF1, IF2 and IF3) and occurs in two major phases {Gualerzi, 1990; Gualerzi, 2001; Boelens, 2002}.

The first phase is assembly of the 30S initiation complex (30SIC).During this phase, the initiator tRNA (fMet-tRNA $_{f}^{Met}$) binds the 30S P-site and its anticodon pairs with the start codon of mRNA. Initiation factors maximize the accuracy of tRNA selection at the translation initiation step {Antoun, 2006}. Removal of IF1, IF3 or IF2 reduces *in vitro* the initiation efficiency by seven, nine or 600 folds respectively, indicating their different effects on rate of initiation. It was also later suggested that the essential nature of IF1 and IF3 is their ability to dissociate 70S ribosomes to recruit them again for initiation {Antoun, 2006}.

The second phase includes the docking of the 50S subunit onto the 30SIC, IF2-dependent GTP hydrolysis, and dissociation of the initiation factors. This generates a 70S initiation complex (70SIC) ready for the first round of elongation. The rate of subunit joining that lead to form functional 70SIC entering elongation and the dissociation of IF3 is strongly influenced by the nature of codon used for initiation, structural elements of the TIR and also by IF1 {Milon, 2008}. The efficiency of translation initiation is determined by many *cis*-acting elements located in the translation initiation region of the mRNA like the initiation codon AUG and the SD {Shine, 1974}.

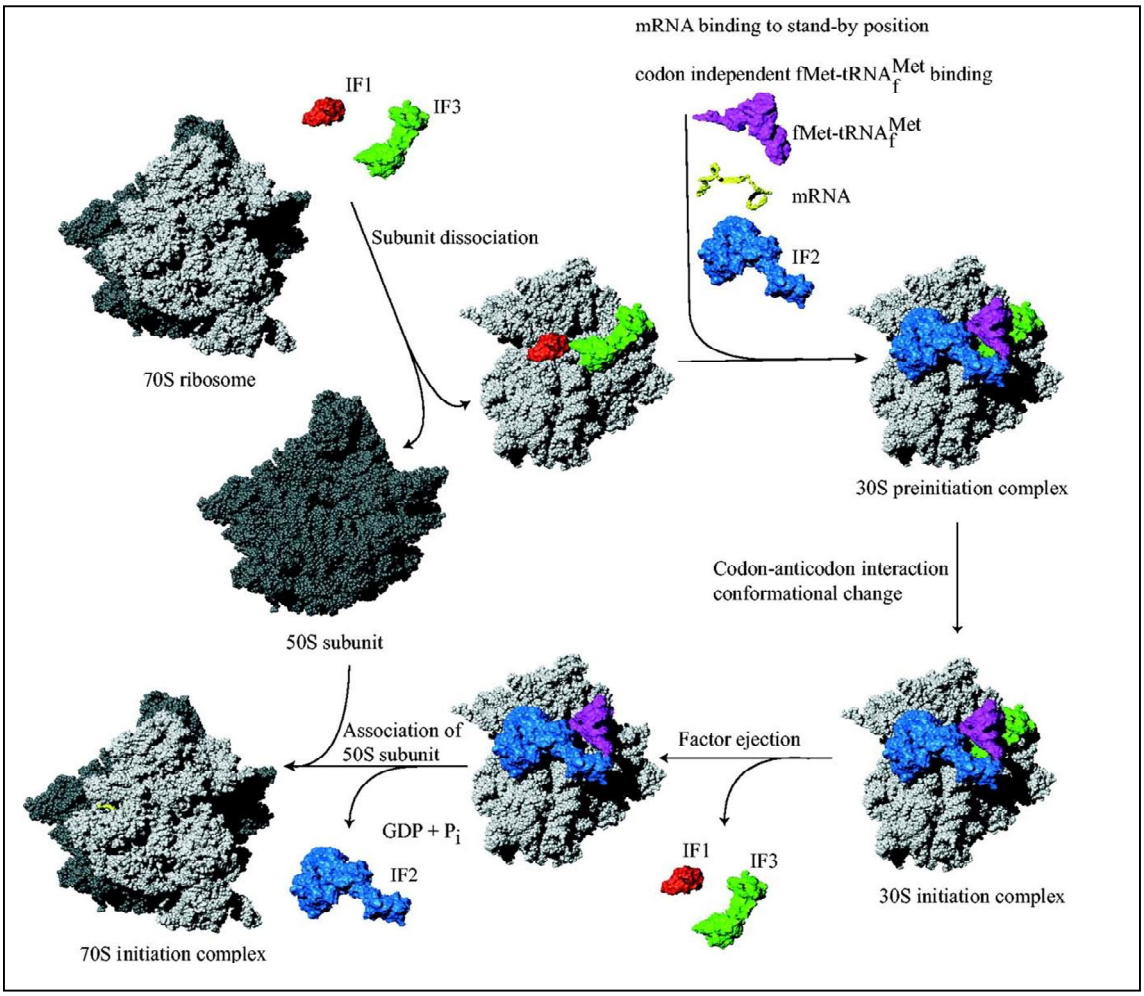


Figure 1.2.1-1: 30S binding mode of translation initiation in prokaryotes (canonical model). *30S and 50S ribosomal subunits are shown in light and dark grey, respectively. Translation initiation factors IF1, IF2, and IF3, the mRNA, and the fMet-tRNA* $_f^{Met}$ *are shown in red, blue, green, yellow, and magenta, respectively {Laursen, 2005}.*

1.2.2 Elongation

Elongation of the peptide chain {Spahn, 1998} is the central event in protein synthesis. The elongation cycle directly involves two main protein elongation factors, EF-Tu and EF-G. EF-Tu forms a ternary complex with GTP and an aminoacyl-tRNA, directing the tRNA to the ribosomal A site. After peptidyl-transfer, EF-G is involved in translocating the mRNA•peptidyl-tRNA complex from the A site to the P site. This enables the next EF-Tu ternary complex access to the new codon present in the A site and thus the elongation cycle is repeated. The elongation cycle ends when ribosome encounters a termination codon into the A site, as a result complete polypeptide is released from the peptidyl-tRNA, mediated by the RFs.

1.2.3 Termination

The synthesis of the polypeptide chain continues until a stop codon (UAA, UAG or UGA), is encountered. Release factors (RF), come into action, releasing nascent polypeptide chain from the ribosome and recycling the ribosomes for the next initiation. Two classes of release factors are distinguished: Class I factors that do not consume energy and are responsible for the hydrolysis of the peptidyl-tRNA. RF1 and RF2 belong to this group, and recognize UAG and UGA respectively; also both recognize termination codon UAA. RF1 and RF2 have a high sequence similarity at the amino acid level {Craigen, 1985}. The Class II release factors are energy consuming and RF3 belongs to this class. The main function of RF3 is to support removal of the class I RFs from the ribosome using GTP hydrolysis, once the peptide hydrolysis has taken place {Za*via*lov, 2001}.

We aim to test a new concept of translation initiation mode existing is bacteria and in this context we add the novel and essential role of translation initiation factors But before, it's an important prerequisite to have pre understanding of the structure and reported function of these factors as per the literature wisdom.

1.2.4 Components of 30S initiation complex

1.2.4.1 The messenger RNA

The messenger RNA (mRNA) is the central molecule in the translation of a genetic message into protein. It is at the ribosome binding site (RBS) which extends over about 30 nucleotides {Steitz, 1969}, the ribosome docks. The recognition and binding of the 30S ribosomal subunit to the correct start site of the mRNA depends to various degrees on structural elements of the translational initiation region (TIR) of the

mRNA. The main elements of a canonical TIR includes the purine-rich Shine-Dalgarno (SD) sequence complementary to the 3´ end region of the 16S rRNA anti-SD {Shine, 1974}, the initiation codon (most frequently AUG), and a spacer of variable length separating the SD sequence and the initiation codon {Gualerzi, 1990}. In *E. coli* the mRNAs usually have the SD sequence AGGAGG located 5-7 nucleotides upstream of the start codon. In general, the spacer between the SD and the start codon varies between 3 and 12 nucleotides and has been shown to be crucial for initiation efficiency {Gold, 1988}.

In the canonical initiation complex the start codon can be AUG, GUG or UUG {Mccarthy, 1994}. These initiation codons occur in *E. coli* at a frequency of 90% (AUG), 8% (GUG) and 1% (UUG) {Blattner FR, 1997}. The exceptional AUU initiation codon is used in *infC* and *pcnB* genes, encoding IF3 {Sacerdot, 1996} and poly(A)-polymerase {Binns N, 2002} respectively.

1.2.4.2 The initiator tRNA

Escherichia coli contains a major and a minor form of initiator tRNA, namely $tRNA_{f1}^{Met}$ (~75%) encoded by three tandemly repeated structural genes (*metZ*, *metW*, *metV*) {Kenri, 1994; Ikemura, 1977} and $tRNA_{f2}^{Met}$ (~25%) by *metY* gene {Ikemura, 1977}. Both tRNAs contain 76 nucleotides and differ in the presence of either 7-methyl-G or A at position 46 {Gualerzi, 1990}.

The initiator tRNA has some unique structural features namely the presence of three consecutive G-C base pairs in the anticodon stem {Schweisguth, 1997}, absence of a Watson-Crick base pair between positions 1 and 72 in the acceptor stem {Thanedar, 2000} and presence of a purine-11: pyrimidine-24 in contrast to the pyrimidine-11:purine-24 base pair found in other tRNAs {Varshney, 1993} but have the same CAU anticodon as elongator tRNA and is aminoacylated by the same synthetase, which recognizes primarily the bases of the anticodon {RajBhandary, 1994}. Later, the aminoacylated initiator tRNA is formylated by methionyl tRNA transformylase (MTF). The most important determinant for formylation of the initiator tRNA is the absence of a 1:72 base pair. Formylation favors selection of the fMet-$tRNA_{f}^{Met}$ by IF2 {Sundari, 1976}, and blocks binding to the elongation factor EF-Tu and thus function as initiator tRNA {Nissen, 1995}.

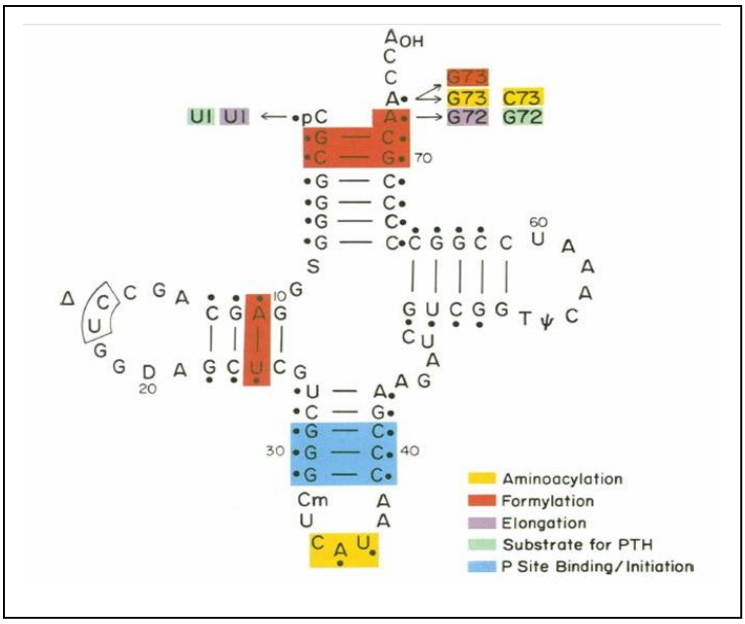


Figure 1.2.4.2-1: Regions of* E. coli *tRNA* $_2^{fMet}$ *important for specifying the various special properties of the tRNA highlighted in color {RajBhandary, 1994}.

1.2.4.3 Initiation factor IF1

In the survey for the IF1, we found that it has been described until now only in context with the 30S-binding mode initiation.

It is the smallest translation initiation factor with the molecular weight of 8.2 kDa. IF1 is essential for cells viability {Cummings, 1994} and is required for optimal translation initiation in *E. coli* {Sands, 1987}. In *E. coli* IF1 is encoded by *infA* gene and contains 71 amino acid residues of known sequence {Pon CL, 1979}. The gene *infA* has been cloned and mapped at 19.9 min on the *E. coli* chromosome {Sands, 1987}. Its concentration is 0.15-0.2 copy per ribosome {Howe, 1983}. Two promoters (P1 and P2) regulate the transcription of the *E. coli* gene as monocistronic mRNAs and both follow ρ-independent transcriptional termination {Cummings, 1991}. The 5´-untranslated region of P1 transcripts is approximately 200 nucleotides longer compared to the P2 transcripts. At normal growth temperatures the P2 promoter is more active than P1, making P2 transcript roughly two-fold more abundant than the P1. In contrast, transcription from the *infA* P1 promoter is highly activated during cold shock {Ko, 2006} suggesting that there is differential activation of the *infA* promoters in response to diverse environmental changes.

The structure of IF1 in solution has been determined by NMR spectroscopy {Sette, 1997}. This structure is characterized by a remarkable rigid five-stranded β-barrel. The N- and C-termini outside the β-barrel are disordered and highly flexible. The loop

connecting strands β3 and β4 of the β-barrel contains a short 3_{10} helix (residues 38-44). The fold of IF1 classifies it to oligomer-binding (OB) family, a class of proteins that interact with oligonucleotides and oligosaccharides {Sette, 1997}. Structures of the archaeal and eukaryotic IF1 homologues (aIF1A and eIF1A respectively) have also been determined {Battiste, 2000}. These structures are highly similar to bacterial IF1 regarding the OB fold (**Figure 1.2.4.3-1**).

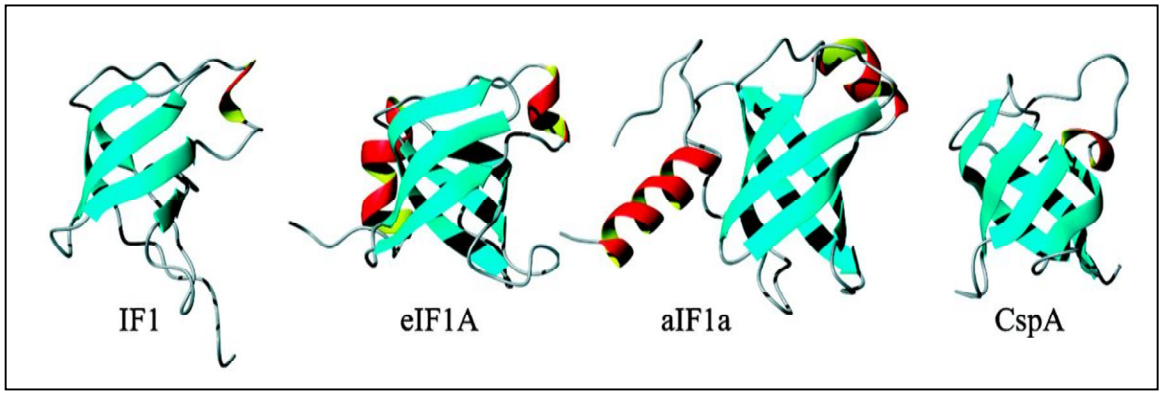


Figure 1.2.4.3-1: Initiation factor IF1 and its structural homologues*: IF1 from* E. coli*, human eIF1A (residues 40 to 125), aIF1A from* Methanococcus jannaschii *and cold shock protein A (*CspA*) from* E. coli *are shown {Laursen, 2005}.*

The OB fold family includes RNA binding proteins such as ribosomal protein S1 {Gribskov, 1992}, the cold shock proteins CspA and CspB {Bycroft, 1997}, eukaryotic initiation factors eIF1A {Battiste, 2000}, eIF2α {Gribskov, 1992} and N-terminal domain of aspartyl tRNA synthetase {Ruff M, 1991}.

Another study indicated that IF1 mimics domain IV of the elongation factor G. This was achieved by the comparison of the hydrophilicity plot of the domain IV of EF-G and homologous region of IF1 {Brock, 1998}.

Not only there is considerable similarity in the structure of eIF1A and IF1 in terms of OB folds, there is also great deal of conservation pattern observed in terms of amino acid identity. With our analysis, we find IF1 about 45 to 90% conserved in bacterial kingdom and it also has a very high percentage identity with archeal and eukaryotic counterparts (details provided in the Results section 3.1).

This immense structural resemblance with high percentage of amino acid identity supports the view of the universal role of IF1, which could be correlated in terms of having similar function (our hypothesis, see section 1.2.6). eIF1A is a part of macromolecule 40S scanning complex and {Pestova, 2001}, eIF1A with eIF1 induces an open conformation of the 40S making it scanning competent {Passmore, 2007}. All

the more both of them are required to locate initiation codon in good context *i.e.* Kozak consensus sequence in eukaryotes {Mitchell, 2008; Pestova, 1998}.

In *B. subtilis* cellular defects caused by *cspB-cspC* double deletion {Weber, 2001} could be cured by heterologous expression of the translation initiation factor IF1 from *E. coli*, but it cannot fully complement CSP's (Cold Shock Proteins) functionality suggesting that both have at least in part overlapping cellular function(s) {Sommerville, 1999}.

Protein-protein crosslinking has identified S12, S18 and IF2 as IF1 neighbors on the ribosome {Boileau, 1983}. In fact, S12 has been found to be important component for decoding, and mutation studies performed in S12 have shown that it likely plays role in tRNA selection process {Sharma, 2007}. Later, chemical probing with dimethyl-sulfate and kethoxal showed that IF1 protects the bases G530, A1492 and A1493 in the 16S rRNA indicating that the factor binds in the A site of the 30S ribosomal subunit {Moazed, 1995}. This set of nucleotides is also protected by the A site bound tRNA {Moazed, 1990} strongly suggesting that they have an overlapping binding site {Baan, 1976}.

Several mutations were made in decoding region of 16S rRNA to characterize the IF1-16S rRNA interaction {Dahlquist, 2000}. It was found that mutation in the 530 loop does not influence IF1 binding in the A site whereas C1407-G1494 base pair and A1408, A1492, A1493 are required for optimal IF1 binding, and mutation of any of the three, deteriorates IF1 binding to the greatest extent {Dahlquist, 2000}. IF1 directly contacts 1400-1500 region of 16S rRNA and affects the 530 loop *via* conformational change. A local change in the conformation of A site caused by the binding of IF1 may be transmitted to the accuracy center of the 30S subunit thus tying the fidelity apparatus used by the A site during elongation to the process of tRNA selection in the P site during translation initiation {Dahlquist, 2000}.

X-ray crystallography clearly confirmed the A-site localization of IF1, and also showed that the factor binds in the cleft between ribosomal protein S12, helix 44 and the 530 loop of the 16S rRNA {Carter, 2001} (**Figure 1.2.4.3-2**). The side of IF1 that interacts with the ribosome is rich in basic residues, whereas most of the acidic residues are on the solvent-exposed surface. A loop from IF1 inserts into the minor groove of helix 44, forms contacts with the backbone of several nucleotides, and flips out bases A1492 and A1493. In turn, these changes induce a rotation of the head, platform and shoulder of the 30S subunit towards the A site {Carter, 2001}. This conformational

change may represent a transition state between subunit association and dissociation {Ramakrishnan, 2002}. However crystallographic data did not confirm that IF1 mimics A site bound tRNA {Brock, 1998}.

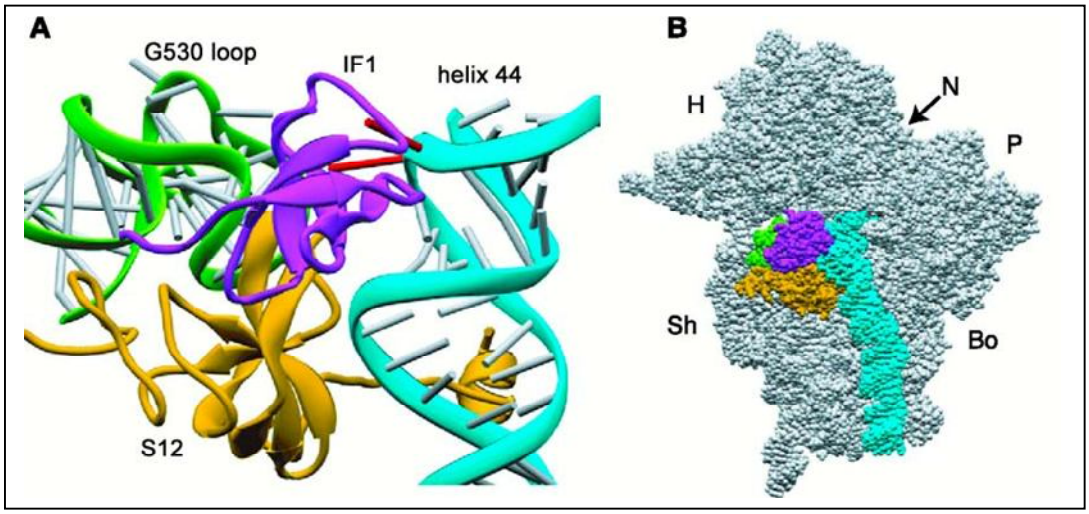


Figure 1.2.4.3-2: Interaction of IF1 with the 30S subunit*: (A) View of the IF1 binding site, with IF1 in purple, helix 44 in cyan, the G530 loop in green, and protein S12 in orange. (B) Overview showing the position of IF1 (purple) with respect to the 30S subunit (gray). H44, G530 loop, and S12 are coloured as in (A). H, head; Bo, body; N, neck; Sh, shoulder; P, platform, {Carter, 2001}.*

IF1 is known to be involved in several functions. Previous data indicates that IF1 stimulates kinetically the formation of initiation complex by increasing (approx. 2.5 times) the limiting V_{max} of the process and thus increase fMet-tRNA binding {Pon, 1984}. It also stimulates IF2 dependent fMet-tRNA binding to 30S or 70S ribosome in the presence of mRNA {Cummings, 1994}.

IF1 function is that of modulating the affinity of IF2 for the ribosome by favoring its binding to the 30S subunit. And vice-versa, the release of IF2 is indirectly promoted when IF1 is ejected {Celano, 1988; Stringer, 1977}. The influence of IF1 on the affinity of IF2 for the 30S ribosomal subunit may result from a physical contact between these two factors on the ribosome as suggested by cross-linking studies {Boileau, 1983} and phylogenetic comparisons {Choi, 2000} or may be indirectly caused by the IF1 induced conformational change as described above. IF1 cooperates with IF2 to ensure that only the initiator tRNA binds to the P site and that it interacts with the initiation codon of the mRNA {Hartz, 1990}. Similar is the function of *B. steaorothermophilus* IF1 and hence can substitute *E. coli* IF1 *in vitro* and *in vivo* {Kapralou, 2008}. However there is one notable difference between *E. coli*, *B.*

steaorothermophilus and *T. thermophilus* IF1, namely that the latter two do not promote the binding of active core of IF2 (IF2ΔN) to 30S which suggests that an existence of IF1-IF2 interaction stimulating IF2 binding to ribosome is at least, not universal {Kapralou, 2008}. In addition, the synergistic action of IF1 and IF2 promotes dissociation of peptidyl-tRNAs with polypeptides of short length from the P site of translating *E. coli* ribosomes {Karimi, 1998}.

By preventing the access of elongator tRNA to the A site, IF1 may participate in conferring specificity to the formation of the 30S initiation complex and subsequent ejection of IF1 opens the A site for incoming aminoacyl-tRNAs {Moazed, 1995}. IF1 while occupying the A site has no codon discriminatory functions at the second codon position, {Croitoru, 2005}.

IF1 affects the association/dissociation rate of ribosomal subunits {Dottavio-Martin, 1979}, favoring the ribosome dissociation activity of IF3 {Grunberg-Manago, 1975}. Recently it was reported that IF1 together with IF3 enhance splitting of 70S ribosomal complex with mRNA having strong SD {Pavlov, 2008}.

In spite of an extensive number of publications concerning IF1 functions reported here, there is not one that describes why IF1 is an absolute requirement for bacteria. There has to be an IF1 specific role, which when disrupted lead to lethal phenotype explaining its essentiality.

1.2.4.4 Initiation factor IF2

IF2 is an essential protein {Cole, 1987} encoded by the *infB* gene and is a part of the polycistronic *nusA* operon {Mortensen, 1995}. Its concentration is 0.15-0.2 copy per ribosome {Howe, 1983}.

IF2 consists of three major segments, these are a variable N-terminal region, a highly conserved 40 kDa part containing in its center a G-domain (GTP binding) and a 25 kDa C-terminal part which contains the fMet-tRNA binding site {Boelens, 2002}. Additionally, the factor can be divided in six domains based on interspecies homology {Mortensen, 1998} (**Figure 1.2.4.4-1**).

Three isoforms of the initiation factor, named as IF2-1 (97.3 kDa), IF2-2 (79.7 kDa), and IF2-3 (78.8 kDa), exist in *E. coli* and other members of the family Enterobacteriaceae {Laursen, 2002}.

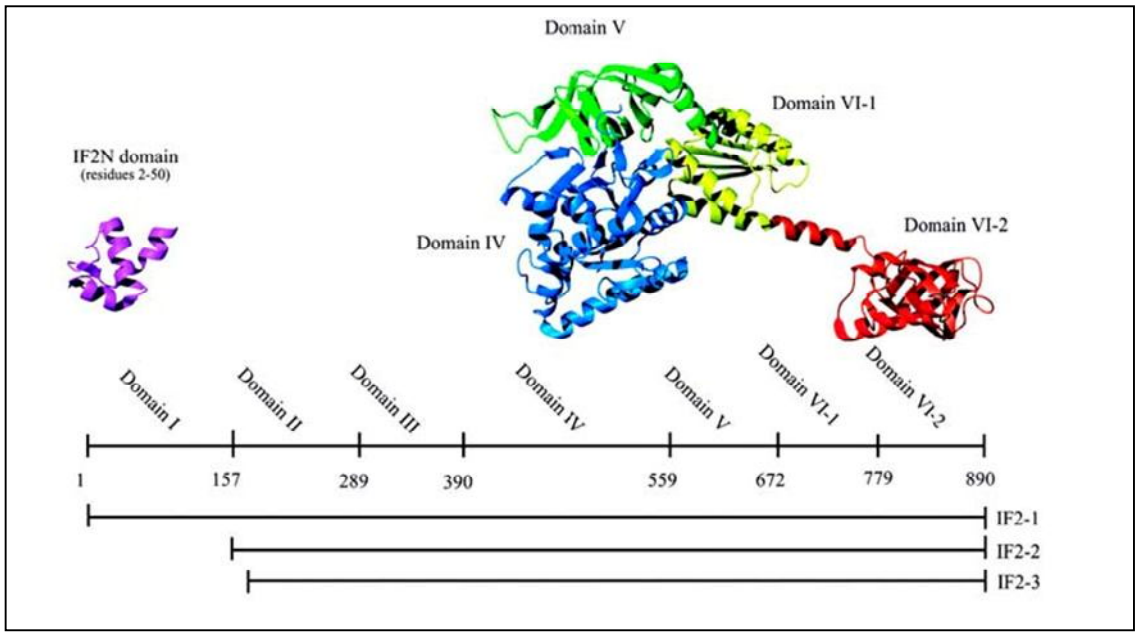


Figure 1.2.4.4-1: Ribbon illustration of the IF2N domain from* E. coli *and the IF2 homologue aIF5B from* M. thermoautothrophicum. *Schematic representation of the E. coli IF2 primary structure, with the domain borders and the length of the three IF2 isoforms {Laursen, 2005}.***

The three isoforms are translated from *infB* mRNA with three different in frame start sites termed as tandem translation {Nyengaard, 1991}. N-terminus lacking forms become strongly dependent on GTP and IF1 for binding to 30S where as full length isoform can independently bind to 30S {Kapralou, 2008}.

Homologues of IF2 have been found in archaea and eukaryotes, where the factor is referred to as aIF5B and eIF5B, respectively {Kyrpides, 1998}. This homologues do not bring the initiator tRNA to the P site but have other functions. Highest homology is observed in G-domain segment for bacterial IF2, eukaryotic eIF5B and archaeabacterial aIF5B {Laursen, 2005}. Mammalian mitochondria have IF2 and IF3 homologs of eubacteria, but a homolog of IF1 is lacking. Recently it was found that mitochondrial IF2 has an extension of conserved 37 amino acids, which is functionally equivalent to IF1 {Gaur, 2008} suggesting IF1 remains essential here also. Cryo-EM structure of a translation initiation complex from *E. coli* showed that IF2-GDPNP binds at the inter-subunit cleft of the 70S ribosome {Allen, 2005}. It contacts the 30S and 50S subunits as well as fMet-tRNA $_{f}^{Met}$.

The main function of IF2 is stimulation of initiator tRNA binding to the ribosome. In spite of the common opinion that IF2 is the carrier of initiator tRNA to the ribosome, as EF-Tu is the carrier of the elongator aminoacyl tRNAs, some data suggest that IF2 is already bound to 30S when it performs its activities {Canonaco, 1986}. Additional

functions reported for IF2 are that of promoting subunit association {Grunberg-Manago, 1975; La Teana, 2001} and upon formation of the 70S initiation complex, favoring the first transpeptidation by inducing the correct positioning of fMet-tRNA in the ribosomal P site {La Teana, 1996}.

1.2.4.5 Initiation factor IF3

Initiation factor IF3 consists of 180 amino acids in *E. coli* and is encoded by the essential *infC* gene mapped at 37.5 min {Sacerdot, 1982}. The gene encoding IF3 is a member of the *infC-rpmI-rplT* operon {Chiaruttini, 1996}. Its concentration is 0.15-0.2 copy per ribosome {Howe, 1983}.

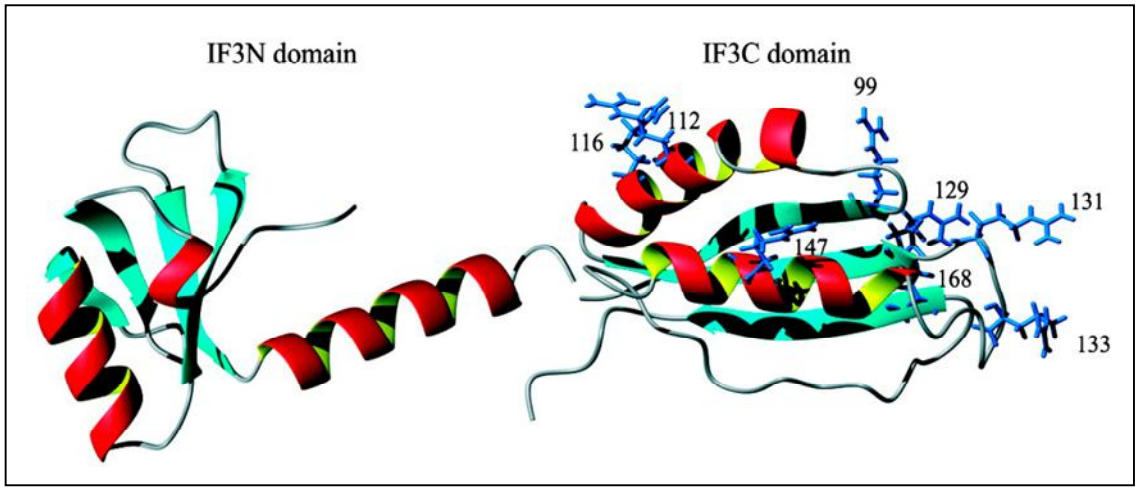


***Figure 1.2.4.5-1: Structure of the IF3N domain from* B. stearothermophilus *and the IF3C domain from* E. coli**. *The side chains of arginine residues in IF3C involved in binding to the 30S subunit (99, 112, 116, 147 and 168) or mRNA (129, 131 and 133) are shown, {Laursen, 2005}.*

IF3 is a 20.5 kDa protein containing two domains of approximately equal size {Moreau, 1997} that are connected *via* a ~45 Å long, lysine-rich and flexible linker {Moreau, 1997}. The linker is essential for IF3 function, but variation of its length and composition does not considerably change the activity of the factor. Crystallographic structures of the two domains IF3N and IF3C of *B. stearothermophilus* have been determined {Biou, 1995} (**Figure 1.2.4.5-1**) and when their binding was investigated it was found that the two domains binds to the 30S subunit {Sette, 1999} and gets removed from it {Moreau, 1997} independently of each other. N-terminus of IF3 binds to E site part of 30S {Dallas, 2001} and this result was obtained *via* protection study. The protected residues of 16S rRNA by IF3 binding identified, in the publication were selected by us and mapped on 30S subunit as shown in **Figure 1.2.4.5-2 A.** In fact

the localization of IF3 on the 30S ribosomal subunit has been extensively investigated by protein-protein {Boileau, 1983} and protein-RNA crosslinking {Ehresmann, 1987}, by chemical probing {Moazed, 1995}, by cryo-electron microscopy {McCutcheon, 1999} and X-ray crystallography {Pioletti, 2001}.

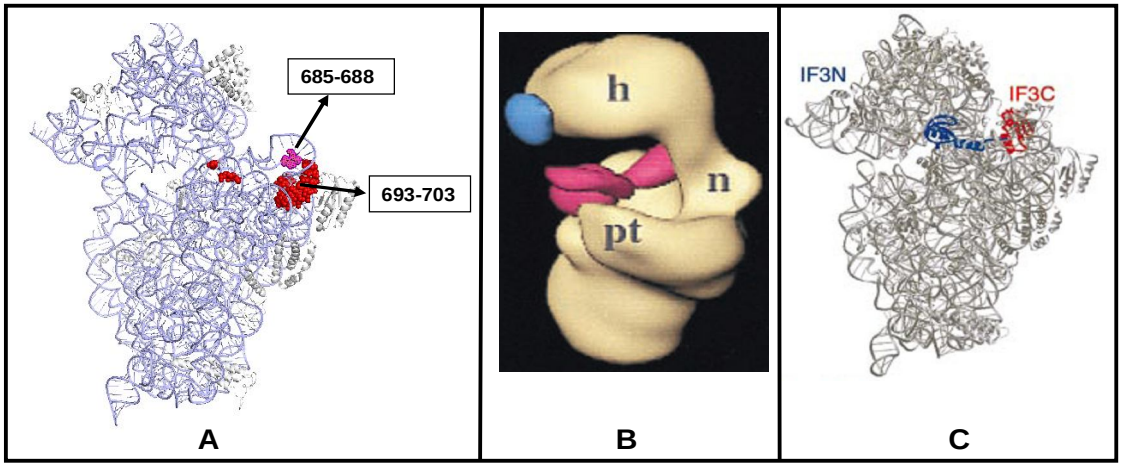


Figure 1.2.4.5-2: Binding of IF3 to the small subunit*, A: N-terminal of IF3 binds to 30S part of E site, highlighted residues were the ones that were mapped on 16S rRNA structure by IF3 obtained via protection study {Dallas, 2001} B: 27-Å cryo-EM reconstruction of the 30S subunit bound to IF3, view from the platform side, with the 50S subunit interface on the left. The positive (magenta) and negative (blue) difference densities on IF3 binding are overlaid on the three dimensional cryo-EM map of the native 30S subunit (yellow). The labels on the 30S subunit indicate the head (h), neck (n), and platform (pt) {McCutcheon, 1999}, B: the model of the complete 30S-IF3C complex (IF3C in red) and the docking of IF3N (blue), {Pioletti, 2001}.*

As a result of these studies there is a general agreement that IF3 binds in the vicinity of ribosomal proteins S1, S7, S11, S13, S18, S19, S21 and of nucleotides 674-713 (helix 23), 783-799 (helix 24), 819-859 (helices 25 and 26), and 1506-1529 (helix 45). Nevertheless, the precise location of IF3 on the ribosome remains hard to pin down since the cryo-EM studies place IF3 on the interface side of the 30S subunit {McCutcheon, 1999}, while the X-ray crystallography data place it on the opposite side of the platform, in contact with proteins S18, S2, S7 and S11 as well as with rRNA helices 23, 26 and 45 {Pioletti, 2001} as shown **Figure 1.2.4.5-2 B** and **C**.

Translation initiation factor IF3 is essential for cell viability {Olsson, 1996} and performs several functions. In particular, IF3 promotes the dissociation of the 70S ribosome by antagonizing association of the 30S subunit to the 50S subunit {Grunberg-Manago, 1975}. In fact, for this activity where the factor supplies the pool of free 30S subunits required for translation initiation, the amount of IF3 to 70S has to be very high from 20 to 200 fold excess {Petrelli, 2001}.

Initiation at the correct start codon is critical for establishing the translational reading frame. A number of mutations have been isolated in *Escherichia coli* that increase spurious initiation (*i.e.* translation from non-canonical start codons such as ACG, AUU, AUC and CUG). These mutations were mapped to *infC*, the gene encoding IF3, strongly implicating the factor's role in start codon selection {Sacerdot, 1996} making it more dependent on cognate codon–anticodon interaction {Antoun, 2006}. IF3 also inhibits formation of a stable 70SIC when the mRNA contains a non-canonical start codon such as AUU {Grigoriadou, 2007; Milon, 2008}.

Finally, a role for IF3 in recycling of ribosomal subunits has been investigated {Singh, 2005}. IF3 was observed to enhance the dissociation of deacylated tRNAs from post-termination complexes and to dissociate 70S ribosomes into subunits after post-termination complex release by ribosome recycling factor RRF {Singh, 2005; Karimi, 1999}.

Having reported the published data for the translation initiation factors and their suggested functions, hereby we describe a new concept of translation initiation which defines more convincing role for some factors and adds higher authenticity to their existence in bacterial cell.

1.2.5 Test of a new concept for bacterial translation-initiation

As described above, the classically acclaimed steps of protein synthesis consists of four phases, initiation, elongation, termination and ribosome recycling. In this context a bacterial 70S ribosome that has encountered the stop signal of the translated cistron, has to dissociate into its 30S and 50S subunits (recycling phase). The 30S subunit is then ready to enter the initiation phase, where it recognizes initiation signals on mRNA such as the AUG codon and upstream of it, the Shine-Dalgarno (SD) sequence with the help of three initiation factors IF1, IF2 and IF3, and with the initiator fMet-tRNA$_f^{Met}$ to form 30S initiation complex. The next step is to get associated with the large 50S subunit yielding the 70S initiation complex, from which the ribosome enters the elongation phase for synthesizing the protein. This initiation view, named as "30S *de novo* initiation type", is well documented in literature but in our opinion describes some, not all initiation events correctly. However, the present unsatisfying situation exists in not finding appropriate answer for three questions raised in issues enlisted here, in the context of 30S initiation type model:

First issue:

(a) In spite of the hundreds of papers dealing with the function of IF1 not one experiment essentially describes why this factor, with ~70 amino acids the smallest universal translation factor, is essential for the viability of bacterial cells.

Only one study indicates indirectly a specific function of IF1 for forming the 70S initiation complex. IF2 and IF3 are reported to provide selective recognition of the initiator tRNA on 30S ribosomes, whereas on 70S it critically requires IF1, *i.e.* discrimination on 70S is supported by IF1. In fact all the three factors contribute to the selection of the initiator tRNA during formation of the 70S initiation complex. This result provides evidence that IF1 functions are mainly attributed to 70S and to lesser extent to 30S {Hartz, 1989}.

In fact an old study shows that 30S initiation complex can be formed even without IF1. IF1 functions act as a catalyst to stimulate the binding of initiator tRNA to much greater extent in case of 70S initiation complex and this may again support the essential role of IF1 on 70S {Benne, 1973}.

Second issue:

In bacteria most mRNAs exist as polycistrons and contain several translation initiation sites, one for each cistron. In *E. coli* polycistronic mRNAs contain 3.3 cistrons on average (details provided in Result section 3.1).

Theoretically, the initiation codons of, for example, cistron n and cistron n+1 of a bacterial polycistronic mRNA, can be recognized independently of one another, aided by the SD sequences, where 30S initiation complexes can form.

It has been observed that in order to be accessible for the formation of 30S initiation complex, an initiation region of mRNA (including the start codon and the SD motif) must be in a single-stranded, non-hydrogen bonded state, not buried in secondary structure. However in polycistronic mRNA encoding for many ribosomal proteins the existing situation is that only the first one is accessible for 30S *de novo* initiation, the second and the following ones are sequestered in secondary structure. A translating ribosome may unfold the secondary structure of the mRNA to expose the second initiation site for the formation of next 30S pre initiation complex {Nomura, 1984}.

But this is impossible to happen because both a 70S ribosome and a 30S subunit cover about 40 nucleotide of the mRNA, roughly 20 nucleotide upstream and 20 downstream of the P-site codon. If the start codon is located in the region of 40 nucleotides covered by empty terminated 70S, 30S would be prevented to bind

because of steric clash and initiate the translation of adjacent cistron (see **Figure 1.2.5-1**).

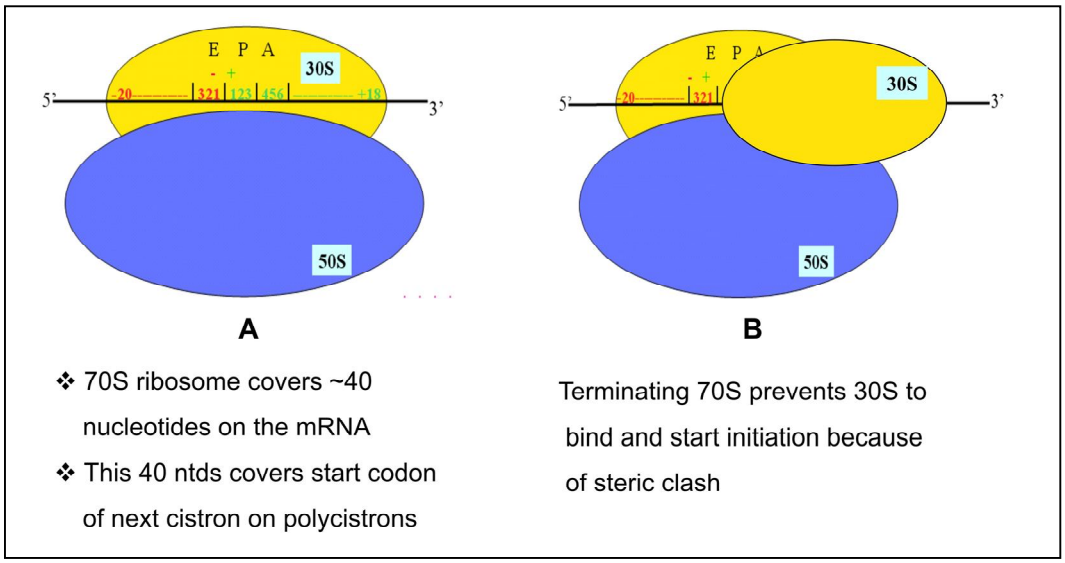


Figure 1.2.5-1: A; Cartoon showing region covered by 70S, B; Simultaneous binding of 30S is not possible when 70S is still present due to steric clash

We therefore assume that the terminating ribosome does not necessary falls off the mRNA immediately after translating a cistron “n”, but rather has a limited capacity to scan for a time window upstream and downstream the mRNA searching for an adjacent initiation site of cistron “n+1”. In this perspective the length of the intercistronic region plays a very important role in determining the type of initiation.

To this end, when analyzed it was found that 75% of 1700 intercistronic regions fall in the range of -15 to +30 nucleotides (details provided in Results section). As we observe in the above figure, that this short intercistronic region is covered by empty 70S, it is very unlikely to imagine the initiation in these sites by 30S.

So the question arises, how does the translation initiation take place in these cistrons? In all cases of r-protein mRNAs, where equimolar synthesis is observed (exception is the L7/L12 mRNA), the intercistronic distance between the two coupled cistrons is relatively small. For *e.g.* in the case of L11-L1 the two cistrons are separated only by three nucleotides. Thus, physical proximity of the translation termination sites and the subsequent initiation sites might guarantee re-initiation by the same ribosome {Nomura, 1984}. In the same way this would be applied to our hypothesis and strongly support that these sites located in intercistronic region of -15 to +30 are re-initiated by empty 70S by scanning upstream or downstream on the polycistronic mRNA.

Third issue:

This issue is related to the second one but is not identical. Translation coupling is a phenomenon that renders the translation of the various cistrons in a polycistronic mRNA interdependent on each other. In many cases, 'downstream' cistrons may not be expressed efficiently if the 'upstream' ones have not been previously translated, because in this situation the initiation region of a downstream cistron is sequestered within a double-helical structure masking its initiation codon and resolved only during translation the preceding one {Nomura, 1984}.

For polycistrons that code for ribosomal protein, each of these proteins are translationally coupled and made precisely in 1:1 stochiometric ratio. If all of these cistrons were to be initiated independently by 30S then their amounts made will be very different from each other and cannot be in strict and precise 1:1 stochiometric ratio. So the question arises how is the precise stochiometry in there is achieved? We assume that the regulation could only be achieved if the first cistron is initiated by 30S and then the empty terminating ribosome re-initiates the downstream cistron as shown in **Figure 1.2.5-2**.

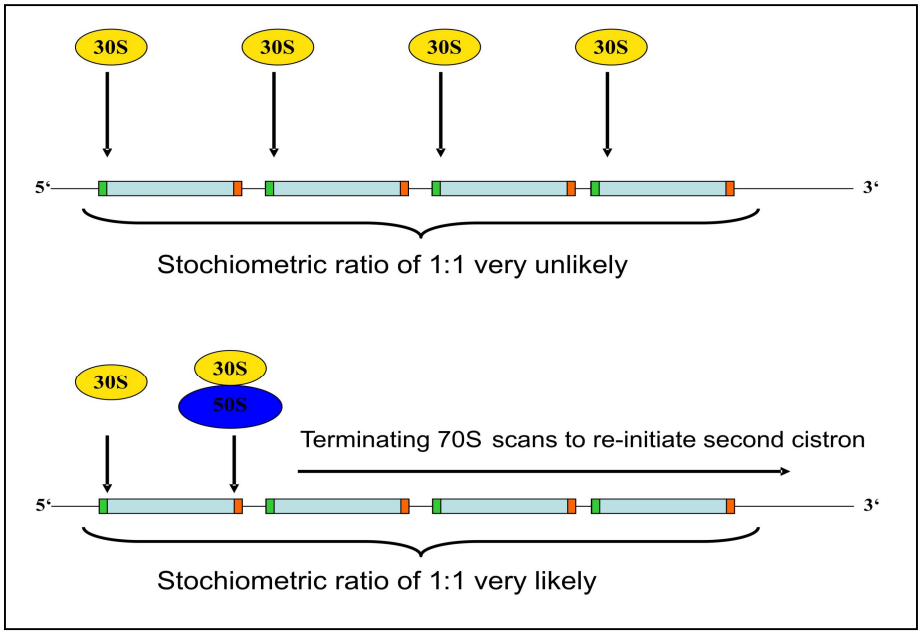


Figure 1.2.5-2: Cartoon showing the translation initiation possibilities at various cistrons in polycistronic mRNA which would then determine their stochiometric amounts.

What might happen is that a 70S ribosome after termination on a stop codon of a preceding cistron and peptide release does not dissociate from such a polycistronic mRNA but proceeds to re-initiate the downstream cistrons by scanning. This aids them to be translationally coupled and enhances their probability to be synthesized in precise 1:1 stochiometric amounts which is prerequisite for ribosomal subunit

assembly. In order to provide an answer for the above enlisted important questions in bacterial translation world, here we summarize our hypothesis, which could efficiently provide a satisfactory solution.

Our hypothesis:

It is general wisdom that 30S type of initiation is the major initiation mode existing in bacteria. Following textbook nomenclature, 30S type of initiation from here on we refer it as 30S binding type of initiation. The reason is that 30S recognizes SD-AUG start region in 5' UTR of mRNA, binds at it directly and starts initiation. It does not scan on mRNA to locate the start sites as the eukaryotic 40S subunit does.

We introduce a second initiation mode, namely 70S-scanning type of initiation, which is taking over the function of translation initiation of the downstream but the first cistron existing in many if not most of the polycistrons.

We suggest that in second mode of initiation, the terminating 70S locates the correct start codon of the next cistron downstream on the mRNA. While scanning and reaching the next initiation site it encounters a problem namely interference by aminoacyl-tRNA•EF-Tu•GTP ternary complex which, might enter the A site depending on the A-site codon in any frame, leading to premature false "initiation" ultimately resulting to abort translation .

We think that such a false "initiation" is prevented by IF1 that binds to the decoding center at the A site. Since IF1 occupies precisely the site of the correct aminoacyl-tRNA in decoding center at the A site {Carter, 2001}, we hypothesize that during scanning of the empty 70S ribosome over the intercistronic region of the mRNA, IF1 binds to the A site of 70S ribosomes and blocks a premature binding of an aminoacyl-tRNA•EF-Tu•GTP ternary complex in any reading frame, favoring correct translation initiation of the downstream cistron on the polycistronic mRNA.

The objective of this work is to employ *in silico* and *in vivo* experiments, which could test our new hypothesis of the 70S-scanning type of initiation.

2 Materials and Methods

2.1 Materials

2.1.1 Sets of biological components (Catalogue numbers in parenthesis)

Qiagen:

Ni-NTA Spin Kit (50), (31314)

Plasmid Maxi Kit (121163)

Plasmid Midi Kit (100), (12145)

QIAprep® Spin Miniprep Kit (250), (27106)

QIAquick® Gel Extraction Kit (50), (28706)

Roche:

Rapid Translation System 100, RTS 100 *E. coli* HY Kit (3 186 156)

Rapid Translation System 500, RTS 500 *E. coli* HY Kit (3 246 949)

2.1.2 Chemicals and simple biological components

Amersham:

[γ-^{32}P]-Adenosine-5'-triphosphate (PB 10218)

Bovine Serum Albumin (BSA, RNase/ DNase Free), (27-8914-02)

Hybond *N*-plus membrane (RPN 203N)

Beckman:

Ultracentrifuge tubes (Ultra-Clear) SW40 and SW28, (344060, 344058)

Biorad:

2x Laemmli Sample Buffer (161-0737)

2x Native Sample Buffer (161-0738)

Premixed 10x SDS-Tris-Glycine Buffer (161-0732)

Premixed 10x Tris-Glycine Buffer (161-0734)

Sodium-dodecyl-sulfate (SDS), (161-0301)

Calbiochem:

HEPES, Free Acid, ULTROL® Grade (391338)

Difco:

Bacto™ Agar (214010)

Bacto™ Peptone (211677)

Bacto™ Yeast Extract (212750)

Invitrogen:

Agarose Electrophoresis Grade (15510-019)

Sucrose (15503-022)

TEMED (15524-010)

Fermentas:

2x Loading Dye Solution for RNA electrophoresis (#R 0641)

6x Loading Dye Solution (#R 0611)

GeneRuler™ 100bp DNA Ladder RNA ladder (#SM 0241)

GeneRuler™ 1kb DNA Ladder (#SM 0311)

RNA Ladder, Low Range (#SM 0411)

T4 DNA Ligase, (#EL 0015)

Fluka:

Spermidine trihydrochloride (85578)

Spermine tetrahydrochloride (85605)

Merck:

2-mercaptoethanol (8.05740.0250)

2-Propanol (1.09634.2500)

Acetic acid glacial 100% (1.00063.2500)

Ammonium acetate (1.01116.1000)

Ammonium chloride (1.01145.1000)

Ammonium peroxodisulfate (APS), (1.01201.1000)

Boric acid (1.00165.1000)

Ethanol (1.00986.2500)

Ethidium bromide (1%), (1.11608.0030)

Formamide (1.09684.1000)

Glycerol 100% (1.05819.1000)

Glycine (1.04201.1000)
Hydrochloric acid 32% (1.00319.2500)
Magnesium acetate (1.04936.1000)
Magnesium chloride hexahydrate (1.05833.1000)
Methanol (1.06002.2500)
Potassium acetate (1.04820.1000)
Potassium chloride (1.04936.1000)
Potassium hydroxide solution 1 mol/L (1.09108.1000)
Sodium carbonate monohydrate (1.06386.1000)
Sodium chloride (1.06404.1000)
Sodium citrate (1.06448)
Sodium hydroxide (1.06498.1000)
Sodium hydroxide solution 1 mol/L (1.09137.1000)
Triplex III GR (EDTA), (1.08418.1000)
Tris(hydroxymethyl) aminomethane (1.08382.1000)
tri-Sodium citrate dihydrate (1.06448.1000)
Urea (1.08487.1000)

New England BioLabs:
Restriction endonucleases with buffers
T4 DNA Ligase (M0202S)

Promega:
Steady-Glo® Luciferase Assay System

Qiagen:
Ni-NTA Agarose (100 ml), (30 230)

Roche Pharmaceuticals:
1,4-Dithiothreitol (DTT) (1 583 786)
DNase I, RNase-free (10 776 785 001)
dNTPs: dATP, dCTP, dGTP, dUTP (1 051 440, 1 051 458, 1 051 466, 1 420 470)

Roth:

IPTG 25 g (2316.4)

Phenol (0040.2)

Roti-Mark STANDARD 1 ml (T851.1)

Trichloroacetic acid (TCA), (8789.1)

Serva:

Aluminiumoxid Alcoa A-305 (12293)

Coomassie® Brilliant Blue G-250 (17524)

Coomassie® Brilliant Blue R-250 (17525)

Sigma:

3-(N-morpholino)propanesulfonic acid (MOPS), (M-1254)

Albumin, bovine (A-7906)

Dextran sulfate (D-6001)

Ficol 400 (F-4375)

Formaldehyde (F-8775)

Lysozyme (L-6876)

Polyvinylpolypyrolidone (P-6755)

ReadyMix™ Taq PCR Reaction Mix with $MgCl_2$ (P-4600)

Spectrapor dialysis membrane (MW 3500)

2.1.3 Non-typical laboratory machines

· RTS ProteoMaster Roche

· PhosphorImager STORM 820 (Molecular Dynamics, Amersham Biosciences) and PhosphorImager cassette BAS 2325 (Fujifilm)

· Luminometer Centro LB 960 (Berthold technologies, Germany)

· Beckmann Coulter DU®640B Spectrophotometer

· Sorvall RC 5B plus centrifuge

· Beckmann L7-55 ultra-centrifuge

· New Brunswick Scientific GmbH Innova 4400 incubator shaker

2.1.4 Bacterial strains of *E. coli*

2.1.4.1 For general purpose

XL-1 Blue: recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacIqZDM15 Tn10 (Tetr)],

BL21 (DE3): F^- dcm ompT hsdS(rB^- mB^-) gal I(DE3),

Can20-12E: derived from *E. coli* K12, is deficient in five RNases: RNase I, RNase II, RNase D, and RNase BN {Deutscher, 1984}. The strain is tetracycline resistant and grows in the presence of 10 μg/ ml tetracycline.

2.1.4.2 Bacterial strains for IF1 cloning

PMF1A*:* wild type strain MG1655 but with a 200 bp chromosomal *infA* deletion.

MRE600: *E.coli* B, RNase I^-

DH5α: *E. coli* K, F^- *Φ80dlacZΔ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(rk,mk+) phoA*

2.1.5 Plasmids

Rapid Translation System RTS pIVEX His tag, 2nd Generation Vector Set
(Roche, 3 269 019)
pET23c (+), (Novagen, 69747)
pSS12-C: It has AraB promoter which is induced in presence of arabinose and repressed in the presence of glucose.
pRK04: has *infA* gene under native promoter.
pXR101: used as template DNA to PCR amplify the *infA* gene.
pACY177: cloning vector with p15A origin of replication

2.1.6 Antibiotics

Ampicillin in H_2O (Roth, K029.2)
Chloramphenicol in 100% ethanol (Boehringen-Mannheim GmbH, 634433)
Kanamycin A (KAN) in H_2O (Roth, T832.3)
Tetracycline (TET) in 100% ethanol (Fluka, 87128)
Thiostrepton (THIO) in 100% DMSO (Sigma, T8902-1G)

2.2 Buffers

2.2.1 Buffers and Electrophoresis solutions

2.2.1.1 Buffers used for DNA work:

Agarose gel solution

10 X TAE	5 ml
Agarose	0.8-2% w/v
Ethidium Bromide (1%)	3 µl
MQ-H_2O	Make up to 50 ml

Agarose gel sample buffer (5X) (for DNA)

EDTA	50 mM
Ficoll 400	10% w/v
Bromophenol blue	0.25% w/v
Xylencyanol	0.25% w/v

Ethidium bromide staining solution

Ethidium bromide 1%	30 µl
MQ-H_2O	300 ml

TAE (10X)

Tris	48.4 g
Glacial acetic acid	11.4 ml
EDTA	3.7 g
MQ-H_2O	Make up to 1000 ml

TBE (10X)

Tris	108 g
Boric acid	55 g
EDTA	7.4 g
MQ-H_2O	Make up to 1000 ml

TE buffer

Tris-HCl, pH 8.0	10 mM
EDTA	1 mM

2.2.1.2 Buffers used for RNA

Agarose gel sample buffer (5X) (for RNA)

EDTA	10 mM
Sucrose	60% w/v
Bromophenol blue	0.1% w/v
Xylencyanol	0.1% w/v

RNA denaturing sample buffer (for AA gel electrophoresis with Urea)

Tris-HCl pH 8.0	10 mM
EDTA	1 mM
Urea	7.5 mM

8 % Urea gel

40% Acrylamide	10 ml
Urea	21 g
10X TBE buffer	5 ml
MQ-H_2O	Make up to 50 ml

10X Phosphorylation buffer (for labeling of 5'-hydroxyl termini of RNA with [γ-^{32}P]-ATP)

Tris-HCl	500 mM
$MgCl_2$	100 mM
β-mercaptoethanol	60 mM
EDTA	10 mM

2.2.1.3 Buffers used for Protein

APS solution 10%

Ammonium peroxy disulphate	10% w/v

Coomassie blue staining solution

Coomassie blue R-250	0.25 % w/v

Methanol	50% v/v
Glacial Acetic acid	10% v/v

Destaining solution for Coomassie gels

Methanol	50% v/v
Glacial acetic acid	10% v/v
Bromophenol blue	0.05% w/v
Xylencyanol	0.05% w/v

Cracking buffer

0.5 M Tris-HCl, pH 6.8	25 ml
β-mercaptoethanol	500 µl
SDS	10 ml
Urea	18.15 g
0.2% (w/v) Bromophenol blue	250 µl
MQ-H_2O	Make up to 50 ml

SDS-PAGE protein sample buffer

SDS	2% w/v
Tris-HCl (pH 6.8)	90 mM
Glycerol	10% v/v
ß-mercaptoethanol	29 mM
Bromophenol blue	0.1% w/v

SDS-PAGE separation buffer (pH 8.8)

Tris	90.86 g
SDS	1 g
MQ-H_2O	Make up to 250 ml

SDS-PAGE stacking buffer (pH 6.8)

Tris	6.1 g
SDS	0.4 g
MQ-H_2O	Make up to 100 ml

Tris-Glycine SDS buffer 1X Running Buffer (Protein SDS-PAGE) pH 8.3

Tris	100 mM
Glycine	760 mM
SDS	0.8% w/v

SDS-separating gel for proteins (various acrylamide concentration)

30% Acrylamide	5 ml - for 15% 5.8 ml - for 17.5% 6.6 ml - for 20%
1.5 M Tris-HCl, pH 8.8	2.5 ml
10% ammonium persulfate	50 µl
TEMED	7.5 µl
MQ-H_2O	Make up to 10 ml

Stacking gel for proteins

30% Acrylamide	0.85 ml
1.5 M Tris-HCl, pH 6.8	1.25 ml
10% ammonium persulfate	25 µl
TEMED	5 µl
MQ-H_2O	Make up to 5 ml

2.2.1.4 Buffer for Western blotting

Transfer Buffer (Western blotting)

Tris	25 mM
Glycine	190 mM
Methanol	20% v/v

Phosphate buffer saline- PBS- 5X pH 7.1

Na_2HPO_4(anhydrous)/$Na_2HPO_4 \bullet 2H_2O$	57.5 g / 72.09 g
NaH_2PO_4 anhydrous/ $NaH_2PO_4 \bullet H_2O$	14.8 g / 17.02 g
NaCl	29.2 g
MQ-H_2O	1 L

Phosphate buffer saline- PBS- 1X pH 7.1

5X PBS	50 ml
MQ-H_2O	200 ml

Phosphate buffer saline-1X PBS-T - pH 7.1

5X PBS	50 ml
MQ-H_2O	200 ml
Tween 20	100 µl

Blocking Buffer

1X PBS-T	100 ml
5% milk powder	5 g

Primary antibody preparation (1:100,000)

Blocking buffer	100 ml
Serum with primary antibody	1 µl

Secondary antibody preparation (1:10,000)

Blocking buffer	10 ml
Serum with secondary antibody	1 µl

Stripping buffer

SDS 10%	20 ml
β-mercaptoethanol	0.7 ml
1 M Tris pH 6.8	6.25 ml
MQ-H_2O	100 ml

2.2.1.5 Buffers for microbiological and molecular methods

P1 (resuspension buffer for plasmid preparation)

Tris-HCl pH 8.0	50 mM
EDTA	10 mM

P2 (Cell-lysis buffer for plasmid preparation)

NaOH	200 mM
SDS	1% w/v

P3 (Neutralization buffer for plasmid preparation)

Potassium acetate, pH 5.5	3 M

QBT (Equilibration buffer for Qiagen column)

MOPS-KOH pH 7.0	50 mM
NaCl	1000 mM
Ethanol	15% v/v

QC (washing buffer for Qiagen column)

MOPS-KOH pH 7.0	50 mM
NaCl	1250 mM
Ethanol	15% v/v

QF (Elution buffer for Qiagen column)

Tris-HCl, pH 8.5	50 mM
NaCl	1250 mM
Ethanol	15% v/v

2.2.1.6 Growth Medium

LB medium

Bacto tryptone	1%
Yeast Extract	0.5%
NaCl	1%

LB-Agar plates

Bacto tryptone	1%
Yeast Extract	0.5%
NaCl	1%
Bacto Agar	2%

M9 minimal medium

Na_2HPO_4	6.0 g
KH_2PO_4	3.0 g
NaCl	1.0 g
NH_4Cl	1.0 g
1 M $MgSO_4$	1.0 ml
1 M $CaCl_2$	0.1 ml
MQ-H_2O	Make up to 1000 ml

Phosphate buffer saline- 10X

Na_2HPO_4	80 mM
NaH_2PO_4	20 mM
NaCl	700 mM

2.2.1.7 Buffers for Ribosome work

Binding buffer 10X

Hepes-KOH pH 7.5	200 mM
$Mg(Ac)_2$	45 mM
NH_4Ac	1500 mM
ß-mercaptoethanol	40 mM
Spermidine	20 mM
Spermine	0.5 mM

Binding buffer composition for polysomes work- 10X

Hepes-KOH pH 7.5	200 mM
$Mg(Ac)_2$	45 mM
NH_4Ac	1500 mM
ß-mercaptoethanol	60 mM
Spermidine	20 mM
Lysozyme	0.4 mg/ ml

30% Sucrose- used for making sucrose gradient for ribosome profiling

Sucrose	30 g
1X binding buffer	100 ml

10% Sucrose- used for making sucrose gradient for ribosome profiling

Sucrose	10 g
1X binding buffer	100 ml

2.2.1.8 Buffers for Northern blotting

10X MOPS

MOPS	0.4 M
Sodium Acetate	0.2 M
EDTA	10 mM

20X SSC

Sodium chloride	3 M
Sodium citrate	0.3 M

50X Denhardt's solution

Ficoll 400	1 g
Polyvinylpyrrolidone	1 g
BSA fraction V	1 g
MQ-H_2O	Make up to 50 ml

Pre-hybridization buffer

SSC	5X
Formamide	50%
Denhardt's solution	5X
SDS	1%
Salmon sperm DNA	100 µg/ ml

Hybridization buffer

SSC	5X
Formamide	50%
Denhardt's solution	5X
SDS	1%
Dextran sulphate	5%

2.2.1.9 Buffers for Thin Layer chromatography

Electrophoresis buffer

Pyridine	40 ml
Glacial acetic acid 100%	80 ml
MQ-H_2O	Make it up to 2 L

Adjust the pH with 37% HCl to 2.8 and then make it up to 2 L with MQ-H_2O

Stoddard solvent

Readymade bought from Roth

Developing reagents

Ninhydrin	0.1 mg
Glacial acetic acid 100%	21 ml
Ethanol	70 ml

2.2.2 Analytical methods

Determination of ribosome and nucleic acid concentrations

The concentration of 70S ribosomes, 30S and 50S ribosomal subunits was determined measuring the absorption at 260 nm.

70S: 1 A_{260} unit = 24 pmol

50S: 1 A_{260} unit = 36 pmol

30S: 1 A_{260} unit = 72 pmol

Conversion factors for the quantification of DNA and RNA

1 base pair in double stranded DNA 660 g/ mol

1 A_{260} unit of double stranded DNA 50 µg

1 A_{260} unit single stranded DNA or RNA (more than 100 bases)	40 µg
1 A_{260} unit of single stranded DNA (less than 25 bases)	20 µg
1 A_{260} unit of single stranded DNA (30-80 bases)	30 µg
A_{260}/A_{280} ratio for pure DNA	1.8
A_{260}/A_{280} ratio for pure RNA	2.0
1 triplet (codon) of RNA	~1000g/mol

2.2.3 Software

Sequence analysis and RNA structure prediction:

Gene Runner, RNA fold

Gel documentation:

Image Quant packet version 5.2 (Molecular Dynamics, Amresham Biosciences)

Molecular structure visualization:

Pymol, PDB Viewer

2.3 Methods

2.3.1 Cell cultures

2.3.1.1 Spot test

This test is performed in order to have more stringent selection of a required clone. Here series of LB plates are chosen in an order where the first one provides the growth favorable plates like antibiotics and sugar, followed by unfavorable plates which would inhibit the growth and then again the plates which can facilitate its growth.

The polystyrene pad has numerous cavities where each colony can be picked up, dissolved in minimal medium and put in each of these cavities. These colonies are then dipped at the stamp end and are stamped on the series of these plates with the one time single inoculation pick up. These colonies pass through different condition and only the colony that grows well at the end of the series is selected as correct clone.

2.3.1.2 Growth curve

Overnight culture is made by inoculating a single colony in 5 ml LB with appropriate antibiotics. The main culture is inoculated by the inoculums obtained by overnight culture to final concentration of 0.05 OD_{600}. The cells are grown and the growth is measured after every 30 minutes at wavelength 600 nm. After the cells reach the stationary phase, the reading is plotted on sigma plot using semi logarithmic scale. Generation time is calculated by measuring the doubling time of cells in log phase.

2.3.1.3 Preparation of *E. coli* competent cells for electroporation

A liter culture of the *E. coli* strain of interest was grown to an OD_{600} of 0.5-1.0. The cells were chilled on ice, and then pelleted by centrifuging, at 4,000 rpm for 15 min at 4 °C in a GSA rotor, and resuspended in 1 volume to the culture volume of pre-chilled sterile MQ-H_2O. Bacterial cells were repeatedly centrifuged and washed in decreasing volumes of cold MQ-H_2O: two washes with 0.5 volume, one wash with 0.02 volume and final resuspension in 2-3 ml filter sterilized 10% (v/v) glycerol. Finally, the cell suspension was aliquotized in 40 µl portions, shock frozen in liquid nitrogen and stored at -80 °C.

The competence of the cells was assessed by transformation using any test plasmid; using 10 ng of plasmid in 40 µl of competent cells. The transformation efficiency determined should be in the range of 10^6-10^8 transformants per µg of plasmid DNA. This level of competence was considered to be sufficient for our purpose.

2.3.1.4 Preparation of chemical competent cells

200 ml culture of the *E. coli* strain of interest was grown to an OD_{600} of 0.5-1.0. The cells were chilled on ice, and then pelleted by centrifuging, at 4,000 rpm for 15 min. The cells are washed twice with 50 ml of 100 mM pre-chilled sterile $CaCl_2$. Bacterial cells after washing finally were resuspended in 2 ml filter sterilized 100 mM pre-chilled sterile $CaCl_2$ with 10 % (v/v) glycerol. Finally, the cell suspension was aliquotized in 50 µl portions, shock frozen in liquid nitrogen and stored at -80 °C.

2.3.1.5 Calculating transformation efficiency

Definition: (cfu on control plate) / (pg of super coiled vector) x (10^6 pg / µg) x (final dilution) = cfu / µg DNA (Note: cfu = colony forming units).

For example, if the control reaction plate has 50 colonies when ~5µl of diluted reaction is plated then,

(50 cfu) / (10 pg) X (10^6 pg) / µg) X (1 ml) / (0.05 ml plated) X 10^2 = 1.0 x 10^{10} cfu per µg DNA.

2.3.2 Nucleic acid- DNA and RNA

2.3.2.1 Transformation by Electroporation

The competent cells are thawed on ice. Then transformed with the required plasmid (1 µl approx 10 ng of DNA) by adding the DNA in the tube, mixing it well and keep it on ice for 2-5 minute. Electroporate it by transferring it in electroporation chamber (The voltage adjusted to 1.5 kV with the resistance 200 ohm and capacity C= 25 microfarad) by applying pulsed electrical field for about 4-5 ms which causes pore formation and enables foreign plasmid to enter into the cells. If the display shows the reading between 3-5, the transformation has succeeded. 1 ml fresh LB medium is added into the cuvettes and then incubated at 37 °C for 1 hour in 1.5 ml Eppendorf tube and mixed at intervals. 0.1 to 0.2 ml is plated on LB plates with required antibiotics and incubated over night for colonies to grow.

2.3.2.2 Chemical transformation

DNA is added to thawed competent cells and incubated on ice for 30 minutes. This is followed by giving heat shock at 42 °C for 1 minute and immediately transferring the tubes on ice. Let it be on ice for 5 minutes and then to it LB or SOC (1 ml or 200 µl) is added. It is then kept on shaking at 37 °C for an hour. After this the cells are plated onto LB agar plates with respective antibiotic or additional requirement like sugar etc. The plates are incubated for minimum of 12 hours at 37 °C to see the colonies.

2.3.2.3 Agarose gel electrophoresis of DNA and RNA

This technique was used for analysis of plasmid DNA after analytical and preparative isolation, as well as for restriction analysis and for the qualitative evaluation of mRNAs and rRNAs from 70S ribosomes, 50S and 30S ribosomal subunits. The percentage of agarose used depends on the expected size of the nucleic acid to be separated: 0.8% for rRNA and 3 kb plasmids, and 1.5-2% for restriction fragments of

600 base pairs or less. The buffer systems used was TAE for DNA samples and TBE for RNA samples.

The agarose solution was heated in a microwave oven for not more than one min. After the agarose is completely dissolved, the solution was cooled to room temperature and before it starts to solidy, 3 µl of ethidium bromide to 50 ml of agarose solution was added, mixed properly and poured into the gel chambers.

The length of the nucleic acid molecules was estimated according to the migration of standards of known molecular weights.

2.3.2.4 Plasmid isolation-mini-prep

Small scale preparations of plasmid DNA from 3-5 ml overnight cultures of bacteria was performed using QIAGEN mini-prep kit. Three ml of bacterial culture (grown for 15-20 h) were centrifuged for 10 min at 5,000 rpm. The cells were sedimented and resuspended in 0.3 ml Buffer P1, then lysis was carried out for two min at room temperature by addition of 0.3 ml of Buffer P2. After neutralization by addition of 0.3 ml of Buffer P3, the samples were mixed by inversion in order to precipitate the total proteins and the chromosomal DNA. This mixture was centrifuged for 20 min at 13,000 rpm (room temperature) and the DNA (supernatant) was taken out and precipitated with 0.7 ml of isopropanol (room temperature) and then centrifuged 12,000 rpm for 30 minutes. The pellet is washed with 70% ethanol and dried. Finally the plasmid is obtained by dissolving the pellet in 30-50 µl of TE buffer. The yield of plasmid obtained by this protocol was between 15-100 µg.

2.3.2.5 PCR

PCR is performed with ReadyMix™ Taq PCR Reaction with $MgCl_2$ from Sigma. It contains everything needed for a PCR reaction except the specific primers and template. The mix includes Sigma's high quality Taq DNA Polymerase, $MgCl_2$, 99% pure deoxynucleotides and buffer in a 2X optimized reaction concentrate. Primers are added to the final concentration of 1 mM and the reaction requires minimum of 10 ng of template DNA. Standard condition includes initial denaturation at 94 ºC for 5 minutes, followed by denaturation at each cycle, *i.e*. 94 ºC for 30 second. This is then followed by annealing of the primers and temperature is chosen in such a way that it should be 5-8 ºC less than Tm of both the primers. Extension is performed at 72 ºC and the time required is 1 minute for the amplification of 1 kb fragment size. This

cycle is repeated for 30 times which, is finally followed by the extension at 72 ºC for 10 minutes. After the reaction is over, the temperature is reduced to 4 ºC.

Every reaction follows the same condition except the annealing temperature which depend on the primers and also extension time which depends on the fragment length to be amplified.

2.3.2.6 Cloning

Steps of cloning

A. Preparation of insert:

1. Insert can be made by PCR amplification of insert which is then cleaned using PCR clean up kit
2. Insert can also be prepared by double digesting the required fragment from the existing construct.

B. Vector DNA preparation *via* Quiagen midi preperation protocol

C. Double digestion of vector and insert

D. Gel purification of double digested product

E. Estimation of amount of vector and insert *via* absorption at A_{260}

F. Ligation

G. Transformation

H. Colonies are checked *via* gene specific primer

I. Confirmation *via* sequencing

2.3.2.6.1 Cloning strategy for preparation of pBER-GFPcyc3

pET23c-BER-GFP was achieved in few cloning steps. Original T7 promoter on the pET23c was removed by using *Bgl*II and *Xba*I restriction sites. In that place using the same enzymes a new fragment containing T7 promoter, Berlin sequence and fragment of chloramphenicol acetyl-transferase (CAT) has been introduced: AGATCTTAATACGACTCACTATAGGCCTTATTTATTTATTTATTTATTATTTACTAT GAAAATTATTTCTAGA (*Bgl*II and *Xba*I are restriction sites present at 5' and 3' end respectively). The method of introduction of the insert is based on the hybridization (annealing) between two complementary ssDNA-oligomers: (1) 5' - GATCTTAATACGACTCACTATAGGCCTTATTTATTTATTTATTTATTATTTACTATG AAAATTATTT – 3' and (2) 5' – CTAGAAATAATTTTCATAGTAAATAATAAATAAATAAATAAATAAGGCCTATAGTGA

GTCGTATTAA – 3' which contain sticky ends for the ligation. Both ssDNA-oligomers were mixed together to the final concentration of 5 pmol/ µl each in the total value of 100 µl. The mixture was incubated for 5 min at 95 ºC and then slowly cooled down to room temperature. This mixture has annealed double stranded insert which, is diluted in water to the final concentration of 1 pmol/µl. It is now ready for cloning and ligated with the open pET-23(c) plasmid previously digested by *Bgl*II and *Xba*I. The plasmid was opened in two steps digestion. (1) digestion by *Bgl*II as follow: 10 µg DNA, 5 µl *Bgl*II, 1x NEB buffer 3, 37 ºC for 5 hours incubation.

Purification of DNA by phenol extraction and ethanol precipitation in the conditions as follow: 1:1 phenol is added, vigorously shaken and upper-water phase collected and moved to the fresh tube. To it again 1:1 chloroform is added, vigorously shaked, upper-water phase moved to the fresh tube. To this 1:10 3 M ammonium acetate is added followed by the addition of 2.5 times volume of absolute ethanol. Mixture was incubated 3 h at -20 ºC to precipitate DNA, finally centrifuged at +4 ºC for 30 min. The pellet was washed by 100 µl of 70% ethanol, centrifuged again for 10 min at +4ºC, dried and dissolved in water.

Plasmid was re-digested by the second enzyme *Xba*I in the same way as described in the upper section. Finally, this double digested plasmid was purified from agarose gel using QIAquick Gel Extraction Kit from Qiagen. Purified DNA was eluted from the Qiagen column by addition of 20 µl of water and the concentration was measured by absorbance at 260 nm. Plasmid and new inserts were mixed in the molar ratio of 1:15.

The ligation was performed overnight at 16 ºC with 400 U of the T4-ligase in 1x ligation buffer in the total value of 10 µl as proposed by Fermentas. Afterwards the reaction components were dialysed on the Millipore filter (0.05 µm) by the simple diffusion on the petri dish filled with water at room temp 15-20 min in order to eliminate salt (ions). This ligation mix was transformed into XL-1 Blue cells by electroporation method (0.1 cm cuvette, 1.5 kV pulse). Correct clones were verified by PCR colony test.

Second part of the cloning was an introduction of DNA fragment between *Xba*I and *Xho*I restriction sites. This fragment has CAT leader peptide and GFP. Two primers were used for this: (1) 5'-

GCTCTAGAAAGGAGTAC*TAATG*ACTAGCAAAGGAGAAG-3' (as italic emphasize the transition from CAT leader and GFP) which contains CAT leader peptide

sequence and some complementary sequence to 5' end of GFP with *Xba*I restriction site (2) 5'-CCTT*GAAGAC*TCTCGAGCCGGATCCCGGGTTTGTA-3' (shown as italic is *Bbs*I restriction site) has sequence complementary to 3' end of GFP with *Bbs*I restriction enzyme. In this situation, *Bbs*I enzymes produces the same sticky ends as *Xho*I and hence makes the two compatible [*Xho*I enzyme cannot be used due to *Xho*I recognition site is located on GFP]. PCR products were digested by *Xba*I and *Bbs*I along with pET-BER plasmid and cloning was performed in the same way as described above.

2.3.2.6.2 Cloning strategy for preparation of pET-GFPcyc3

pET23c-GFP was achieved by insertion of GFP in pET-23(c) using *Nhe*I and *Not*I using two primers: (1) 5'-CTA*GCTAGC*ATGACTAGCAAAGGAGAAGAACTT-3' (as italic shows *Nhe*I restriction site) and (2) 5'-ATAGTTTA*GCGGCCGC*CCGCCGGATCCCGGGTTTG-3' (italics shows *Not*I restriction sites). The conditions for cloning and plasmid preparation are same as described in previous section.

2.3.2.6.3 Cloning strategy for preparation of pBER-int-GFPcyc3 (Berlin intermission GFP)

pET23c-BER intermission-GFP was achieved in few cloning steps. PCR amplification is done using long oligo forward primer which encompasses the *XbaI* site in 5' end, intermission sequence of 39 nucleotides after the stop codon of chloramphenicol acetyl transferase (CAT) leader peptide and the start codon of GFP. Reverse oligo has complementary sequence to 3' end of GFP with *BbsI* restriction site. The PCR fragment is then digested with both the enzymes (*XbaI* and *BbsI*) one by one along with vector pET-23(c). In the insert *Bbs*I enzymes cuts in the position identical to *Xho*I [*Xho*I enzyme cannot be used due to *Xho*I recognition sequence is located on GFP]. Finally vector and insert DNA was purified on the agarose gel (1% agarose with 1x TAE buffer) using QIAquick Gel Extraction Kit from Qiagen for removal of internal fragments. Purified DNA was eluted from the Qiagen column by addition of 20 µl of water and the concentration was measured by absorbance at 260 nm. Plasmid and insert are mixed in the molar ratio of 1:15. The ligation was performed overnight at 16 ºC with 400 U of the T4-ligase in 1x ligation buffer in the total value of 10 µl as proposed by Fermentas. Afterwards the reaction components were dialysed on the Millipore filter (0.05 µm) by the simple diffusion on the petri dish filled with water at

room temp 15-20 min in order to eliminate salt (ions). This mixture was transformed in XL-1 Blue cells by electroporation method (0.1 cm cuvettes, 1.5 kV pulse and correct clones are verified by PCR colony test.

2.3.2.6.4 Cloning strategy for preparation of pET-14b MS2 (which has N-terminal His tag)

The insert was prepared by double digesting T vector-MS2 construct with *NdeI* and *XhoI* together in NEB buffer 2 without BSA at 37 °C for 5 hour. The fragment was gel purified and quantitated.

The vector was prepared as follows:

1. By serial single digestion, that means first single cut with *Nde*I overnight in buffer 2 followed by gel purifying the insert and then subject it to second digestion with *XhoI* for 5 hours at 37 °C
2. By putting both the enzymes together in NEB buffer 2 for 5 hours at 37 °C

The vector was gel purified and then was subsequently used for ligation. The vector to insert ratio is taken as 1:10, ligation performed for 1 hour at room temperature, dialysed and then chemically transformed in DH5α competent cells.

Colonies were screened for the presence of MS2 by PCR.

2.3.2.6.5 Cloning strategy for preparation of pET23c MS2 (which has C-terminal His tag)

The insert was prepared by PCR amplification using same 5' forward oligo which has *NdeI* site and 3' reverse oligo which has region complementary to C terminal end of MS2 along with *Xho*I site. PCR product was cleaned *via* PCR cleaning kit. Then double digestion with *NdeI* and *XhoI* was performed at 37 °C for 6 hours followed by gel purification.

The vector is first digested with *NdeI* overnight and gel purified. It is then subjected to second digestion with *XhoI* after which, it is purified and used for ligation. The vector to insert ratio taken is 1:10, ligation performed for 1 hour at room temperature, dialysed and then chemically transformed in DH5α competent cells. Colonies were screened for the presence of MS2 by PCR.

2.3.2.6.6 Cloning strategy for preparation of pFLAG-GFP with secondary structure at 5'UTR, pFLAG-GFP without secondary structure at 5'UTR, pFLAG-BER-GFP

The use of pFLAG vector is that it provides the gene to be under the control of tac promoter and these constructs are then used to make pACY177-GFP with secondary structure at 5'UTR, pACY177-GFP without secondary structure at 5'UTR, and pACY177-Ber-GFP. PCR amplification of +/- GFP (with and without secondary structure at 5'UTR) was done using primers having structured region and unstructured region in 5'UTR with cyc3GFP as template DNA. Forward primer has *AseI* restriction site (*NdeI* could not be used as it was present inside coding region, and also *AseI* is compatible with *NdeI* site in vector) and reverse primer has *BbsI* (with give ends same as *XhoI* which, is compatible with *SalI* site). This PCR amplified insert is then ligated to Topo vector from which, highly efficient double digested insert can be made in subsequent step. Ber-GFP insert was prepared by PCR using oligo which has 9 flaking nucleotides upstream of restriction site and facilitate proper digestion of insert. This PCR amplified product was double digested by both the enzyme together in NEB buffer 2 for 5 hours at 37 °C. All inserts are ligated to pFLAG vector which, is prior digested with *NdeI* in 5'end and *SalI* at 3'end downstream of tac promoter. The procedure is same as described before. pFLAG positive clones from all the three sets were over-expressed in order to verify that expression of GFP from these constructs are happening. After confirming the expression of the GFP from tac promoter next step was to transfer the region with tac promoter and GFP into pACY177 vector.

2.3.2.6.7 Cloning strategy for preparation of pACY177-GFP with secondary structure at 5'UTR, pACY177-GFP without secondary structure at 5'UTR, pACY177-BER-GFP

This vector is a low copy number plasmid, has ampicillin resistance and p15A origin of replication which makes it compatible to Col-E1 origin of replication of pSS12C-IF1 plasmid already present in *Ec* ($IF1^-$)/ pAraIF1 strain. This would aid two plasmids to co-exist in the same cells. The two can be expressed together using different conditions specific for their expression, namely arabinose inducing IF1 expression from pSS12C plasmid and IPTG inducing the expression of GFP.

First step was PCR, performed in order to introduce *AatII* site at the 5'end and *Bsp681* at 3'end *via* oligo's and these sites would help it to get ligated into pACY177. The PCR fragments from all GFP constructs is ligated to Topo vector from where it is double digested with two enzymes mentioned before and ligated to prior double

digested and purified pACY177 vector. After ligation and transformation these colonies are screened for the presence of positive clones.

Positive clones are transformed in *Ec* (IF1$^-$)/ pAraIF1 strain by chemical transformation for the final experiment. These transformed cells were plated onto LB arabinose + ampicillin + kanamycin + chloramphenicol plates and then the colonies were checked for the presence of IF1 and GFP by PCR. The colonies giving the positive PCR result are used for final experiment.

2.3.2.7 Cloning of *infA* gene in pSSC12 vector and generation of *Ec* (IF1$^-$)/ pAraIF1 strain

2.3.2.7.1 Cloning of IF1, *infA* gene in pSSC12-C vector

The vector used for cloning of IF1 gene is called as pSSC12-C. It is 6100 base pair long and harbors kanamycin and chloramphenicol antibiotics resistance gene. It also has AraB promoter which makes it conditionally inducible *i.e.* a gene when under AraB promoter is expressed in presence of arabinose and repressed in presence of glucose. PCR to amplify the *infA* gene was performed using oligos which harbor *KpnI* restriction site in forward primer and *XhoI* site in reverse primer and using pXR101 as template DNA. pSSC12-C vector DNA is doubly digested with *KpnI* and *XhoI* using buffer 1 and enzymes from New England Biolabs for 2 hours at 37 °C .The completely digested fragment is gel purified. Also the insert which is PCR amplified is double digested in the same way and gel purified.

Ligation was performed using T4 DNA ligase and its buffer from Fermentas. 10 ng of double digested vector was used and insert was taken in 1:2 molar excess over vector. The reaction was carried out at 22 ºC for three hours. The sample was then heated at 65 ºC for 10 minutes to inactivate the enzyme and desalted *via* dialysis using Millipore 0.05 µm nitrocellulose filters. The ligated product is then transformed in DH5α *via* electroporation and was plated onto LB arabinose kanamycin chloramphenicol plates. Next day colonies were picked up and their plasmid preparation was made and was sent for sequencing. Four of them showed the correct sequence and was then named as pKPW01.4, pKPW01.9, pKPW01.13, pKPW01.23, pKPW01.4.

2.3.2.7.2 Generation of strain which has chromosomal *infA* gene knocked out and IF1 is conditionally expressed from pSSC12 vector in the presence of arabinose

PMF1A/pRK04 (*Ec* (IF1$^-$)/ pIF1 strain) was obtained from the laboratory of Prof. Leif *A.* Isaksson, Department of Genetics, Microbiology and Toxicology, Stockholm University. The selected IF1 clones in pSSC12-C vector were transformed in strain *Ec* (IF1$^-$)/ pIF1 by chemical transformation and were selected onto LB arabinose + kanamycin + chloramphenicol plates. The new final strain having both the plasmids was named as *Ec* (IF1$^-$)/ pIF1/ pAraIF1. This strain was streaked on LB arabinose + kanamycin + chloramphenicol plate and a single colony was inoculated LB arabinose + kanamycin + chloramphenicol medium. It is then grown overnight at 37 °C. Next morning cell culture was diluted 1:40 into Minimal medium (MM) arabinose + kanamycin + chloramphenicol and grown for 6 days at 37 °C. After the growth 100 µl of 1:100 and 1:1000 dilution was plated on LB arabinose + kanamycin + chloramphenicol plate and LB glucose ampicillin plates. Growth was visible on both the plates.

Thereafter further 50 colonies from LB arabinose + kanamycin + chloramphenicol plate were selected and replicated twice on MM arabinose + kanamycin + chloramphenicol plates. After two replication, clones 32 and 36 were selected and inoculated in LB arabinose + kanamycin + chloramphenicol medium and grown overnight at 37 °C .The cell culture next day was diluted to 1:40 in MM arabinose + kanamycin + chloramphenicol medium and were grown for 12 days at 37 °C . During the growth, samples from the cell culture (1:10,000 dilution) were plated on to MM arabinose + kanamycin + chloramphenicol plate and MM glucose ampicillin plate for three times. At the third time no growth was visible on MM glucose ampicillin plate.

Ten clones from MM arabinose + kanamycin + chloramphenicol plate after this step were selected and inoculated in LB arabinose + kanamycin + chloramphenicol and grown overnight at 37 °C .

Using replicator, a spot test of all the clones was preformed. Here after picking inoculum once, it is serially plated onto first LB plate, second LB arabinose + kanamycin + chloramphenicol plate, third LB glucose ampicillin plate and then finally to LB plate again. It was observed that there is no growth observed on LB glucose ampicillin plate. This lead to the obtaining a strain which has lost pIF1 plasmid and has only got pAraIF1 and therefore it was named as *Ec* (IF1$^-$)/ pAraIF1.

2.3.3 *In vivo* experiments

2.3.3.1 Growth comparison of the *Ec* (IF1$^-$)/ pAraIF1 and *Ec* (IF1$^-$)/ pIF1 strains

Large scale:

To start the growth curve first of all the glycerol stock of *E. coli* (IF1$^-$)/ pAraIF1 and *E. coli* (IF1$^-$)/ pIF1 strain were freshly streaked onto LB arabinose + kanamycin + chloramphenicol and LB glucose ampicillin plates respectively. Overnight culture was prepared by picking single colonies and growth was allowed in LB arabinose + kanamycin + chloramphenicol broth or LB glucose ampicillin broth. Next day the cultures were diluted to the final concentration of 0.05 OD_{600} in either 200 ml LB arabinose or LB glucose. The growth was performed for both, *E. coli* (IF1$^-$)/ pAraIF1 and *E. coli* (IF1$^-$)/ pIF1 strain with arabinose at 37 °C and glucose at 30 °C. In each condition cells were grown and reading was measured and plotted on semi-logarithmic sheets

Small scale:

Two different mother glycerol stocks of *E. coli* (IF1$^-$)/ pAraIF1 and of *E. coli* (IF1$^-$) / pIF1 were streaked onto LB arabinose + kanamycin + chloramphenicol and LB arabinose ampicillin plate respectively. A single colony is picked up and inoculated to have the overnight culture in 2 ml. From the overnight culture, 5 µl is transferred to 1 ml fresh LB medium with glucose or arabinose with or without respective antibiotics for each of the strains. This diluted culture is then transferred into 96 well Elisa plate in 200 µl volume, growth is performed in Growth reader machine for 12 hours at 37 °C and the readings are automatically plotted after every minute.

2.3.4 Experiments with Green fluorescent protein constructs

2.3.4.1 *In vivo* expression of GFP

Overnight cultures were prepared from the single colonies of BL21 (DE3) cells containing pET-GFPcyc3, pBER-GFPcyc3, and pBER-int-GFPcyc3 LB (Luria-Bertani) medium with 100 µg/ ml ampicillin (Amp) is the requirement for the growth of these transformed bacterial cells. From the overnight cultures dilution of 1:50 is done for the main culture. Cells were incubated up to A_{595} equal to 0.3-0.4 (about two hours). Then IPTG was added to the final concentration of 1 mM and cells were incubated for

additional four hours. 100 µl of cells were transferred into 1.5 ml Eppendorf tube and centrifuged for 3 min at 4800 rpm at 4 ºC. Dissolve the cell pellet in the protein sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 5M urea, 25% glycerol, 0.01% Bromophenol blue, 5% 2-mercaptoetanol) and incubate 15-20 min at 50 ºC with vigorous shaking. Centrifuge 10 min at 14,000 rpm at room temperature.

Apply 5 µl of the supernatant fraction (prevent aspiration of DNA from the lower part) on 15% protein gel (PAGE). The electrophoresis conditions are as followed: 90 V for 10 min and then 150 V for 2 hours in the cold room.

2.3.4.2 *In vitro* expression of GFP

In vitro experiments were done as a coupled transcription-translations assays using the *E. coli* lysate-based system (RTS 100 *E. coli* HY Kit from Roche) for the expression of GFP from pET-GFPcyc3, pBER-GFPcyc3.

Reactions were executed as per manufacturer's instruction. In brief, the lyophilized components supplied in RTS 100 kit were reconstituted and mixed as shown below.

1. Reaction mix

Master mix of RTS for 10 reaction:

24 µl	*E. coli* lysate
20 µl	Reaction Mix
24 µl	Amino acids
2 µl	Methionine
10 µl	Reconstitution buffer

Running reaction mix:

8 µl	Master mix
1 µl	plasmid (0.2 µg/µl) pBER-GFPcyc3, pET-GFPcyc3
1 µl	different concentrations of IF1 or BSA

Standard Conditions: 30°C, 900 rpm shaking, 10 hours.

Reactions were incubated for 10 hours at 30 ºC with shaking (900 rpm) in the RTS Proteo Master Instrument (Roche). Afterwards, 1.5 µl from the reaction mix was mixed with 3.5 µl of water and 5 µl SDS sample buffer (62.5 mM Tris-HCl, pH6.8, 2% SDS, 25% glycerol, 0.01% Bromophenol blue, 5% 2-mercaptoethanol) denatured at 95 °C for 2-3 minutes and loaded onto SDS PAGE. To 1.5 µl of reaction samples 3.5 µl water is added along with 5 µl native sample buffer (62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 0.01% Bromophenol blue) for native PAGE after maturation of GFP for 24h at +4 °C and were applied on native 15% gel and the conditions for electrophoresis were the same as for SDS PAGE but the run is performed in cold. None of the components are to be used twice after thawing. The fluorescence of the

GFP is measured *via* Laser induced Fluorescence Scanner instruments (Amersham Biosciences). Using image quantification program these bands are quantified and plotted using excel.

2.3.4.3 Comparison of different GFP's expression in *Ec* (IF1$^-$)/ pAra IF1 strains under optimized growth reader conditions

pACY177-GFP with secondary structure at 5'UTR, pACY177-GFP without secondary structure at 5'UTR, pACY177-BER-GFP are chemically transformed in *Ec* (IF1$^-$)/ pAraIF1 strain which is plated onto LB arabinose + kanamycin, + chloramphenicol + ampicillin plate. The colonies are picked up and confirmed for the presence of GFP *via* PCR. The positive clone is then used for further experiment. Overnight culture is prepared by inoculating a single colony in 2 ml of LB having ampicillin 40 µg/ ml- final concentration, Kanamycin 15 µg/ ml- final concentration, chloramphenicol 10 µg/ ml- final concentration and also arabinose 0.2% - final concentration at 37 ºC at 140 rpm shaking. From the overnight culture take 10 µl and transfer into 1 ml of fresh LB ampicillin + arabinose. From it, 200 µl is transferred in triplicates in 96 well Elisa plate. Let the culture grow up to 0.6 OD_{600} at 37 °C. From this, 10 µl of cells are transferred to 1 ml of LB arabinose and other 10 µl to LB glucose with + kanamycin + chloramphenicol + ampicillin. Let it grow at 25 °C to 0.25- 0.3 OD_{600} and then add IPTG to final concentration of 1 mM (2 µl from 100 mM stock). Let it grow for 3 hours after induction. Pellet down the cells and then lyse in SDS loading dye, denature at 85 °C for 5 minutes and then check the expression on 15% SDS gel loading both un-induced and induced fraction. Stain the gel with Coomassie and then de-stain in order to visualize the gel. The loading is normalized with control band by quantitating them from each lane and calculating correction factor. This is then multiplied to individual GFP bands density measured using image quantification software and then pico moles estimated by comparing it with purified GFP protein band intensity. These data are then plotted in excel sheet with uninduced GFP amounts subtracted from the induced one.

General scheme for performing experiment in Growth reader machine for expressing pACY177 GFP with and without secondary structure along with pACY177 BER-GFP in *Ec* (IF1$^-$)/ pAraIF1 strain is shown in result section.

2.3.5 Methods: With Proteins

2.3.5.1 Casting SDS gels

Depending on the size of the protein the SDS gels are made (smaller proteins requires gels having higher percentage of acrylamide and vice-versa). It consists of two layers, upper layer almost one-fifth the size of gel as stacking. The composition is same irrespective of whatever percentage acrylamide gel is selected in separating layer.

The second layer almost occupying four-fifth of the gel is separating layer and it is here where the percentage selection is made depending upon the amount of acrylamide is used in solution (as mentioned in buffer table).

The gel plate is fixed in the apparatus supplied by Biorad. The bottom is sealed by just pouring a very little amount of the separating solution around 0.6 ml at the base and let it solidify. That would prevent the leaking of gels.

After it solidifies, separating gel is poured and kept for sometime to solidify. On the top of it a thin layer of isopropanol is added to prevent the uneven layer at the top to be formed. After it solidifies stacking solution is added and then comb is fixed. After this solidifies the comb is removed and then the gel is placed in running chamber with 1X Tris glycine SDS buffer to start running the gel at room temperature. The gel is run at around 100 V for 15 minutes and then increased to 120 V and run till the dye from reaches the bottom.

2.3.5.2 Coomassie staining

The SDS gels are stained with Coomassie in order to visualize the protein band in the samples. Gel is incubated with Coomassie solution for 30 minutes with slow mixing at room temperature.

2.3.5.3 Destaining of Coomassie stained gels

The Coomassie stained gel is subjected to detaining solution and constantly stirred at room temperature till the gels get completely destained. After complete destaining, the gels are kept in water.

2.3.5.4 Protocol for analytical protein test

Pellet down the cells and to it add cracking buffer, generally for 8 ml of pellet add 200 μl of cracking buffer. After that, incubate it at 95 °C for 5-10 minutes. Pellet down the cells at 4 °C, 10,000 rpm for 10 minutes. Take out the supernatant and run it on SDS

gel. Estimation is done by taking 1 OD at A_{230} of the known protein in the same gel, and comparing its band intensity with our protein band which is prior normalized in terms of loading with control band. This value is then multiplied by 220 μg/ ml (1 OD at A_{230} = 220 μg/ ml), which would finally provide the rough estimate our protein concentration.

When protein is made for the functional Assay, then the cells are lysed by adding the Lysis buffer.

Buffer composition:

50 mM NaH_2PO_4

300 mM NaCl

For 10 ml of culture pellet add 1 ml of Lysis buffer along with lysozyme to final concentration of 1 mg/ ml. Incubate at ice for 30 minutes and do the sonication. It is sonicated 4 times 1 sec each sonication cycle with 10 second pause between each. Pellet down the cells after sonication at 4 °C, 10,000 rpm for 10 minutes. Take out the supernatant, mix it with loading dye and load it on gel. It amount is estimated the same way as mentioned above.

2.3.5.5 Large scale protein over-expression and purification

2.3.5.5.1 Translation initiation factor- IF1

Overnight culture is made and is then inoculated in larger volume (200* 5 ml).It is grown till 0.4 OD_{600}, IPTG added to final concentration of 0.8 mM, and further grown for 2.5 hours. Check the OD_{600} and make a gel for over expression with equal number of cell of un-induced and induced fractions. After confirming over expression, pellet down the cells, lyse it using lysis buffer and lysozyme as mentioned in Quiagen protocol. Perform centrifugation to separate the pellet and supernatant (save a small amount). 1 liter culture pellet is lysed in 35 ml of lysis buffer and from it 10 ml was loaded onto Ni-NTA column for FPLC. Before loading the samples the supernatant was centrifuged at 40,000 rpm for 1 hour in order to remove ribosomes. FPLC was performed, which encompasses,

1. Stabilization of the reading by passing column using buffer A (50 mM imidazole in lysis buffer)
2. The collection of flow through followed by wash of 10 ml with 50 mM imidazole.
3. Started collecting the elution fraction each of 200 μl from 100 mM imidazole (20% of buffer B which is 500 mM imidazole) to the final of 500 mM.

All elution fractions are checked for the presence of protein by loading 20 μl from each on 20% SDS page and Coomassie staining it. Pool in the fraction (approx 4 ml) which has good amount of protein and perform dialysis 45 minutes three times 100 volume 1X binding buffer at 4 °C. Make an aliquot of 20 μl, shock freeze in liquid nitrogen and store at –80 °C. After dialysis different amount of purified protein samples are checked (5 and 10 μl) onto 20% SDS page by Coomassie staining and their concentration calculated *via* Bradford. It is also checked for RNase contamination.

2.3.5.5.2 Translation initiation factor - IF3

Overnight culture is made and is then inoculated in larger volume (2x1 L).It is grown till 0.5 OD_{600}, IPTG added to final concentration of 0.8 mM, and further grown for 3 hours. Check the OD_{600} and also over expression with equal number of un-induced and induced cells. After confirming over expression, pellet down the cells, lyse it using micro fluidizer in lysis buffer without lysozyme. Perform centrifugation to separate the pellet and supernatant (save a small amount). 1 liter culture pellet is lysed in 35 ml of lysis buffer and the total amount was loaded onto Ni-NTA column for FPLC. Before loading the samples the supernatant was centrifuged at 40,000 rpm for 1 hour in order to remove ribosomes. FPLC was performed, which encompasses,

1. Charging the Nickel column with Nickel salt.

1. Stabilization of the reading by passing column using buffer A (0 mM imidazole in lysis buffer)

2. The collection of flow through followed by wash with 50 mM imidazole.

3. Start collecting the elution fraction each of 2 ml after wash in 0 to 100 % buffer (no imidazole in 0% of buffer A and 500 mM imidazole in 100% buffer B).

The peak fractions are checked for the presence of protein by loading 20 μl from each on 15% SDS page, and Coomassie staining it. After confirming the fractions that have good amount of protein, they are pooled and dialysis is performed for 45 minutes three times in 100 volumes 1X binding buffer at 4 °C. Aliquot of 20 μl is made, shock froze in liquid nitrogen and stored at –80 °C. After dialysis different amount of purified protein samples are checked (5 and 10 μl) onto 15% SDS page by Coomassie staining and their amounts estimated by comparing it with the known amounts of control protein BSA loaded in same gel. The protein is also checked for RNase contamination.

2.3.5.5.3 MS2 protein purification

MS2 protein purified is used for binding experiments with the specific MS2 binding region that was introduced in various constructs used during the study. MS2 is purified under denaturating condition using Qiagen protocol. The pellet from 1 litre culture was lysed in 8M urea and phosphate buffer in way mentioned in Qiagen protocol book. The pellet and supernatant was separated by centrifuging at 10,000 rpm for 40 minutes. The supernatant was then subjected to centrifugation at 42,000 rpm for 1 hour in 60Ti rotor. The supernatant is then mixed with Ni-NTA slurry which was prior properly mixed by shaking it up and down several times. After adding the slurry to the supernatant, it was kept for mixing for 1 hour in cold room. The empty column is prior equilibrated with 8 M urea buffer – 10 ml. The supernatant is now loaded on to column and the flow through was collected. It is then washed with washing buffer which is same basic 8M urea buffer. This step was performed two times 5 ml each with 10 mM imidazole, and one time 10 ml with 20 mM imidazole. The elution is performed with 50 mM, 100 mM, 150 mM, 200 mM, 250 mM, 300 mM and 500 mM imidazole each 4 ml. Each of the fractions was then tested on 15% SDS gel.

The first three elution fractions are pooled as one and the rest are pooled as other having two batches of purified protein. Serial dialysis is performed.

Same basic buffer is used only the concentration of urea changes. Also 1 M K-acetate is added in all steps.

First step is done with 6 M urea at room temperature overnight

Second step is done in 4 M urea at room temperature

Then it is immediately taken in cold room and the dialysed 4 times in buffer

Hepes 20 mM, K-acetate 1 M, Mg-acetate- 4.5 mM, glycerol 10%, and β-mercapto-ethanol 4 mM, pH 7.6.

The second batch goes on with dialysis very nicely without precipitating at all.

The first batch precipitated very fast and there after 5 times centrifuging at 7,000 rpm for 6 minutes, the sample is diluted 4 times, *i.e.* to 8 ml of sample 24 ml of dialysis buffer is added to avoid anymore precipitation of proteins and the dialysis was further proceeded. Then after fourth dialysis both the samples were shock frozen taking out small aliquot to check on gel and also measure OD at 230nm.

The gel was run and it was found that all the pellets from the first batch have good amount of protein so it was decided to pool all pellets of the first batch sample, in total 2 ml in dialysis buffer and then freeze the sample.

The large diluted volume of first batch of purified protein was further concentrated. First the entire amount about 32 ml was taken for dialysis in another 1 litre of dialysis buffer, overnight in cold. The buffer used is described here: Hepes 20 mM, K-acetate 500 mM, Mg-acetate- 4.5 mM, Spemine-0.05 mM and spermidine 2 mM and beta mecaptoethanol-4 mM, pH 7.6. There should be no glycerol. After lyophilizing the whole 32 ml after dialysis, the pellet was suspended in final volume of 3 ml in MQ water, and then it was dialysed again in same buffer in 300 ml overnight. It is again lyophilised and suspended finally in 1 ml of MQ, and then finally dialysed for 45 minutes again in the same buffer and the checked onto gels and quantified.

2.3.5.5.4 Maltose binding protein-MS2: MBP-MS2 over expression and purification

MBP-MS2 protein has tac promoter and is expressed in DH5α. A single colony (small one) is picked and overnight culture is made in 20 ml with 2% glucose and 60 µg/ ml concentration of ampicillin. From the overnight culture, main culture is made where inoculation is done to the final OD_{600} of 0.05 in 200 ml LB with 2% glucose and 60 µg/ ml concentration of ampicillin. The cells are grown to 0.5 OD_{600} and then they are induced with 0.5 mM IPTG and kept growing for 3 hours. The cells are pelted and resuspended in lysis buffer:

Composition of lysis buffer:

Hepes 20 mM pH- 7.5, KCl 200 mM, EDTA 1 mM and PMSF:100 µM.

The cells are lysed by sonication and then centrifuged for 30 minutes at 12,000 rpm to separate pellet and supernatant. The supernatant is again centrifuged in 60Ti for 1 hour at 42,000 rpm to separate ribosome and then this supernatant is used for purification. The supernatant is mixed with Amylose slurry in cold for 40 minutes and is then loaded onto column. Wash is performed with 0 mM, 0.02 and 0.05 mM maltose and step elution with 1, 2, 5, 10, 20, 50 and 100 mM maltose in buffer B is performed.

Buffer B composition

Hepes 20 mM pH 7.5, KCl 20 mM, EDTA 1 mM and glycerol 10%.

All the fractions are checked on 12% SDS gel along with supernatant, wash and flow through and then fractions are pooled and dialysed in dialysis buffer.

Dialysis buffer composition:

Hepes 20 mM pH 7.5, KCl 100 mM, glycerol 10%.

The dialyzed samples are again checked on gel and then aliquots are stored in -80 °C.

2.3.5.6 Removal of His tag by thrombin cleavage after purification of His tagged proteins

Prepare 1X PBS buffer and dissolve the thrombin lyophilized powder in 500 μl of this buffer in order to get 1unit per μl. Take 1X binding buffer and add calcium chloride dehydrated salt to the final concentration of 2 mM and this is going to be used for dialysis buffer. Take 200 μl of IF1 where concentration of 1 μl is approx 0.4 μg (according to Bradford estimation) so the total amount is approx 80 μg and to this add 1 unit of thrombin. Mix it well and put it in dialysis bag which has cut off is 3.5 kDa, and put in measuring cylinder with 100 ml dialysis buffer (1X binding buffer with 2 mM $CaCl_2$) and let is spin slowly in the buffer. Keep it for overnight in cold room. Take it out and run SDS gel 20% with uncut IF1, thrombin and marker as control. Coomassie stain it and check for the migration difference. Make small aliquots of this cut protein and store it in –80 ºC

2.3.5.7 Check for the RNase in the purified protein sample

Procedure 1

Incubate the sample with labeled [^{32}P]-mRNA (about ≥2,000 cpm) at 37 °C for 5 minutes. Mix the reaction with 2X RNA loading dye and load it on urea PAGE, which has been pre run for 15-20 minutes. Run the sample to 3/4th of the gel-length and expose it to Phospho-Image cassette for 1.5-2 hours or overnight if the labeled mRNA count is very low and then scan the gel.

Procedure 2

Incubate the sample with mRNA at 37 °C for 5 minutes. Mix the reaction with 2X RNA loading dye and load it on urea PAGE, which has been pre run for 15-20 minutes. Run the sample to 3/4th of the gel-length and stain it with toludine blue for 10 minutes and then de-stain it with water. If the protein sample has RNase contamination, the mRNA will be degraded and will be observed as smear after destaining in comparison to intact mRNA, which does not have any RNase contamination.

2.3.6 Experiments with Northern Blotting

2.3.6.1 Labeling of primer with γ32P

2.3.6.2 Protocol for preparing sample

1. Take the sucrose gradient samples to it add 10% of 1M sodium acetate and double the volume absolute ethanol, keep the sample in -80 °C for minimum 1 hour. Centrifuge the tube at 8,000 rpm for 45 minutes and suspend the pellet the 2X RNA loading dye.
2. Take 20 μl from each sucrose gradient fraction and add equal amount of 70% phenol. After mixing and spinning it down, take 10 μl aqueous phase and equal volume of 2X RNA loading dye.

2.3.6.3 Protocol for running the sample

This sample is then applied on 2% agarose gel with 2% formaldehyde and ethidium bromide. The electrophoresis is performed for 1 hour at 100 V in 1X MOPS buffer. Prior to the transfer of RNA from gel to membrane the control UV radiation photo was made. The gel is washed 3 times for 5 minutes in water to remove the contamination of formaldehyde.

2.3.6.4 Protocol for transfer

The mRNA from agarose is transferred onto Hy-bond n-plus membrane using capillarity forces made by buffer 10X SSC going through stack of towels. The transferred is performed at room temperature for 24 hours. After the transfer the membrane is washed in 2X SSC buffer and placed on UV lamp for 5 minutes (the side without the RNA being in front of the lamp) in order to crosslink mRNAs. The membrane is baked at 80 ºC for 1 hour.

2.3.6.5 Protocol for hybridizing with labeled primer

The membrane is incubated in the pre hybridization buffer containing salmon sperm, which covers the entire membrane and eliminate the nonspecific hybridization of labeled primer. It is performed at 42 °C for 4 hours. It is then removed and is then incubated with hybridization buffer containing labeled primer overnight at 42 °C. After that membrane is washed first with 2X SSC at room temperature for 15 minutes, then with 2X SSC 45 minutes with 0.1% SDS at 60 °C and the last washing with 2X SSC

for 15 minutes at 60 °C. Then the membrane is wrapped with saran and placed in phospho imager cassette for at least 2 hours, and then scanned followed by quantification of bands.

2.3.7 Western Blotting

General protocol:

2.3.7.1 Preparation of the PAGE

Apply samples with reference purified protein of known concentration along with pre-stained protein to the 15%-20% SDS-gel and run under following conditions: 10-15 min at 100 V and then at 120 V until there is a good separation of the pre-stained marker protein bands. It is important to not let the lowest band (~10 kDa) of the pre-stained marker to run out of the gel.

2.3.7.2 Preparation for blotting

Polyvinylidene difluoride (PVDF) membrane is cut out 1x and 4x Whatmann 3 mm filter paper of an appropriate size (same as gel). Shortly before the PAGE run is finished, moisten the membrane in methanol for a few seconds (1-3 sec), incubate the membrane in water for 1-2 minute to remove methanol; soak membrane in transfer buffer for a few minutes to displace H_2O. Now the membrane is ready for blotting.

2.3.7.3 Transfer of proteins to the membrane (electro-blotting)

Prepare the gel for blotting: incubate it for 15-20 min in transfer buffer. Assemble the BioRad Apparatus. In the tray filled with transfer buffer assemble the following “Sandwich”: onto the black side of a clamp place 1-2 sponge pieces and 2x filter Whatman papers soaked in transfer buffer, gel (after incubation), the membrane (cross-cut one of the edges of the membrane in order to recognize the protein-side latter on) in a way that it lays on the gel and does not move over it, because the absorption of the proteins begins immediately; place another 2x Whatman filter papers, “iron” the batch over with the help of glass reaction tube for few times in various directions to get rid of air bubbles, now place another 1-2 pieces of sponge and close the clamp (transparent size is on the top). Place the clamp-sandwich into the red and black frame so that the black side of the clamp faces the black side of the

frame; put this all into the Bio-Rad tank, place the ice-block as well, and fill in with transfer buffer. Perform the protein transfer under following conditions: 40 V for Tris-glycine transfer buffer overnight in the cold room, or 100 V for 1 (one) hour in the cold room as well.

Transfer is also done using semi dry transfer apparatus. Here two 2x filter Whatman papers soaked in transfer buffer is placed at the bottom onto which gel is placed. The methanol treated PVDF membrane is placed onto gel avoiding any air bubble between them and then on the top 2x filter Whatmann papers soaked in transfer buffer is placed. The cover of the apparatus is put and on the top of the apparatus, weight of approximately 2 kg is kept as to have efficient transfer. The transfer is performed with 100 milli ampere for 1 hour for 8*6 cm dimension gel. Stop the transfer, disassemble the “sandwich” and verify the progress of the protein transfer with Coomassie/Silver staining for the gel and with Ponceau Red for the membrane (max. 1 minute), until the bands are seen, then wash the membrane with water). Incubate the membrane in a small tray with 1x PBS-T of 5% milk powder (Blocking buffer) either at room temperature (RT) for 2 (two) hours or at +4 °C (in the cold room) overnight (O/N).

2.3.7.4 Reaction with antibodies

Wash the membrane 3x 50 ml with the Wash buffer (1x PBS-T) at RT for 10 min. Add primary antibodies: anti-IF1 or primary antibody of any protein diluted 1:10000 in 1x PBS-T- 5% milk powder. Incubate the membrane with gentle shaking at RT for two hours or at +4 °C (in the cold room) overnight (O/N).

(The anti-IF1 antibody solution is still OK for re-use if stored at +4 °C for 1 (one) week since the preparation). Wash the membrane with the Wash Buffer 3x50 ml for 10 min. Add secondary antibodies: ECL *anti-rabbit IgG HRP*-linked $F(ab')_2$ Fragment (from goat; GE Healthcare, a former Amersham Bioscience; NA9340-1ML), diluted 1:10,000 in 1x PBS-T- 5% milk powder (5 µl in 50 ml). Incubate the membrane with gentle shaking at RT for one hour. Wash the membrane with the Wash Buffer 3x50 ml at RT for 10 min.

2.3.7.5 Development of the membrane

This step is done using the ECL Kit for Western Blotting detection (GE Healthcare, a former Amersham Bioscience).

Remove the detection reagents from storage at 2–8 °C and allow equilibrating to room temperature before opening. Mix detection solutions A and B in a ratio of 40:1 (*e.g.*, 200 µl solution A + 5 µl solution B). The final volume of detection reagent required is 0.1 ml/cm^2. Drain the excess of the wash buffer from the washed membrane and place it with protein side up on the sheet of Saran Wrap or other suitable clean surface. Pipette the mixed detection reagent on to the membrane. Incubate for 5 minutes at room temperature.

2.3.7.6 Chemi-luminescent detection

Drain the excess of the detection reagent by holding the membrane gently in forceps and touching the edge against a tissue. Place the blots protein side down on to a fresh piece of Saran Wrap, wrap up the blots and gently smooth out any air bubbles. Place the wrapped blots, protein side up, into the x-ray film cassette. Switch off the normal light and work under the red lamp from now on. Place a sheet of autoradiography film (for example, Hyperfilm ECL) on top of the membrane. Close the cassette and expose for 15 seconds. Remove the film and replace with a second sheet of unexposed film. Develop the first piece of film immediately and on the basis of its appearance estimate for how long to continue the exposure of the second piece of film. Second exposures can vary from less than 1 min to 1 hour. The detection can also be done using the LAS1000 instrument.

Western blotting for the detection of IF1 in different types of samples:
Run the 20% SDS gel with quantified protein (purified IF1 = 2.5, 5.0 and 7.5 pmole). Along with load S30 extract of wild type cell and also pre-stain marker. Do the transfer in transfer buffer at 100 V for 1 hour in cold room using PVDF membrane (first rinsed in methanol and then washed in water). Block over night by 5% milk powder solution in cold room with slow stirring. Wash it 3 times 10 minutes each with PBS-T 50 ml each with slow stirring. Incubate it with primary antibody which is made by dissolving required amount of stock into 5% milk powder solution (after dissolving milk powder in 1X PBS-T, it has been centrifuged for 10,000 rpm for 20 minutes, in order to remove un-dissolved milk powder). Incubation is done for 2 hours at room temperature. Standardization is done with 10,000, 20,000, 30,000, 40,000, 50,000, 60,000, 80,000, 100,000 dilution range of primary antibody in order to find out the best which, does not pick up any other protein apart from IF1. Wash it 3 times 10 minutes each with

PBS-T 50 ml each. In the next step, incubate it with secondary antibody 1:10,000 dilution, for 1 hour, room temperature, slow stirring. Wash it 3 times 10 minutes each with PBS-T 50 ml each. Remove the extra wash buffer by slowing drying it in air and then add the reagents after mixing two solutions A and B as suggested in kit protocol. Spread evenly the reagent on the membrane for 5 minutes, air dry and put it in the cassette, which is the exposed to film for different interval of time in order to get the best picture.

After standardization of concentration of primary IF1 antibody as mentioned in above protocol, Western blotting is performed with many samples. It is first of all performed to determine the different amount of IF1 present in *Ec* ($IF1^-$) / pIF1 and *Ec* ($IF1^-$)/ pAraIF1 strain. It is done with old RTS and new RTS *E. coli* RTS batch in order to estimate the amount of IF1 present in these samples and hence determine the variations in them. It is also performed with different ribosome particles and also Polysomes made from Can20-12E cells to determine the presence of different protein onto them. All of these are enlisted in result section.

2.3.7.7 Stripping of membrane to re-probing the proteins with another antibody

Put the membrane in a small plastic bag, add stripping buffer. Incubate in water bath for 30 min 60 °C. After that rinse membrane with tap water several times. Wash membrane with 50 mM Tris pH 7.5 2-3x for 10 min (shaking), then either store membrane in Tris buffer for 1-2 days, or proceed with blocking.

2.3.8 Experiments with sucrose gradient

2.3.8.1 Preparation of S30 extract for Ribosome profile analysis

2.3.8.1.1 First method: lysing cells by using Akowa

In each condition cells were grown and reading was measured and plotted on semi logarithmic growth curve sheet. After the cells reached the OD_{600} of 0.6, the cells were fast cooled in prechilled bottles which have ice (100 g of ice for 100 ml of culture) and pelleted down at 5,000 rpm for 10 minutes. The pellet was immediately stored in -20 °C. Mortar pestle was prior cleaned and baked and chilled. In this equal amount of Alcoa as cell wet weight is taken and cell pellet is crushed for 1 minute in cold room. This helps to break the cells wall and lyse the cell. Care should be taken for the amount of time for crushing the cells as excess crushing would destroy other

cellular components. The lysed cells are the mixed with 1 ml of 1X Tico buffer (suspension with buffer can be done with as minimum volume as possible to get the concentrated S30). It is the carefully transferred to Corex tube which is spun at 10,000 rpm for 10 minutes first and then the clear supernatant is again transferred to fresh Corex tubes and respun at 10,000 rpm for 20 minutes. The clear supernatant obtained from this step is then quantified *via* measuring A_{260}. The obtained A_{260} value is the multiplied with dilution factor and also by 0.8 and this is the final amount of OD present per ml for ($IF1^-$)/ pAraIF1 strain and *Ec* ($IF1^-$) / pIF1 strain. Equal number of A_{260} units is loaded onto 10-30% sucrose gradient made in 1X Tico buffer. SW40 run is performed at 24,000 rpm for 20 hours and the gradient was visualized by gradient runner.

2.3.8.1.2 Second method: lysing cells by using lysozyme

To observe the ribosome pattern in the same condition 200 ml of cells were grown in ideally similar condition to 0.6 OD_{600} and each of the cultures was fast cooled in prechilled bottles, which has ice (100 g of ice for 100 ml of culture) and pelleted down at 5,000 rpm for 10 minutes. The cell pellet was suspended in 1X binding buffer with lysozyme- 0.4 mg/ ml and frozen in liquid nitrogen .The cell is thawed on ice in cold room and the supernatant and pellet are separated by centrifuging at 10,000 rpm for 5 minutes . The clear supernatant obtained from this step is then quantified *via* measuring A_{260}. The obtained A_{260} value is the multiplied with dilution factor and also by 0.8 and this is the final amount present per ml for *Ec* ($IF1^-$)/ pAraIF1 strain and *Ec* ($IF1^-$) / pIF1 strain. Equal number of A_{260} is loaded onto 10-30% sucrose gradient made in 1X Tico buffer. SW40 run is performed at 24,000 rpm for 20 hours and the gradient was visualized by gradient runner. Gradient runner is an instrument that measures the absorbance of samples at A_{260} while the sucrose gradient is pumped out. Based on the absorbance value the curve is plotted.

2.3.8.2 Polysome Preparation

2.3.8.2.1 Preparation of polysomes in presence of antibiotics thiostrepton

Preculture is done by inoculating a single colony in 5 ml LB medium with correct antibiotics. This is then used for the large scale culture of 100 ml. Overnight culture is used as inoculum and added to the fresh medium to the OD_{600} of 0.05. Growth is

performed at 120 rpm shaking at 37 °C and the growth rate is measured at regular intervals till the it reached to approximately 0.5 OD_{600} . Add Thiostrepton to the final concentration of 10 μg/ ml and incubate shake for 20 sec at 37 °C. Fast chilling is done by adding approximately 100 ml of ice measured to the bottles, which are prechilled in –20 °C, and then the grown culture is poured into it. Mix for 2-3 sec and then put it dry ice acetone bath shaking for 5-10 sec. Pellet down the cells at 5,000 rpm for 5 minutes. Remove the supernatant and suspend the pellet in 1 ml of 1X polysomes buffer and also add 5 μl of DNase enzyme. Transfer it to 2 ml Eppendorf, mix and freeze the sample in liquid nitrogen minimum overnight. All this steps should be done on ice in cold room. After overnight, the frozen samples are thawed in cold room in ice water and the centrifuged at 10,000 rpm for 5 minutes at 4 °C, the supernatant is collected in fresh Eppendorf. Check the A_{260} and approx 3-4 A_{260} units is loaded onto 10-30% sucrose gradient made in 1X polysome buffer and the SW60 is performed for 38,000 rpm for 2 hours and the profile is seen.

2.3.8.2.2 Preparation of polysomes in absence of antibiotics thiostrepton

The same experiment is performed without the use of antibiotics, thiostrepton. The important point is that all the steps should be performed very fast and in a very cool environment.

Reagents

1. Thiostrepton, dissolved in DMSO stock 10 mg/ ml
2. DNase enzyme, which is RNase free; 10 U/μl, Roche
3. Polysome Buffer- binding Buffer with 0.4 mg/ ml lysozyme

2.3.8.3 Samples processing obtained from fractionation of sucrose gradient

Fractionation is done *via* fraction collector, which enables to collect constant volume of samples of definite size which is then processed to be used for Western blotting.

To the fractions 1X binding buffer is added in ratio of 1:1. In this TCA to the final concentration of 10% is added and incubated on ice for one hour. It is then centrifuged at 14,000 rpm for one hour at 4 °C. The supernatant is very carefully decanted *via* pipetting. Sample is then washed with 1.5 volume of acetone and then centrifuged at 14,000 rpm for 45 minutes at 4 °C. The supernatant is decanted very slowly and the white pellet is dried in 37 °C incubator till all acetone has evaporated. The pellet is suspended in mix dye (1:1 ratio of high pH SDS dye and normal SDS

loading dye). The sample is the denatured at 95 °C for 2-3 minutes, spun shortly and the proceeded for Western blotting.

2.3.9 Expression of Dual Luciferase in *Ec* ($IF1^-$)/ pAraIF1 and MG1655 strain

Dual luciferase plasmid is designed in such a way that it has renilla luciferase as first cistron and firefly luciferase as second cistron in pFLAG vector. The vector provides the expression of both the luciferase to be from tac promoter with IPTG induction and additionally the native origin of replication is changed to p15A to match with the compatibility of IF1 plasmid (pAraIF1) in special *Ec* ($IF1^-$)/ pAraIF1strain. It is to be mentioned here that presence of glucose inhibits the expression from tac and therefore the amount of protein made will be less as compared to arabinose which is not inhibitory. In order to have the effect of glucose only for IF1 expression and not reporter gene, different IPTG amounts were used for glucose and arabinose. It is 3 mM for glucose and 0.1 mM for arabinose for both the strain in order to have same amounts of reporter protein to be expressed. Having explained here now follows the procedure for experiment.

Transform dual luciferase plasmid in *Ec* ($IF1^-$)/ pAraIF1 and MG1655 strain. MG1655 is mother strain for generating *Ec* ($IF1^-$)/ pAraIF1 and therefore considered as wild type strain. Pick up the colonies and suspend them in fresh LB medium with 0.2 % arabinose and ampicillin. First set of growth of the cells are performed at 34 ºC till OD_{600} is reached 0.35 to 0.4. The growth is performed in small volume of 200 µl in 96 well plate and measured automatically in growth reader instrument. At this point of time cells are harvested, pelleted and suspended in final 1:20 dilution in either LB arabinose with ampicillin or LB glucose with ampicillin. To this IPTG is also added to the final concentration of 0.1 mM for LB arabinose and 3 mM for LB glucose and second set of growth is performed at 30 ºC for almost 5 hours. After this point of time cells are harvested and suspended in lysis buffer which is 1X binding buffer with 10 mg/ ml fresh lysozyme. Lysis is performed by 3-4 times freezing and thawing after which pellet and supernatent are separated by centrifugation at 10,000 rpm for 5 minutes at 4 ºC. Supernatent is now used for measuring both the luciferase. For measurement 10 µl of supernatent is mixed with 10 µl of first reagent and firefly luciferase in measured. After this 10 µl second reagent is added, mixed and renilla luciferase is measured. Both the measurements are done for 10 seconds. Ratio of

both the luciferase is calculated by dividing the values of firefly luciferase to renilla luciferase for both LB arabinose and LB glucose. The same sets of cells are also checked for the amount of IF1 and IF3 protein present in them *via* Western blotting using protein specific antibody along with control S7 ribosomal proteins.

2.3.10 Technique for obtaining picture with high resolution

First make the complete pictures in power point and save it in windows metafile (.wmf) format. Then open this picture in Coral software and export it in JPEG format. This picture is of high resolution and can be incorporated directly into text file.

2.3.11 Primers used for making different constructs

Primers	**Nucleotide sequence**
IF1 forward primer / *Ec* (IF1$^-$)/ pIF1	5’ GCTGTGGTATGGCTGTGC 3’
IF1 forward primer / *Ec* (IF1$^-$)/ pAraIF1	5’ GTCCACATTGATTATTTGC 3’
IF1 reverse primer	5’ GCGACTACGGAAGACAATG 3’
T7 promoter	5’ TAATACGACTCACTATAGGG 3’
T7 terminator	5’ GCTAGTTATTGCTCAGCGG 3’
Berlin Intermission forward primer	5’ TATTTCTAGAAAGGAATACTAATAATACTAATAAGCTAACCTAAGGTAACGTACTAATAAGCAGGAGTACTAATGACT 3’
Berlin Intermission reverse primer	5’ CCTTGAAGACTCTCGAGCCGGATCCCGGGTTTGTA 3’
16S mature (Northern blotting)	5’ GCGCATTTCACCGCTACTCC 3’
23S mature (Northern blotting)	5’ CGGTCCTCTCGTACTAGG 3’
Forward oligo met mut	5’ GATCTTAATACGACTCACTATAGGCCTTATTTATTTATTTATTTATTATTTACTTTGAAAATTATTT 3’

Reverse oligo met mut	5’ AATTATGCTGAGTGATATCCGGAATAAATAA ATAAATAAATAATAAATGAAACTTTTAATAAAGA TC 3’
Forward oligo lys-inframe mutation	5’ GATCTTAATACGACTCACTATAGGCCTTA TTTATTTATTTATTTATTATTTACTATGTAAAT TATTT 3’
Reverse oligo lys-inframe mutation	5’ AATTATGCTGAGTGATATCCGGAATAAATA AATAAATAAATAATAAATGATACATTTAATAAA GATC 3’
Forward oligo lys-outframe mutation	5’ GATCTTAATACGACTCACTATAGGCCTTA TTTATTTATTTATTTATTATTTACTATGATAATTAT TT 3’
Reverse oligo lys-outframe mutation	5’ AATTATGCTGAGTGATATCCGGAATAAATAA ATAAATAAATAATAAATGATACTATTAATAAAG ATC 3’
pFLAG (Sec-Str-GFP) Forward primer	5’ CACGCAATTAATCAAAAGGGGACCCCTTGC GGGGTCCCCCAAAACAAAACAAAACAAAACA AAACGGAGGCACTAATATGACTAGCAAAGGA GAAGAACTTTTCACTGG 3’
pFLAG (No-Sec Str-GFP) Forward primer	5’ CACGCAATTAATCAAAACAAAACAAAACAAA ACAAAACGGAGGCACUAAUAUGACTAGCAAA GGAGAAGAACTTTTCACTGG 3’
Forward Berlin pFLAG primer II	5’ CGGCGGCGGATTAATGGCCTTATTTATTTA TTTATTTATTATTTAC 3’
GFP- Ara reverse primer	5’ CCTTGAAGACTCTCGAGCCGGATCCCGGG TTTGTA 3’

3 Results

IF1 is one of the highly conserved proteins present in all three domains of life. In order to obtain the conservation pattern of the protein, IF1 sequences were obtained from NCBI database for bacteria, archea and eukaryote counterparts and were compared. Comparison can be performed to obtain a less strict conservation pattern called homology and a more strict pattern called identity. When referred to homology it means that a particular position could be occupied by similar kind of amino acid *e.g.* lysine or arginine, the ones falling in same groups, but identity indicates that the position should have exactly identical amino acid. The later is a much strict and sharp comparison and this is the one used in our analysis too. The result is summarized in form of table (**Table 3-1),** which, shows that the conservation pattern in terms of amino acid identity is up to 90% in bacteria. This high percentage value in itself explains the importance of the factor.

	Bacteria	Eukaryotes	Archaea
Bacteria	**45-90%**	**30-40%**	**25-35%**
Eukaryotes	-	35-95%	25-40%
Archaea	-	-	35-75%

Table 3-1: Conservation of IF1*, sequences were obtained from NCBI database, compared and percentage was calculated.*

3.1 Bioinformatic Analysis

With our hypothesis as described in the Introduction section, we first of all started detailed bioinformatic analysis. We searched in for the total number of polycistronic mRNA present in bacteria. It was found that 2,602 *E. coli* genes are organized in

operons and form polycistronic mRNAs. Most of the operons contain two cistrons as seen in **Figure 3.1-1**.

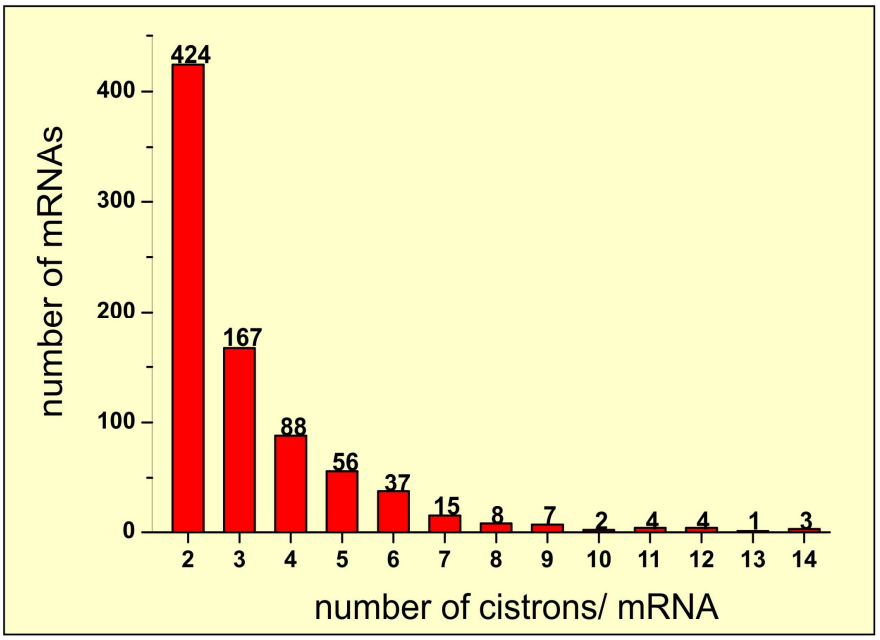


Figure 3.1-1: Bioinformatic analysis *showing bacterial mRNA's existing as poylcistrons with distinct cistron numbers*

From Figure 3.1-1 we calculated that a polycistronic mRNA has 3.3 cistrons on average.

Intercistronic distance is defined by the number of the nucleotide that is present as a space between stop codon of upstream cistron (n) and start codon of the down stream cistron (n +1). The first nucleotide after stop codon is taken as +1 and is counted till the A-base of the start codon is reached. Positive numbers indicates that the AUG of the next cistron is located downstream from the stop codon of the previous one, whereas negative numbers mean that the start codon of downstream cistron is located in the previous cistron, upstream of the stop codon, and therefore it overlaps with the preceding one. This is explained in the scheme given above **(Figure 3.1-2)**, for example in case of the intercistronic distance measured as 8, 'A' of AUG is counted at the 8th position after the stop codon with 7 nucleotide as spacer whereas in case of +1 it is the first nucleotide after stop codon with no spacer. In '-' case the last nucleotide of stop codon is taken as -1 and it is counted in negative till the first nucleotide of start codon upstream is located for *e.g.* in case of -4, 'A' of AUG is at -4 position from the last nucleotide of the stop codon taken as -1 but in case of -1 the last nucleotide of stop codon, is taken as first nucleotide of start codon. Only in

the case of two stop codons *i.e.* UAA and UGA and not UAG, the possibility of -1 reading frame exist because their last nucleotide 'A' can be used as first nucleotide for start codon as AUG.

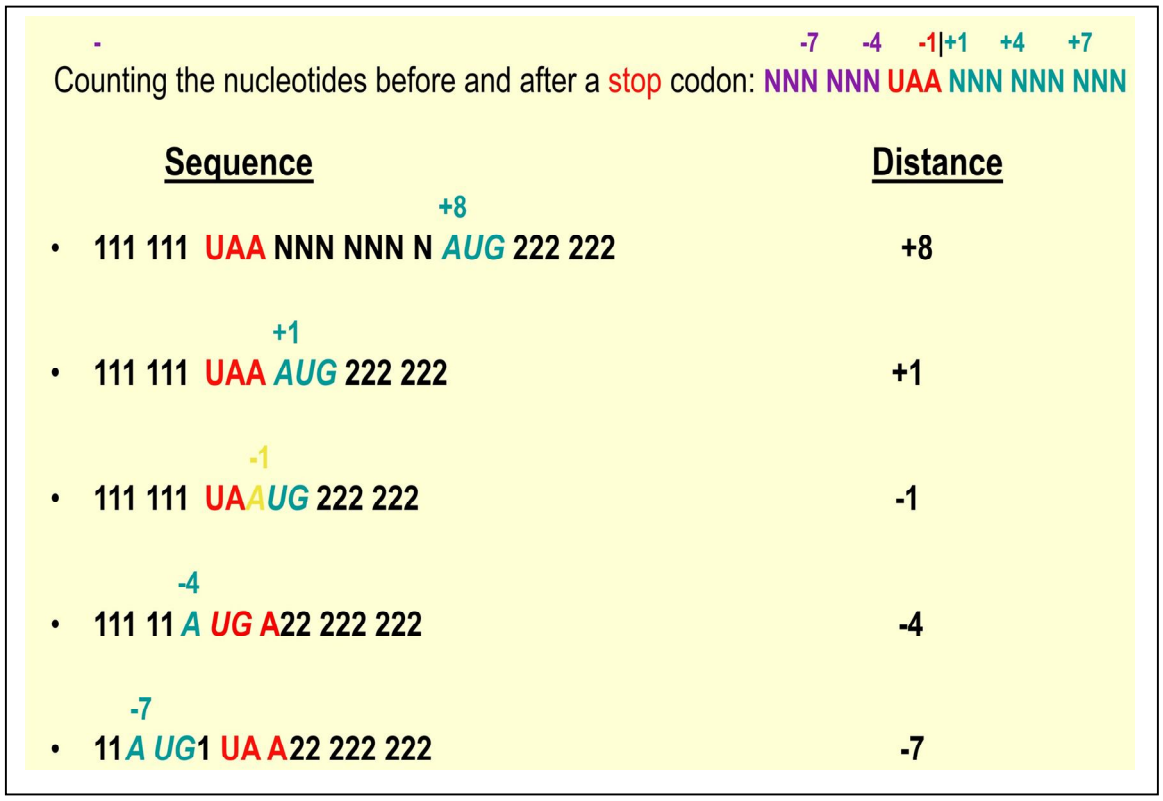


Figure 3.1-2: Examples explaining our nomenclature to define different positions of initiator codon *on poylcistrons which then defines intercistronic distances.*

In line with bioinformatic studies, we found that 75% of the 1700 intercistronic regions in *E. coli* mRNA, are either overlapping with the upstream cistron ('-' cases) or located in +30 nucleotides range **(Figure 3.1-3 B)** and have short intercistronic length of approximately 30-40 nucleotides. The assessment was done up to +200 nucleotides and it was found that only a minority of intercistronic distances have a length from 30 to 200 nucleotides, even less is observed with a length of more than 200 nucleotides as seen in **Figure 3.1-3 A**. It is remarkable that intercistronic distances peak at position -1 and -4 as seen in **Figure 3.1-3 B.** This pattern of intercistronic distance was observed for both ribosomal genes as well as other bacterial genes.

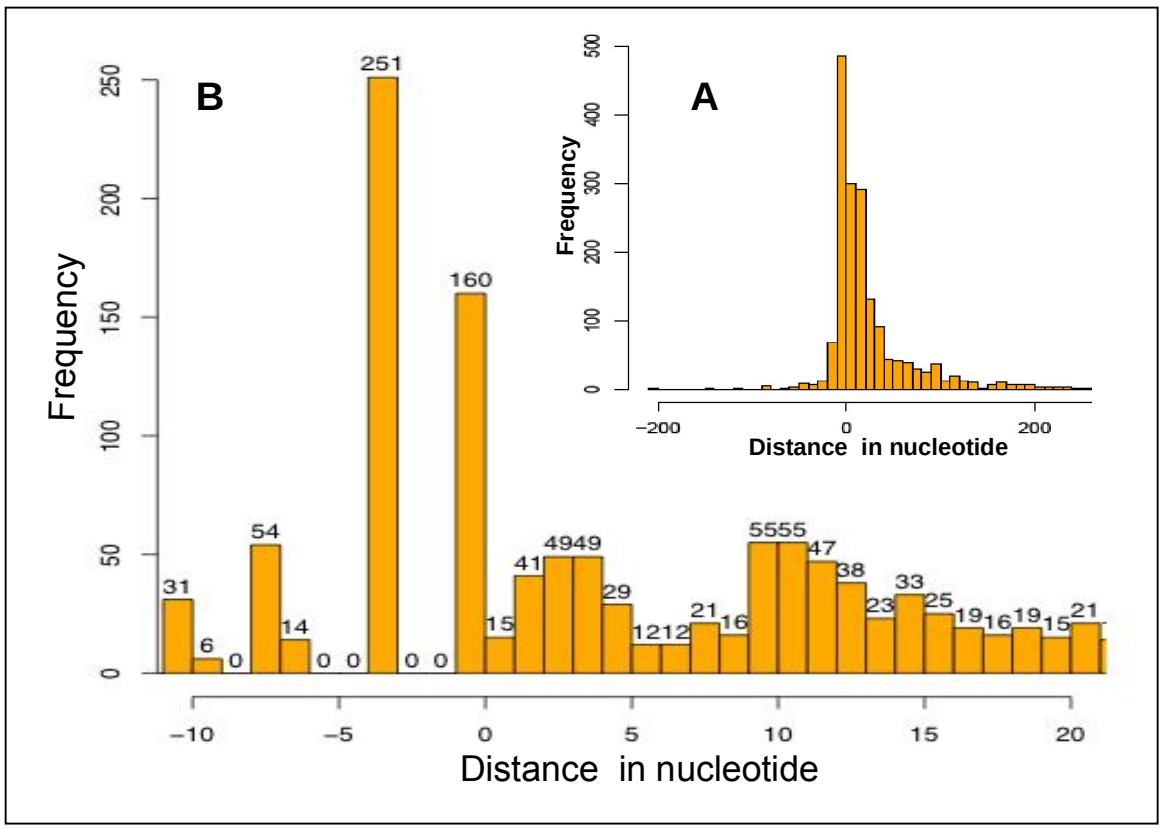


Figure 3.1-3: Bioinformatics study *showing the number of cistrons on Y axis and their corresponding intercistronic distance on X axis*
A: Intercistronic distance up to 200 nucleotides. B: Intercistronic distance between -10 to +20 nucleotides.

As shown in Introduction section the region from -15 to +30 nucleotides on mRNA, during translation is found to be covered by the empty terminating ribosome and therefore its presence will prevent the simultaneous binding of 30S because of steric clash. Therefore, probably initiations in these sites are done by the terminated 70S ribosome *via* scanning. As mentioned before, if 75% of the 1700 intercistronic region are either overlapping or located in +30 nucleotide range than their initiation could only be achieved by 70S. This would then account for 70S scanning type of initiation as the predominant initiation type in contrast to the 30S binding type of initiation. (Knud, this section will be shifted in Introduction after you have checked)

With reference to our hypothesis, 70S scanning type of initiation should require IF1 and possibly also IF3.

In order to analyze this issue, polysomes were prepared from Can20-12E cells and checked for the presence of IF1, IF3 on ribosomal particles *via* Western blotting with S7 as control ribosomal protein.

3.2 Presence of IF1 and IF3 on ribosomal particles

3.2.1 Checking the presence of IF1 and IF3 *in vivo,* on ribosomal particles *via* Western blotting with S7 as control ribosomal protein

Can20-12E cells were grown in LB till 0.4 OD_{600} (log phase) and polysomes were prepared as mentioned in Materials and Methods section, pages 73-74. One of the representative polysome pattern made in LB without any antibiotic usage is shown **Figure 3.2.1-1** with control 70S ribosome**.**

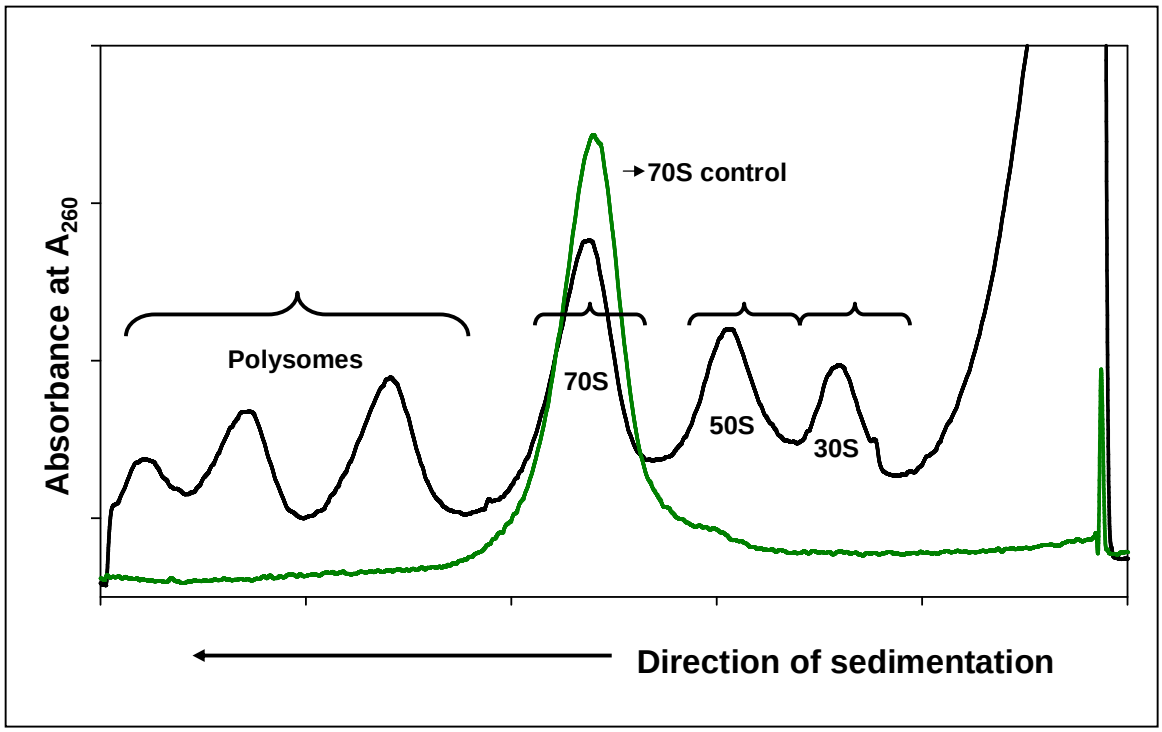


Figure 3.2.1-1: Polysome profile preparation*. Representative polysome profile of Can20 cells. Cells were grown in LB without antibiotic thiostrepton, S30 was prepared and run on 10-30% sucrose gradient in SW40 rotor for 18 hours at 18,000 rpm at 4 °C along with purified 70S ribosome as control shown in green color.*

From each sucrose gradient run small fractions were made, processed as described in Materials and Methods section pages 74-75 and checked for the presence of IF1 and IF3 with control S7 protein *via* Western blotting using protein specific antibody. As shown in **Figure 3.2.1-2,** IF3 is present in highest amount on 30S as expected, but strikingly it is also present on polysomes and 70S. Most interesting, IF1 is exclusively present on 70S and small amount on polysomes but not on 30S. In order to determine the stoichiometry of each of these factors present on ribosomal particles, a plot was made in a way as described in legend, **Figure 3.2.1-3.**

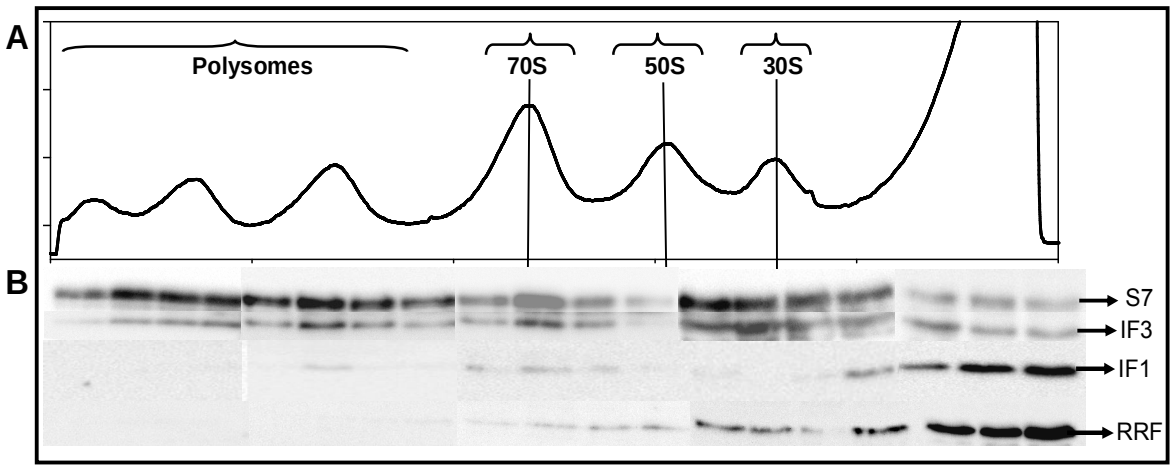


Figure 3.2.1-2: Fractions are checked for S7, IF3, IF1 and RRF by Western blotting. *A: Polysome was made without antibiotic thiostrepton and S30 was run on 10-30% sucrose gradient in SW40 rotor for 18 hours at 18,000 rpm at 4°C. B: Small fraction were collected* via *fraction collector and processed as mentioned in methods section and checked* via *Western blotting for IF1, IF3, RRF and S7.*

The amount of each of the translation initiation factors in pico moles were plotted together with polysome profile pattern in a single graph and shown in **Figure 3.2.1-3.**

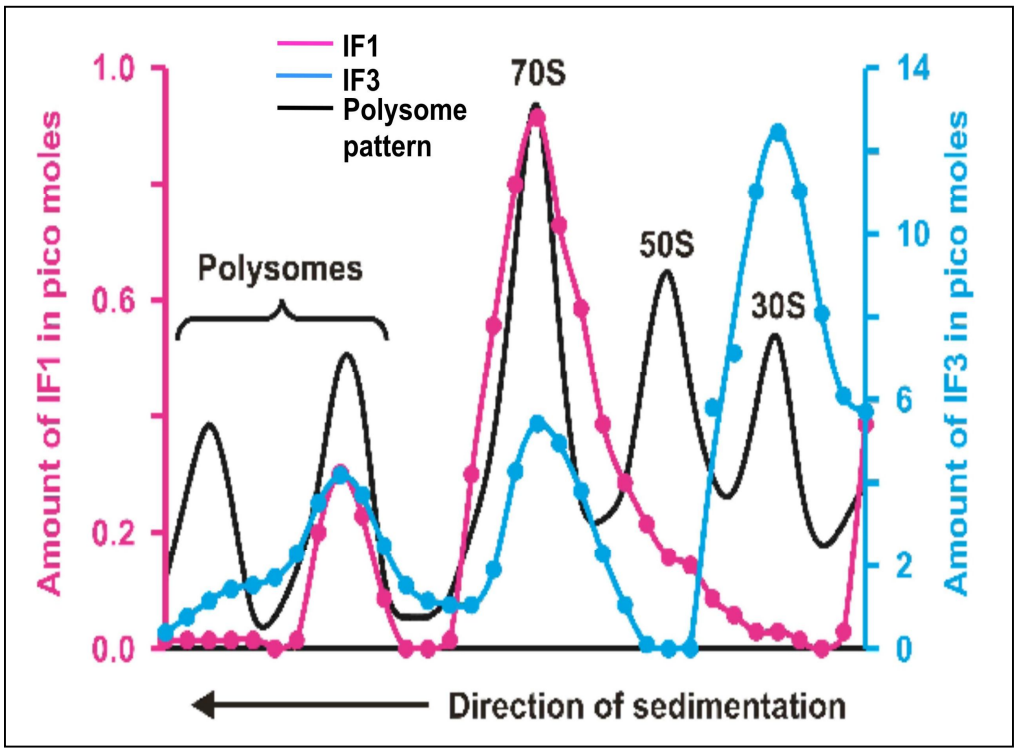


Figure 3.2.1-3: Quantitative plot of each factors present on polysomes, 70S, 50S and 30S. *Each of the protein bands were quantified using image quantification software and pico moles were estimated comparing it with control protein present on the same membrane. It is then aligned with the sucrose gradient pattern.*

Blue curve represents IF3 and has the highest peak at 30S but strikingly 70S too has half the amount as compared to 30S. IF1 which, is represented by pink curve shows peak exclusively at 70S and tiny peak at disomes.

This surprising observation were rechecked in a second similar experiment and the result obtained here was the same confirming that these two factors, IF1 and IF3 are present on 70S ribosome, though in different stoichiometric amounts. This result is the first ever evidence that shows the presence of IF1 and IF3 on 70S ribosome and is an important indication of an exclusive function of IF1 on 70S.

3.2.2 Confirming the presence of IF1 and IF3 *via* Western blotting on ribosomal particles obtained under a different run condition that clearly separates 70S from 50S and 30S

In the previous experiment we employed centrifugation condition which could visualize polysomes, 70S and subunits in single run. As a result, peaks of each of the particles were a bit overlapping and therefore in order to have a better separation of 70S from subunits, the same S30 sample used in the previous experiment was run on 10-30% sucrose gradient and centrifuged at 24,000 rpm for 20 hours. This run condition is optimum to have a very clean separation of 70S, from 50S and 30S and avoid any overlap of the peaks while assessing them in our assay.

Fractions obtained from the run were processed in the same way as described in previous experiment. Western blotting performed with the samples showed that IF3 is present mostly at 30S, but almost half the amount on 70S. IF1 is again exclusively present on 70S as seen lane 1 and 2 in **Figure 3.2.2-1**.

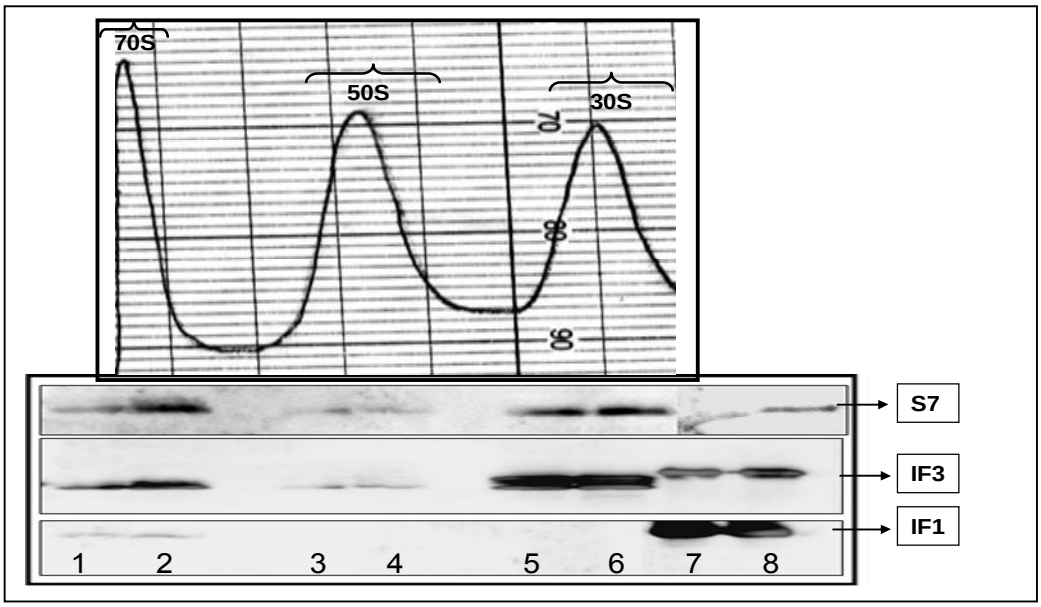


Figure 3.2.2-1: Ribosome profile pattern and Western blotting to detect IF1, IF3 and S7 on fractions. *A: S30 was run on 10-30% sucrose gradient in SW40 rotor for 20 hours at 24,000 rpm at 4 °C. B: Small fractions were collected* via *fraction collector and processed as mentioned in methods section and checked* via *Western blotting for IF1, IF3,and S7; lane1 and 2 = 70S fraction, lane 3 and 4 = 50S fraction, lane 5 and 6 = 30S fraction and lane 7 and 8 = purified S7/ IF3/ IF1 protein*

Quantitative plot was made in this assay also, in order to determine the pico moles of these factors present on 70S, 50S and 30S, using similar calculation described in previous experiment.

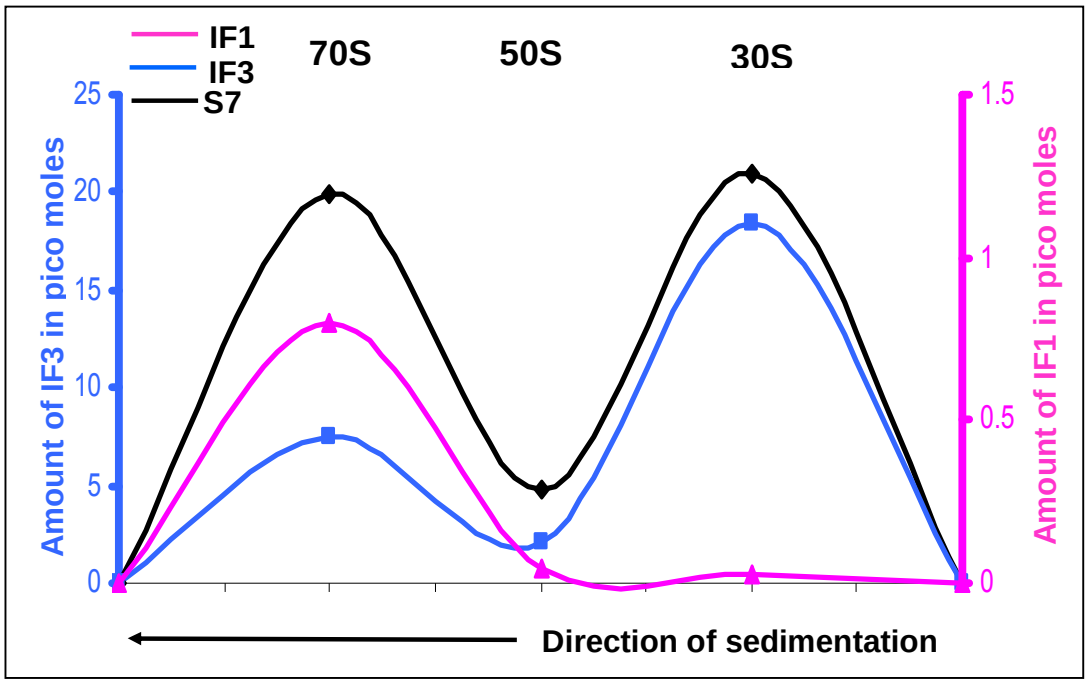


Figure 3.2.2-2: Quantitative plot of each factors present on 70S, 50S and 30S*. Each of the protein bands were quantified using image quantification software and pico moles were estimated comparing with control protein present on the same membrane and plotted.*

The result shown in the **Figure 3.2.2-2** was in harmony with the one obtained from the polysome run, namely both IF1 and IF3 are present on 70S. IF3 has higher affinity for 30S besides being present in almost half the amount on 70S as compared to 30S but IF1 exclusively is a 70S factor.

With these two independent experiments we showed the presence of IF1 and IF3 on ribosomal particles *in vivo*, during log phase growth of bacterial cells. Besides this, we were also interested to analyze various intermediate components produced during preparation of reassociated 70S ribosome from crude with respect to the factors present on them. This would provide us an idea as to what all factors are present on crude 70S obtained from bacterial cells and at which, phase during the purification process, 70S gets rid of these in order to obtain factor free reassociated 70S. This would also indirectly provide us information about the affinity of the associated factor

that we identify at the starting step of purification *i.e.* on crude 70S. If the factor is identified to be present at the later step of purification it would be interpreted as tightly bound to the ribosomes, since it is able to sustain a very high centrifugal force during Zonal centrifugation.

3.2.3 Ribosomes obtained at different stages of 70S purification were analyzed for the presence of IF1, IF3 *via* Western blotting with S7 as control ribosomal protein

The scheme in **Figure 3.2.3-1** shows the methods used in our laboratory to prepare ribosomes. Crude 70S is prepared by pelleting ribosomes from a supernatant obtained from S30 lysate by zonal centrifugation. This is then washed with 1X Tico buffer, and centrifuged to obtain Tico 70S ribosomes. After this, Tico 70S is dissociated in "dissociating buffer" and zonal centrifugation is performed to obtain Tico 30S and Tico 50S. Tico 30S and Tico 50S are suspended in "reassociation buffer" and then subjected to another zonal centrifugation to obtain reassociated 70S. Alternatively crude 70S can be directly subjected to dissociation in dissociation buffer, to give crude 30S and crude 50S**.**

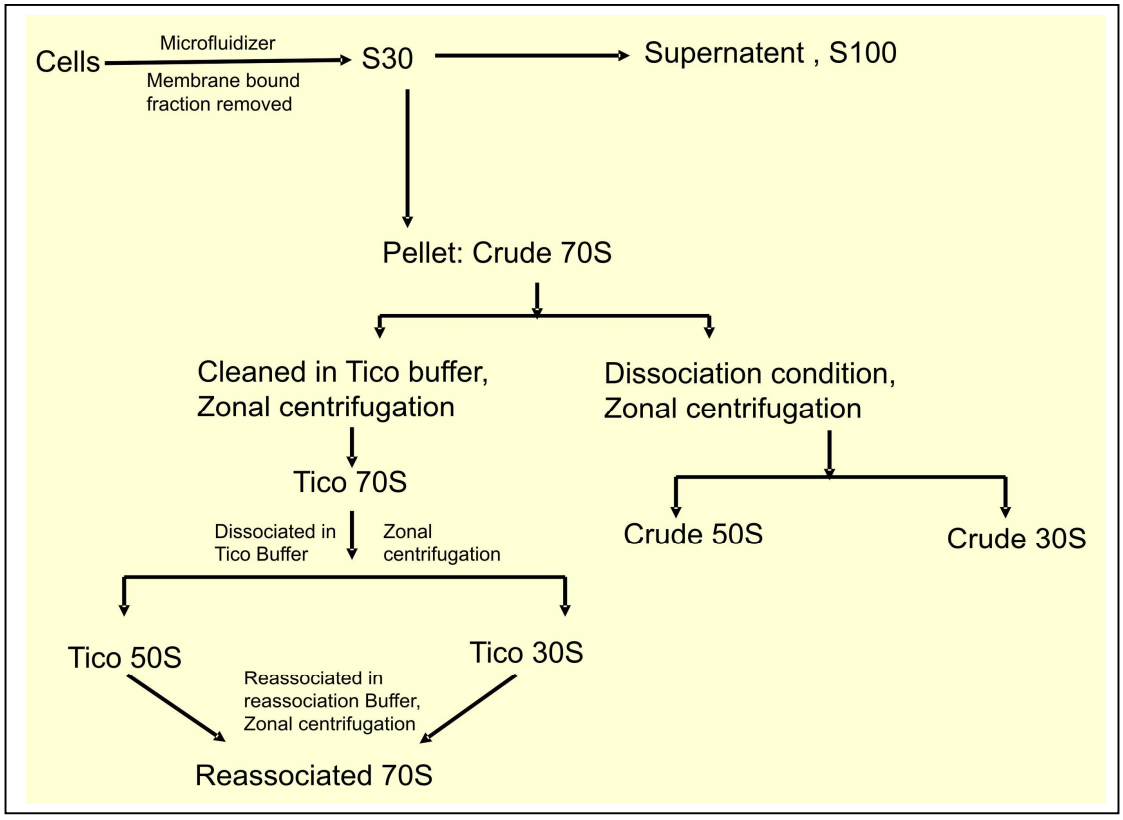


Figure 3.2.3-1: Scheme showing isolation and preparation of factor free reassociated 70S

The results are summarized in **Figure 3.2.3-2.** To start with crude 70S, Western blotting showed that it has many factors present on it like IF3, IF shown by lane 3 and 4 panel B. In next step when Tico 70S formed no factors except IF3 is found to be present shown by lane 1 and 2 panel B. Further when Tico 30S is formed from Tico 70S which, is eventually used for the formation of reassociated 70S, it becomes absolutely factor free shown by lane 1 and 2 and lane 3 and 4 of panel A respectively. This means that IF1 is not so tightly bound to the ribosomes and are removed from the ribosome in the second step of purification when Tico 70S is formed, but IF3 still remains bound which, indicates that it is much more strongly bound to 70S.

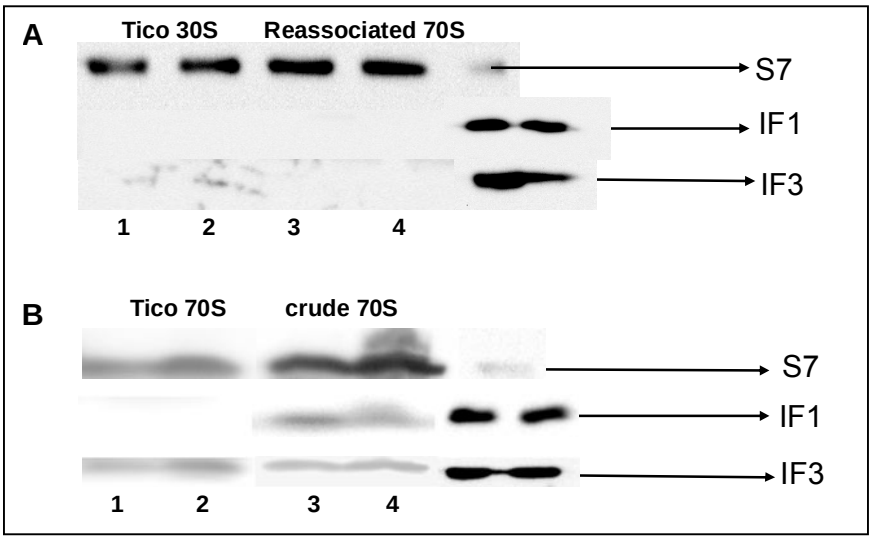


Figure 3.2.3-2: Western blotting with different set of 70S and 30S obtained at various stages of ribosome preparation*. Western blotting is performed with the different ribosomes isolated as described in scheme. Each was detected for the presence of IF1, IF3 with S7 as control ribosomal protein.*

Presence of IF3 on 70S ribosome in all our experiments holds contradiction to the old literature reports according to which, IF3 exclusively is a 30S factor. It promotes dissociation of 70S by antagonizing association of 30S subunits to 50S subunits. To this end, we were curious to know the effective amount of IF3 that is required for the 70S dissociation. Here is the assay which, aims to show this.

3.2.4 Amount of IF3 required for 70S dissociation

To test the amount at which, it favors the dissociation of 70S ribosome, different amounts of purified IF3 was incubated with a constant amount of reassociated 70S and experiment was performed as described in legend. Then the amount of 70S being dissociated was checked. The surprising result was that from 0.1 to10 times excess over 70S, IF3 does not dissociate ribosomes as shown in **Figure 3.2.4-1 panel B to F**. As soon as IF3 amounts were increased in later experiment, we found that it dissociates when present in very high concentration *i.e.* 30-50 times excess over 70S ribosomes as shown in **Figure 3.2.4-2 panel E and F** respectively. Hence

we require IF3, a minimum of 20 times excess over ribosome to dissociate 70S into 50S and 30S subunits. Lower ratio of IF3 over ribosomes does not promote the dissociation of 70S.

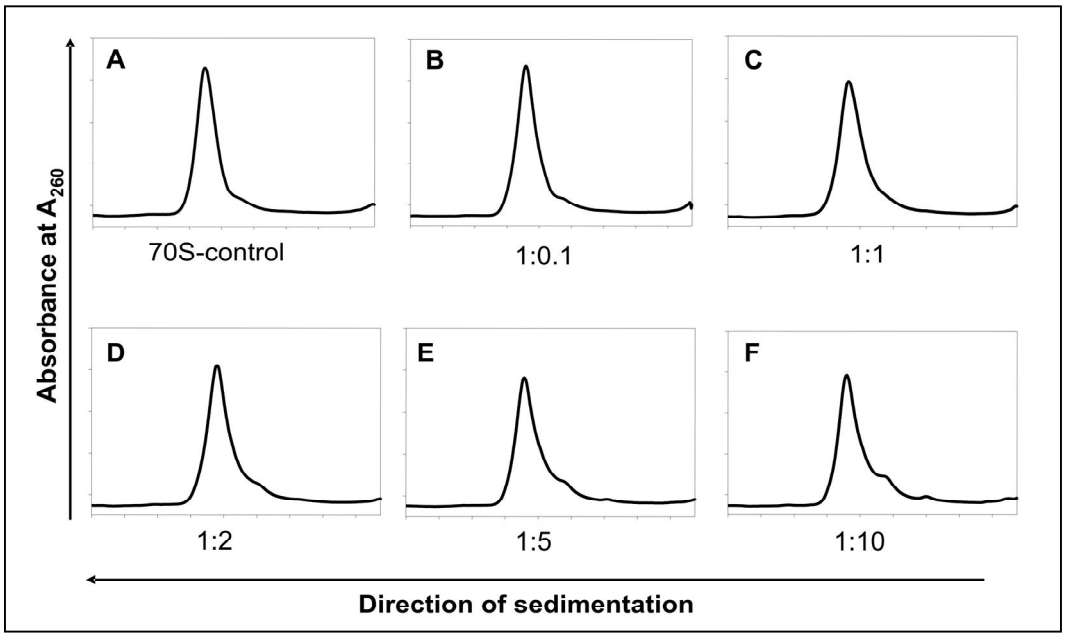


***Figure 3.2.4-1: 70S dissociation by IF3 (lower range)**. Increasing amounts of purified IF3 over 70S was mixed and incubated at 37 °C for 15 minutes in 1X binding buffer. It is then chilled on ice for 10 minutes and then loaded onto SW60 rotor and run on 10-30% sucrose gradient for 3 hours at 38,000rpm A-70S control, B-1:0.1, C-1:1, D-1:2, E-1:5, F-1:10 70S: IF3 ratio*

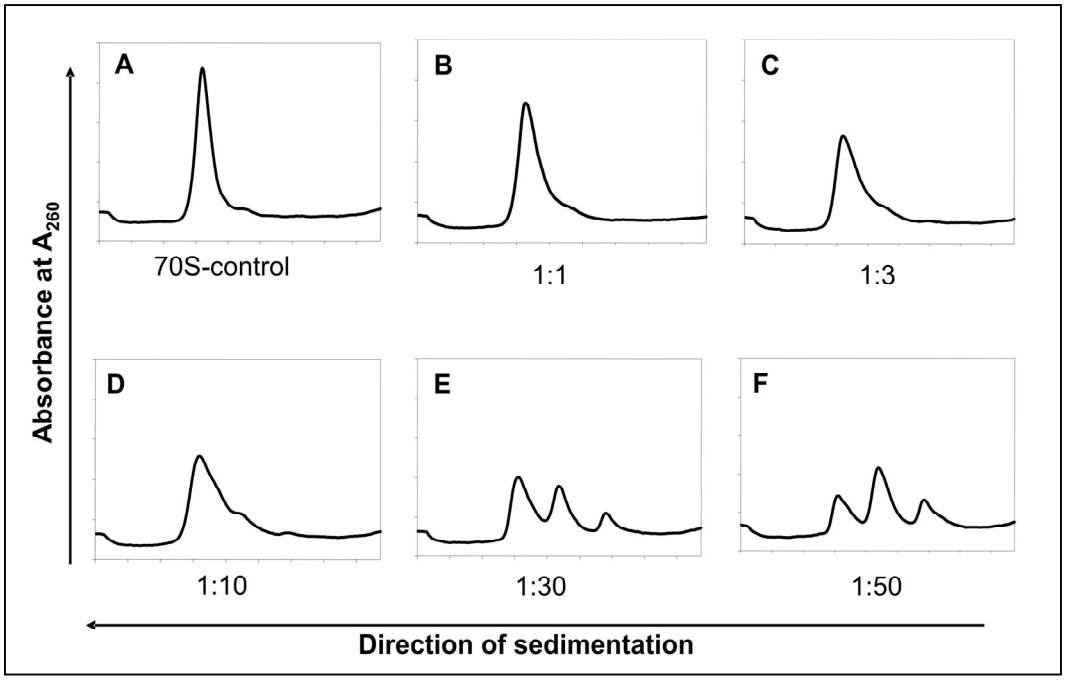


***Figure 3.2.4-2: 70S dissociation by IF3 (higher range)**. Increasing amounts of purified IF3 over 70S was mixed and incubated at 37 °C for 15 minutes in 1X binding buffer. It is then chilled on ice for 10 minutes and then loaded onto SW60 rotor and run on 10-30% sucrose gradient for 3 hours at 38,000 rpm A-70S control, B- 1:1, C-1:3, D- 1:10, E-1:30, F-1:50 70S: IF3 ratio.*

After providing the evidence that IF1 and IF3 are present on 70S and might be involved in 70S scanning type of initiation, we were curious to study what happens in bacterial cell, when IF1 which, is one of the essential gene and probably an important participant of 70S scanning type of initiation is knocked out.

For this study special strain *Ec* (IF1$^-$)/ pAraIF1 is constructed that has chromosomal IF1 gene knocked out and is expressed in *trans* from plasmid conditionally. In the next sections a detailed *in vivo* analysis is described.

3.3 In vivo strategy: Switching on and off the synthesis of IF1

3.3.1 Construction of the special strain *Ec* (IF1$^-$)/ pAraIF1

To study the effect of the knock-out of chromosomal *infA* gene encoding IF1, we had first to clone the gene under a controllable promoter which, can be conditionally switched on and off. Cloning of *infA* gene is described in Materials and Methods section page 57. *E. coli* strains and the plasmids used for the generation of this special strain are enlisted here with a systematic name supporting an easy understanding. This nomenclature is used throughout the Results section. The name reflects the property of the strain for example *Ec* (IF1$^-$)/ pAraIF1 means that the *E. coli* strain has a knocked-out chromosomal IF1 gene, IF1 is expressed in *trans* from the pAraIF1 plasmid. pAraIF1 plasmid means that IF1 is under the control of AraB promoter, whereas in pIF1 plasmid IF1 is expressed from the native promoter.

Table 3.3.1-1: List of* E. coli *strains used for* in vivo *experiment

Systematic Name	Name given from other labs	Genotype of Bacterial strains
MG1655 (IF1$^-$) or *Ec* (IF1$^-$)	PFM1A	As wild type strain MG1655 but with a 200 base pair chromosomal *infA* deletion
E. coli	MG1655	*E. coli* B, RNase I$^-$
	DH5α	*E. coli* K, F$^-$ *Φ80dlacZΔ(lacZYA-argF)U169 deoR recA1 endA1*

		hsdR17(rk,mk+) phoA
Ec (IF1$^-$) / pIF1	PFM1A/pRK04	As wild type strain MG1655 but with a 200 bp chromosomal *infA* deletion and IF1 expressed in *trans* from native promoter in plasmid
Ec (IF1$^-$)/ pAraIF1	PFM1A/pKPW01	As wild type strain MG1655 but with a 200 bp chromosomal *infA* deletion and IF1 expressed in *trans* from AraB promoter in plasmid

Table 3.3.1-2: List of plasmids used for* in vivo *experiment

Systematic name	Name given by other labs	Resistance	Properties
	pSS12-C	chloramphenicol and kanamycin resistance	It has AraB promoter which, is induced in presence of arabinose and repressed in the presence of glucose.
pIF1	pRK04	ampicillin resistance	It has *infA* gene under native promoter.
	pXR101	ampicillin resistance	It is used as templates DNA to PCR amplify the *infA* gene.
pAraIF1	pKPW01	chloramphenicol and kanamycin resistance	It has *infA* gene under AraB promoter which, is induced in presence of arabinose and repressed in the presence of glucose.

The vector used for cloning of IF1 gene is called pSS12-C. It is 6100 base pair long and harbours kanamycin and chloramphenicol antibiotics resistance gene. It also has AraB promoter which, makes it conditionally inducible: a gene under its control is expressed only in presence of arabinose and repressed in presence of glucose. This strain was produced in the following way:

E. coli (IF1$^-$)/ pIF1 is a strain where *infA* gene present on chromosome is knocked out and is encoded by plasmid pIF1 as shown in **Figure 3.3.1-1.**

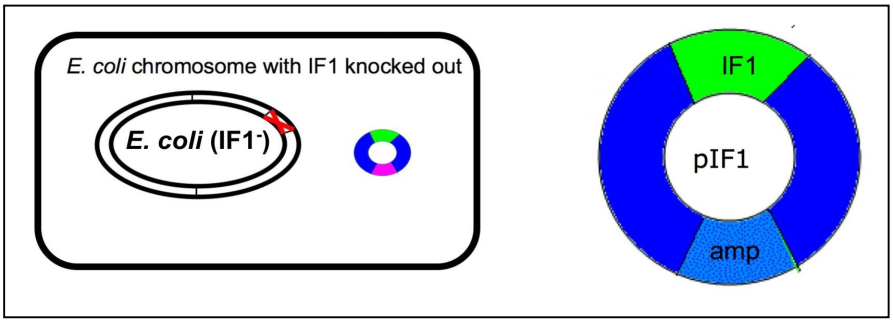


Figure 3.3.1-1: Construction of* E. coli *(IF1$^-$)/ pIF1 strain with knocked out chromosomal* infA *gene and IF1 expressed in* trans *from plasmid*. Initial strain has chromosomal* infA *gene encoding IF1 knocked out, IF1 is expressed constitutively in trans from pIF1 plasmid having native promoter.***

pIF1 plasmid induces ampicillin resistance and is unconditionally expressing IF1 from native promoter (we obtained this strain from the Prof. L. A. Isaksson laboratory in Stockholm). *E. coli* (IF1$^-$)/ pIF1 strain from here on, was then used as a template strain, where plasmid pAraIF1 was introduced *via* transformation. At this point cells have both the plasmids with IF1 expressed unconditionally from pIF1 and conditionally in the presence of arabinose from pAraIF1. This is the Mix strain and was then grown for several generations in minimal media having arabinose, kanamycin and chloramphenicol supporting the pAraIF1 plasmid, which, after continuous growing over four months lead it to loose pIF1 plasmid in the absence of ampicillin. The final strain, having only pAraIF1 plasmid *i.e.* IF1 expressed specifically in presence of arabinose is shown in **Figure 3.3.1-2 B**.

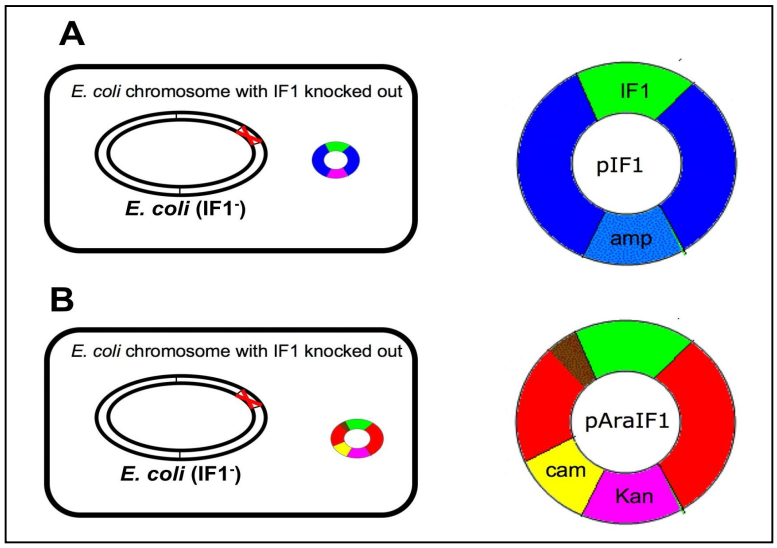


Figure 3.3.1-2: Constructing* E. coli *strain, having conditional expression of IF1 exclusively from plasmid *A:* E. coli *(IF1$^-$)/ pIF1; Initial strain where in background of knocked out chromosomal* infA *gene, IF1 is expressed from pIF1 plasmid having native promoter B: Final strain* E. coli *(IF1$^-$)/ pAraIF1 where IF1 is conditionally expressed from pAraIF1 plasmid in the presence of arabinose.*

The final strain was selected using spot test which helps to screens the clones on selective plates. As shown in **Figure 3.3.1-3**, plate 1 is a LB arabinose plate and in this condition expression of IF1 in any strain will not be hampered and therefore all the cell types could grow. In plate 2, in the presence of arabinose, chloramphenicol and kanamycin only cells containing both plasmids (Mix strain) or only pAraIF1 could grow, because these strains have plasmids which, can express IF1 in the presence of arabinose and antibiotics. In plate 3 where there is LB glucose and ampicillin only *E. coli* ($IF1^-$)/ pIF1 and Mix strain grows because the expression of IF1 from pAraIF1 plasmid in *E. coli* ($IF1^-$)/ pAraIF1 is switched off in presence of glucose and ampicillin, but in the final plate where there is LB arabinose again all 4 sets of cell grows. The experiments very well shows that our final strain *i.e., E. coli* ($IF1^-$)/ pAraIF1 grows specifically in the presence of arabinose with or without antibiotic chloramphenicol and kanamycin.

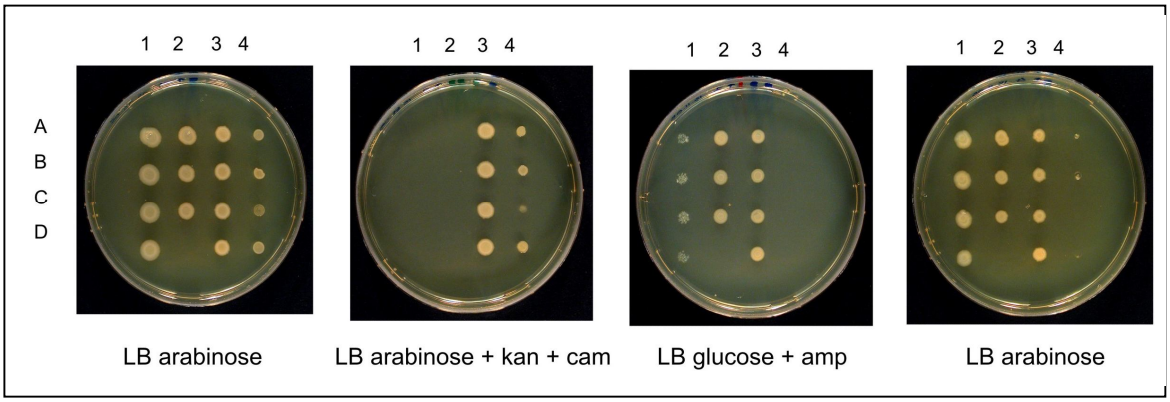


Figure 3.3.1-3: Initial Spot test to isolate* E. coli *($IF1^-$)/ pAraIF1strain*. Clones are screened on LB plates having variable antibiotics and sugar in order to isolate the correct one that grows specifically on arabinose, kanamycin and chloramphenicol. Each column contains a specific strain: wild type MRE600: column 1;* E. coli *($IF1^-$)/ pIF1: column 2;* E. coli *($IF1^-$)/ pIF1/ pAraIF1 (Mix): column 3; and* E .coli *($IF1^-$)/ pAraIF1: column 4. It is done in quadruplet and is indicated by A, B, C, and D. Column 4 in the last plate shows little but significant growth.*

Since IF1 is an essential gene and required for cell viability, only the IF1 expressing cells will grow. Therefore, the first test after the generation of the special strain, would be to detect the effect on growth when IF1 is switched on and off. For this, *E. coli* ($IF1^-$)/ pAraIF1 strain was grown in presence of arabinose and glucose and growth curves were plotted as shown in **Figure 3.3.1-4**.

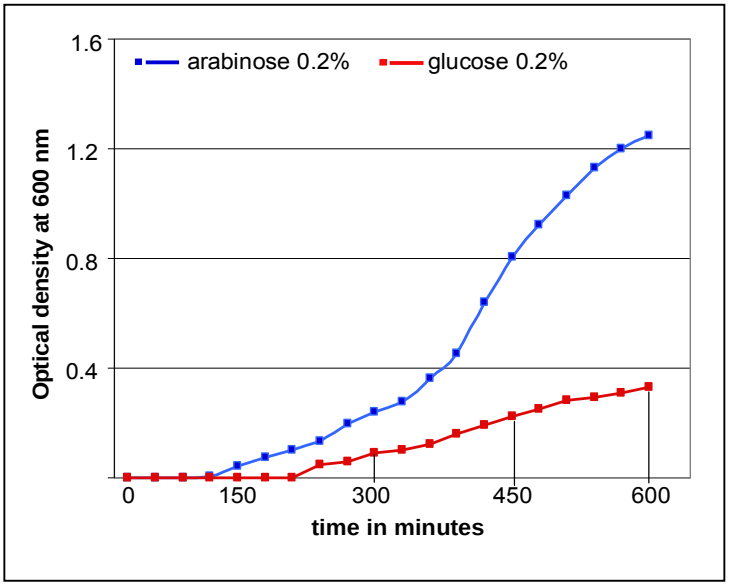


Figure 3.3.1-4: Growth curve for the* E. coli *(IF1⁻)/ pAraIF1 strain*. It is performed in presence of 0.2% glucose and 0.2% arabinose, OD_{600} is measured and the growth curves are plotted.*

It shows that the strain *E. coli* (IF1⁻)/ pAraIF1 grow up to four times slower in presence of inhibitor glucose. After observing the growth difference in the presence of arabinose and glucose we wanted to employ this strain for further experiments but before that we wanted to confirm the properties again.

3.3.2 Confirmation of the properties of *E. coli* (IF1⁻)/ pAraIF1 strain

A glycerol stock made from the first experiment was streaked on an LB plate containing arabinose, kanamycin and chloramphenicol. 16 colonies were picked from *E. coli* (IF1⁻)/ pAraIF1 strain and 4 from *E. coli* (IF1⁻)/ pIF1 strain and a spot test was performed. **Figure 3.3.2-1** shows colonies in red zone as *E. coli* (IF1⁻)/ pAraIF1 strain and colonies on the circumference marked in blue are *E. coli* (IF1⁻)/ pIF1 strain.

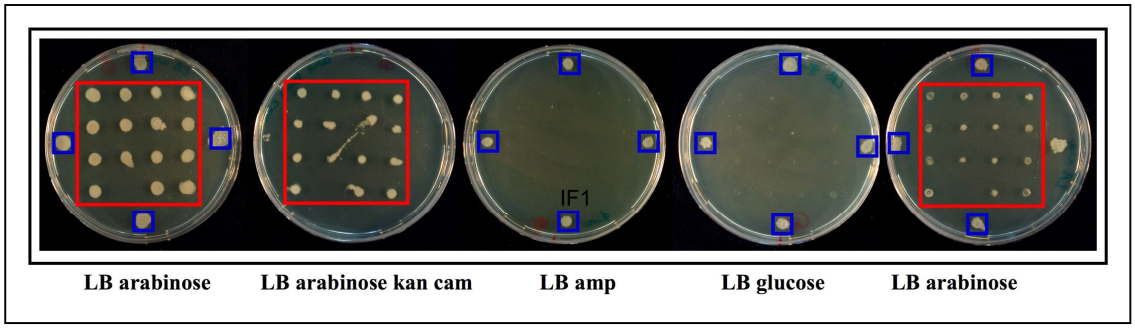


Figure 3.3.2-1: Spot test for genetic screening of different strains. *Screening the colonies obtained from an old glycerol stock by spot test, selecting the correct clone that grows only on LB plates with arabinose + kanamycin + chloramphenicol (*E. coli *(IF1⁻)/ pAraIF1strain). kan = kanamycin; cam = chloramphenicol, amp = ampicillin.*

When these colonies are spotted on plate 1 which, is LB arabinose both the strains grow. In plate 2 in presence of arabinose + kanamycin + chloramphenicol only *E. coli* (IF1$^-$)/ pAraIF1 strain grows because this condition is specific for the expression of IF1 from pAraIF1 plasmid. In plate 3 (LB ampicillin) and also in plate 4 (LB glucose) only *E. coli* (IF1$^-$)/ pIF1 strain grows suggesting that the *E. coli* (IF1$^-$)/ pAraIF1 strain lost the pIF1 plasmid and that presence of glucose inhibits the expression of IF1 in this strain and hence no growth. Finally in plate 5 having LB arabinose both *E. coli* (IF1$^-$)/ pAraIF1 and *E. coli* (IF1$^-$)/ pIF1 strain grow indicating that all plates contained sufficient amounts of *E. coli* cells. From this test finally a single colony of *E. coli* (IF1$^-$)/ pAraIF1 strain was picked up which, showed growth only in specific condition *i.e.* LB arabinose + kanamycin + chloramphenicol. From this colony again many vials of glycerol stock was made which, were used throughout of the following experiments in this work.

Next we tested the effect of temperature on the growth of the various strains. For this *E. coli* (IF1$^-$)/ pAraIF1, *E. coli* (IF1$^-$)/ pIF1 and the Mix strain along with MRE600 cells as a wild-type control were streaked onto series of plates which, have either glucose or arabinose as carbon source and also differ in their antibiotics combinations. The order of streaking is shown in (**Figure 3.3.2-2 A**).

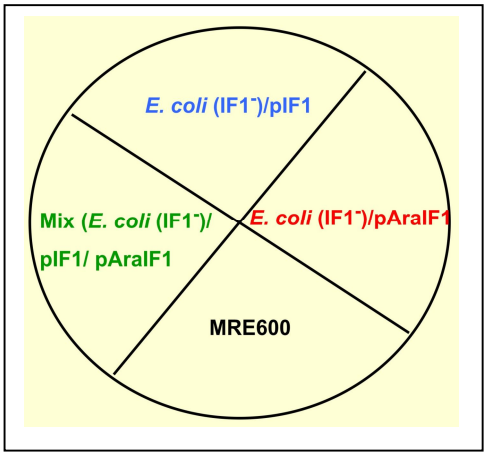


Figure 3.3.2-2 A: Order of streaking on petri plates

The growth on the petri plates was performed both at 37 °C and at 30 °C. There was no growth of the *E. coli* (IF1$^-$)/ pAraIF1 strain on LB glucose plates strain, confirming it to be inhibitor for the expression of IF1 from pAraIF1 plasmid whereas *E. coli* (IF1$^-$)/ pIF1, Mix and MRE600 cells showed no growth difference in presence of either glucose or arabinose and grew normally as shown in **Figure 3.3.2-2 B**.

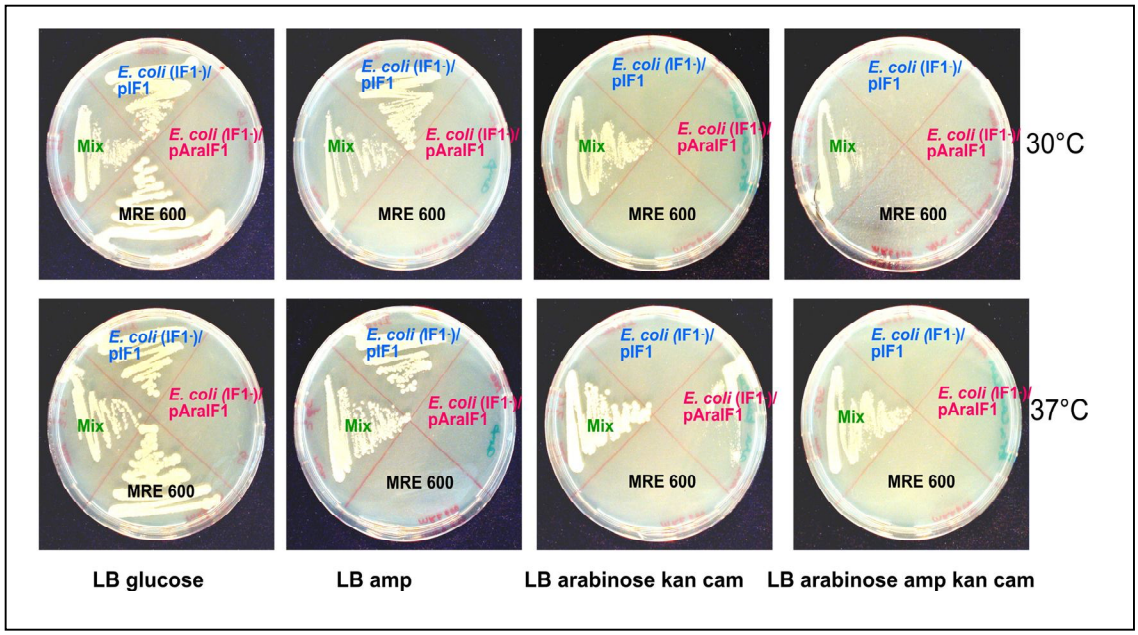


Figure 3.3.2-2 B: Effect of temperature, sugar and antibiotics on growth of different strains, E. coli (IF1⁻)/ pIF1, E. coli (IF1⁻)/ pIF1/ pAraIF1 = mix, E. coli (IF1⁻)/ pAraIF1, MRE600

It was also found that at 30 °C expression of IF1 from AraB promoter is much more tightly controlled, whereas this temperature did not have any effect on *E. coli* (IF1⁻)/ pIF1 strain. As it affected only *E. coli* (IF1⁻)/ pAraIF1 strain, this condition was chosen for performing next growth curve experiments.

This was done in LB medium as described in Materials and Methods section page 59. For each condition the reading was measured and plotted on semi-logarithmic sheets, represented by different colors as shown in **Figure 3.3.2-3.**

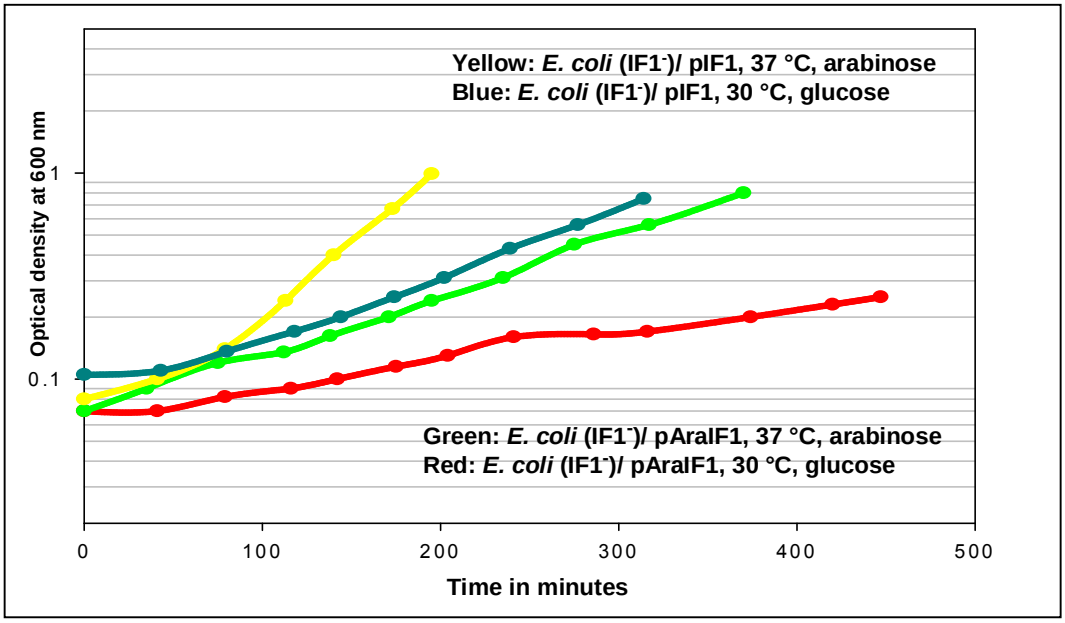


Figure 3.3.2-3: Growth curves for* E. coli *(IF1⁻)/ pIF1 and* E. coli *(IF1⁻)/ pAraIF1 strain. *Growth was performed in the presence of LB 0.2% glucose and LB 0.2% arabinose, OD_{600} was measured and the curves were plotted.*

The growth curves plotted showed that *E. coli* (IF1⁻)/ pAraIF1 strain grows here two times slower in absence of IF1 *i.e.* in glucose as compared to wild type *E. coli* (IF1⁻)/ pIF1 strain where IF1 is expressed in normal amounts in the same condition.

We also observe that *E. coli* (IF1⁻)/ pAraIF1 cells keep growing slowly in the presence of glucose, where IF1 is supposed to be switched off. This being an essential gene, and in the condition where IF1 expression is switched off, logically growth should be completely stopped. In order to understand, why little growth is permitted when IF1 expression is inhibited in presence of glucose, we first wanted to confirm if the individual strains still have the specific plasmid and secondly check the IF1 content of the above grown cell for which, growth curve is plotted *via* Western blotting.

In order to fulfil our first requirement we used PCR as a tool. Forward primer specific for each plasmid was designed as shown in figure below, to perform this experiment. The PCR results confirmed the presence of the respective IF1 plasmids in each strain *i.e. E. coli* (IF1⁻)/ pAraIF1 strain had pAraIF1 and *E. coli* (IF1⁻)/ pIF1 strain had pIF1 plasmid shown as lane 1 and lane 3 respectively (**Figure 3.3.2-4**).

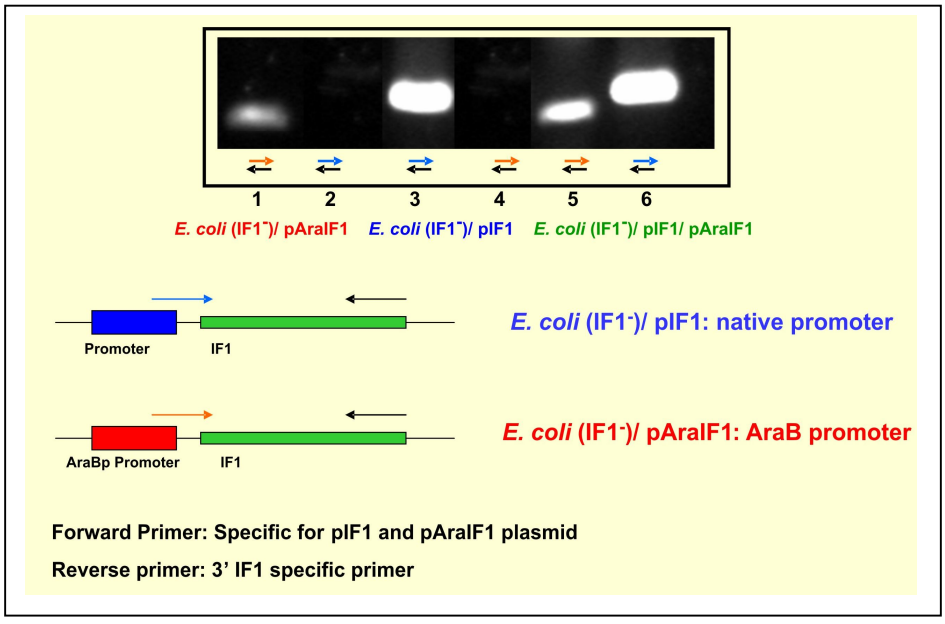


Figure 3.3.2-4: PCR was used to confirm the presence of plasmids in* E. coli *(IF1⁻)/ pIF1,* E. coli *(IF1⁻)/ pAraIF1 and* E. coli *(IF1⁻)/ pIF1/ pAraIF1 (Mix) strain. *PCR was performed using forward primer specific for respective plasmid in individual strain (blue color for pIF1 and red color for pAraIF1) and IF1 reverse primer (black color) as shown in scheme. In Lane 1 pAraIF1 specific forward primer and in lane 2 pIF1 specific primer is used for* E. coli *(IF1⁻)/ pAraIF1 strain. In Lane 3 pAraIF1 specific forward primer and in lane 4 pIF1 specific primer is used for* E. coli *(IF1⁻)/ pIF1 strain. In Lane 5 pAraIF1 specific forward primer and in lane 6*

pIF1 specific primer is used for Mix strain, all the combinations are shown with the different set of primers with distinct color.

Mix strain having both plasmids, showed IF1 specific PCR product when both sets of forward primers are used indicated by lane 5 and 6 **(Figure 3.3.2-4).** This result confirmed that the respective plasmids are present in the strain and therefore this cannot be the reason why *E. coli* (IF1$^-$)/ pAraIF1 strain still grows slowly in glucose.
Then we looked into the second issue with respect to IF1 content and solved this by checking S30 obtained from the cells grown in glucose *via* Western blotting using IF1 specific antibody. It was found that in case of glucose, there is still a residual amount of IF1 that is been expressed because of leakiness of the promoter which, keeps the cells growing slowly.

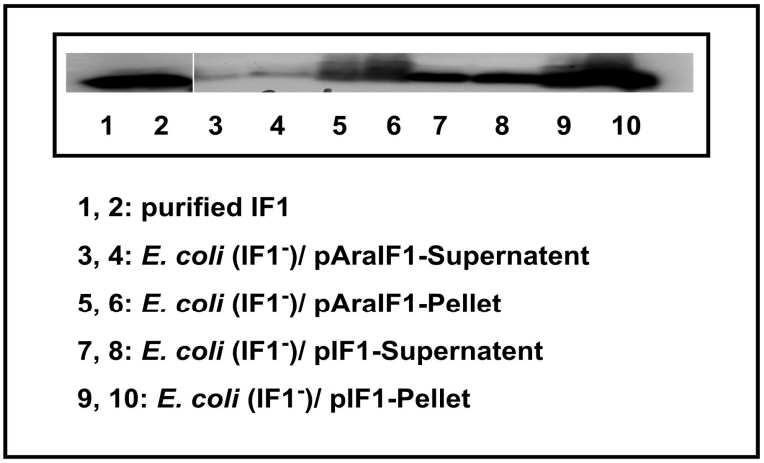


Figure 3.3.2-5: Western blotting to detect IF1 content: E. coli *(IF1$^-$)/ pAraIF1 strain and* E. coli *(IF1$^-$)/ pIF1 strain were grown in glucose at 30 °C, cells were lysed in lysis buffer after normalizing cell density depending on the final OD_{600}, supernatant and pellet was separated. These both were then denatured in SDS loading dye and used for Western blotting with IF1 specific antibody. Band intensity of each was measured, normalised with control protein band and percentage calculated.*

The Western blot represented by **Figure 3.3.2-5** shows that the IF1 amount in case of *E. coli* (IF1$^-$)/ pAraIF1 strain grown in glucose is almost 25% as compared to *E. coli* (IF1$^-$)/ pIF1 strain grown in the same condition.
This observation provided the answer for the slow growth of *E. coli* (IF1$^-$)/ pAraIF1 strain in glucose and was taken into account in all our future experiments.

3.3.3 IF1 and its effect on ribosomal assembly

In the Introduction it is explained that many of the ribosomal genes are present in polycistronic mRNA and due to very short intercistronic regions would be an ideal candidate to be initiated by 70S as 70S scanning type of initiation. We have also suggested the participation of IF1 in this type of initiation and therefore reduced IF1

amount would negatively affect this initiation event. The consequence of it would be improper synthesis of the ribosomal protein which, would be reflected as assembly defect.

In order to study the following, S30 samples were prepared as described in detail in Materials and Methods (pages 72-73). It was then loaded on SW40 and run was performed at 24,000 rpm for 20 hours because this centrifugation run condition allows the best separation of ribosomal particles, to be visualized by gradient runner.

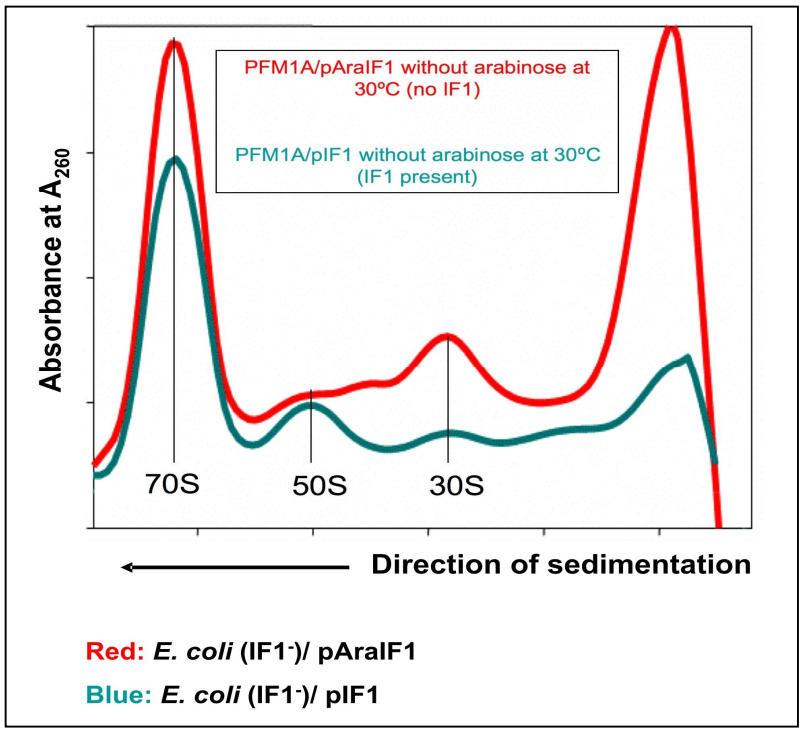


Figure 3.3.3-1: Ribosome profile of* E. coli *(IF1⁻)/ pAraIF1 and* E. coli *(IF1⁻)/ pIF1 strain. *Both the strains were grown in LB glucose at 30 °C and S30 prepared from both was run on SW40 at 24,000 rpm for 20 hours at 4 °C to obtain the profile.*

The alignment of the A_{260} profiles for both the strains grown in glucose at 30 ºC is shown in **Figure 3.3.3-1,** where red illustrates *E. coli* (IF1⁻)/ pAraIF1 profile and blue the *E. coli* (IF1⁻)/ pIF1. The ribosome pattern reflects that *E. coli* (IF1⁻)/ pAraIF1 strain has a strikingly irregular profile as compared to *E. coli* (IF1⁻)/ pIF1 which, has the normal profile of the ribosomal particles,. Complete comparison of ribosome profile for each strain under all growth conditions is shown in **Figure 3.3.3-2.**

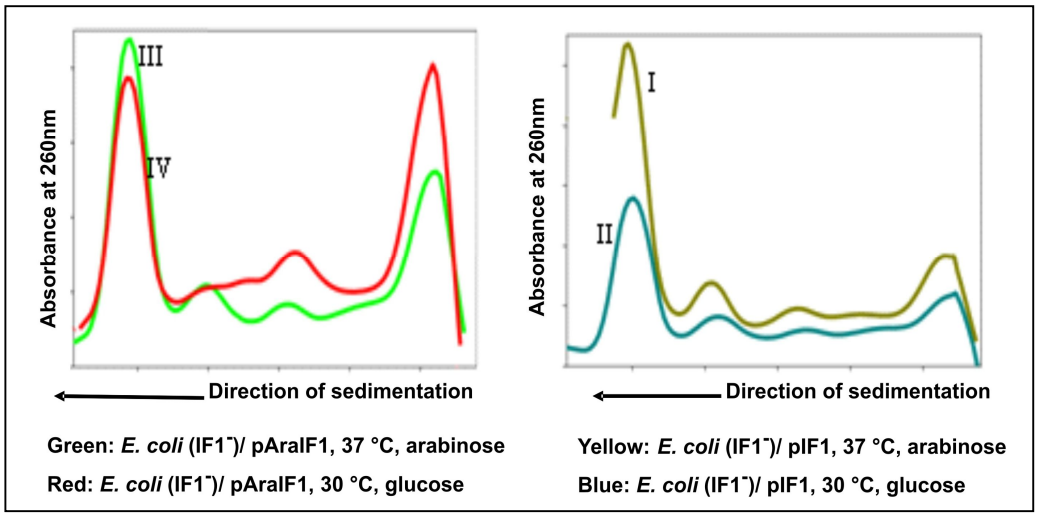


Figure 3.3.3-2: Comparison of Ribosome profile of* E. coli *(IF1$^-$)/ pAraIF1 and* E. coli *(IF1$^-$)/ pIF1 strain under all conditions*. Both the strains were grown in LB glucose at 30 °C and LB arabinose at 37 °C. S30 was prepared at all condition and was run on SW40 at 24,000 rpm for 20 hours at 4 °C to obtain the profile.*

This result is very striking and suggests that there is a 50S assembly defect in the IF1 deficient state which, could be due to accumulation of 31S and 42S precursor for the 50S subunit assembly.

These gradients are plotted on paper sheet and area of the regions specific for 70S and subunits is the index of the relative amount of each of these particles present in that sample. This paper area is cut and weighed. The stoichiometry of 70S, 50S and 30S normally in the bacterial cell is 3: 2: 1 and therefore the weighed value of 70S, 50S and 30S are divided by 3: 2: 1 respectively in order to normalize their stoichiometric amounts. This whole calculation is summarized in **Table 3.3.3-1** which, shows that *E. coli* (IF1$^-$)/ pIF1 strain has 70S to 30S in a 3:1 molar ratio and this is increased to 1:1 in *E. coli* (IF1$^-$)/ pAraIF1 because of abnormal accumulation of increased 30S amount in the cell.

This can be explained by the fact that in IF1 deficient state, there is 50S assembly problem because of which, mature 50S cannot be formed. As a result of decreased amount of mature 50S, functional 70S formation is hampered, leading to the abnormal accumulation of 30S.

Table 3.3.3-1: Determination of ratio of 70S to 30S ribosome *obtained from sucrose gradient of* E. coli *(IF1⁻)/ pAraIF1 and* E. coli *(IF1⁻)/ pIF1 cells grown in glucose at 30 °C*

Strain, growth condition (Medium and temperature)	weight of the area of 70S/ 3	weight of the area of 50S/ 2	weight of the area of 30S/ 1
E. coli **(IF1⁻)/ pIF1, (LB glucose and 30 °C)**	**0.0689/ 3 = 0.023**	**0.0107/ 2 = 0.0053**	**0.0075/ 1 = 0.0075**
E. coli **(IF1⁻)/ pAraIF1, (LB glucose and 30 °C)**	**0.082/ 3 = 0.027**	**0.0178/ 2 = 0.0089**	**0.026/ 1 = 0.026**

In order to verify that 50S assembly defect has any correlation with the amount of 16S and 23S rRNA, RNA was prepared from each fraction as described in Materials and Methods section page 68 and checked on gel. The result for *E. coli* (IF1⁻)/ pAraIF1 strain and *E. coli* (IF1⁻)/ pIF1 strain is shown in **Figure 3.3.3-3 A** and **Figure 3.3.3-3 B** respectively.

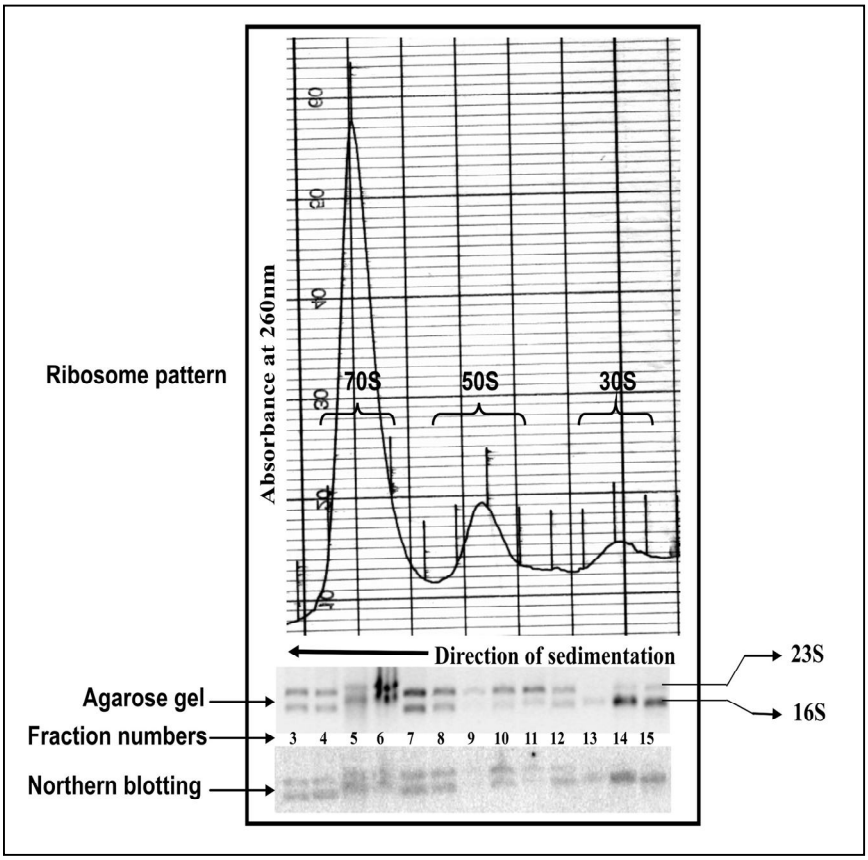


Figure 3.3.3-3 A: Ribosome pattern of **E. coli** ***(IF1⁻)/ pAraIF1 strain with gel showing amounts of 16S and 23S rRNA****. Strain was grown in LB glucose at 30 °C and S30 prepared was run on SW40 at 24,000 rpm for 20 hours at 4 ºC to get the ribosome pattern. Small fractions were taken from the run, processed for RNA, loaded onto 1.5% agarose gel and were also tested for the amount of mature 16S and 23S rRNA* via *Northern blotting. Upper band corresponds to 23S and lower to 16S. The gel fractions are aligned to gradient.*

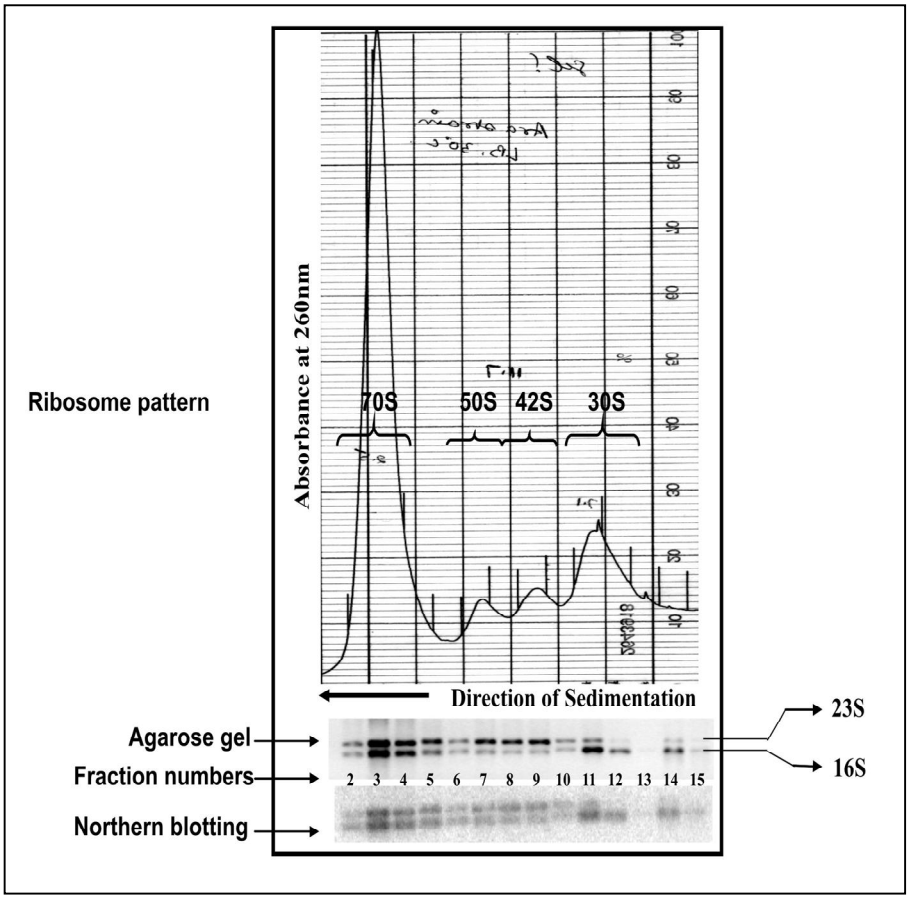


Figure 3.3.3-3 B: Ribosome pattern of* E. coli *(IF1⁻)/ pIF1 strain with gel showing amounts of 16S and 23S rRNA*. Strain was grown in LB glucose at 30 °C and S30 prepared was run on SW40 at 24,000 rpm for 20 hours at 4 ºC to get the ribosome pattern. Small fractions were taken from the run, processed for RNA, loaded onto 1.5% agarose gel and were also tested for the amount of mature 16S and 23S rRNA* via *Northern blotting. Upper band corresponds to 23S and lower band to 16S. The gel fractions are aligned to gradient.*

As we observe in the **Figure 3.3.3-3 A**, the intervening peak between 30S and 50S in the *E. coli* (IF1⁻)/ pAraIF1 profile contains 23S rRNA and thus represents 42S, precursor of 50S. The large 30S peak contains significant amounts of 23S rRNA indicating that 32S precursor of 50S assembly also contributes to this peak. The gels for 16S and 23S rRNA were processed for Northern blotting, procedure described in Materials and Methods section pages 68-69 using radiolabeled primers to detect mature 16S and 23S rRNA. The result obtained here was similar as seen in agarose gels represented by **Figure 3.3.3-3 A and 3.3.3-3 B** bottom panel.

Overall it is clear that 50S assembly is heavily affected by the shortage of IF1 in contrast to the 30S assembly (see Discussion section).

In the next step, we wanted to analyze whether a low IF1 amount also affects the polysome profile of the cell. For this, polysomes were prepared from *E. coli* (IF1⁻)/ pAraIF1 and *E. coli* (IF1⁻)/ pIF1 strains using the protocol described in Materials and Methods section pages 73-74.

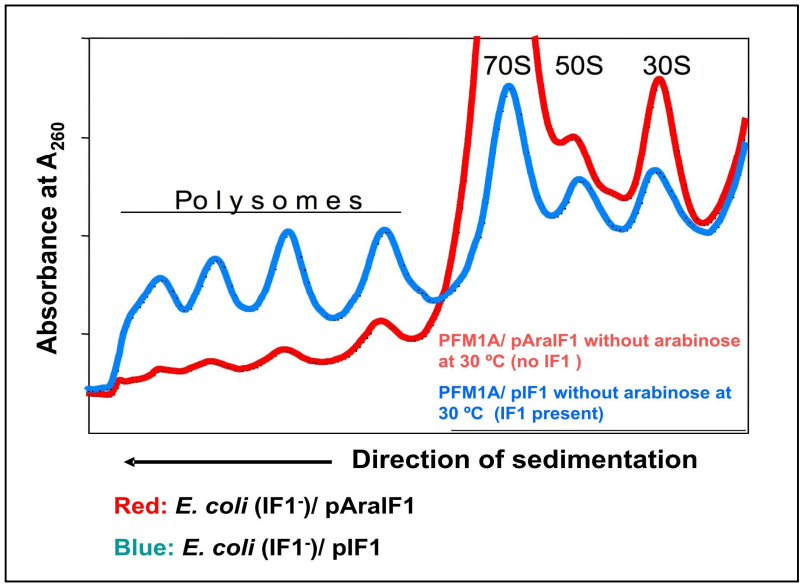


Figure 3.3.3-4: Polysome profile for* E. coli *(IF1⁻)/ pAraIF1 and* E. coli *(IF1⁻)/ pIF1 strain. *Polysomes were prepared from both the strains grown in LB glucose at 30 °C and were run on SW40 at 18,000 rpm for 18 hours at 4 °C to get the polysome pattern.*

The result is clear as seen in **Figure 3.3.3-4** that *E. coli* (IF1⁻)/ pIF1 strain had a distinct polysome profile in contrast to the *E. coli* (IF1⁻)/ pAraIF1 strain. The strong reduction of the polysome in the *E. coli* (IF1⁻)/ pAraIF1 strain indicates that the impaired formation of the 50S subunits strongly impacts their capability to form polysomes.

Since the above experiments are performed in glucose at 30 °C, the condition best chosen for the very tight repression of IF1 from pAraIF1 plasmid, a final growth curve was performed at the 37 °C in order to confirm that the previously observed growth difference, ribosome and polysome profile patterns obtained from both the strains, are not the function of temperature, but rather a property of IF1 deficiency. This experiment is done in growth reader machine with 200 µl volume. The procedure of the experiment is described in detail in Materials and Methods section page 59.

This result for *E. coli (*IF1⁻)/ pAraIF1 strain is represented by the curves shown in **Figure 3.3.3-5 A** and was in harmony with the earlier observed result, namely growth in arabinose medium is about 1.5 to 3 times faster than in glucose at 37 °C, similar to one seen in growth curve experiment performed at 30 °C and also there is not much difference between presence and absence of antibiotics.

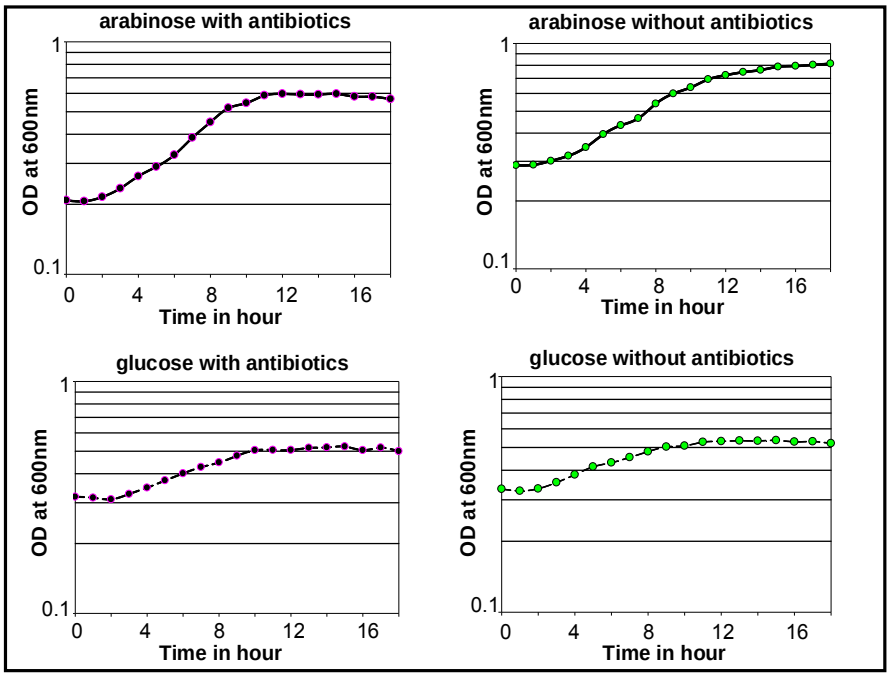


Figure 3.3.3-5 A: Confirmation of growth rate of* E. coli *(IF1⁻)/ pAraIF1 strain *at 37 °C in 0.2% glucose and 0.2% arabinose in growth reader instrument. OD was measured at 600nm and growth curves were plotted.*

Figure 3.3.3-5 B represents *E. coli* (IF1⁻)/ pIF1 strain which, shows no difference with either glucose or arabinose as expected. With this we could conclude that the effects observed in earlier section are not the function of temperature due to IF1 deficiency in bacterial cells.

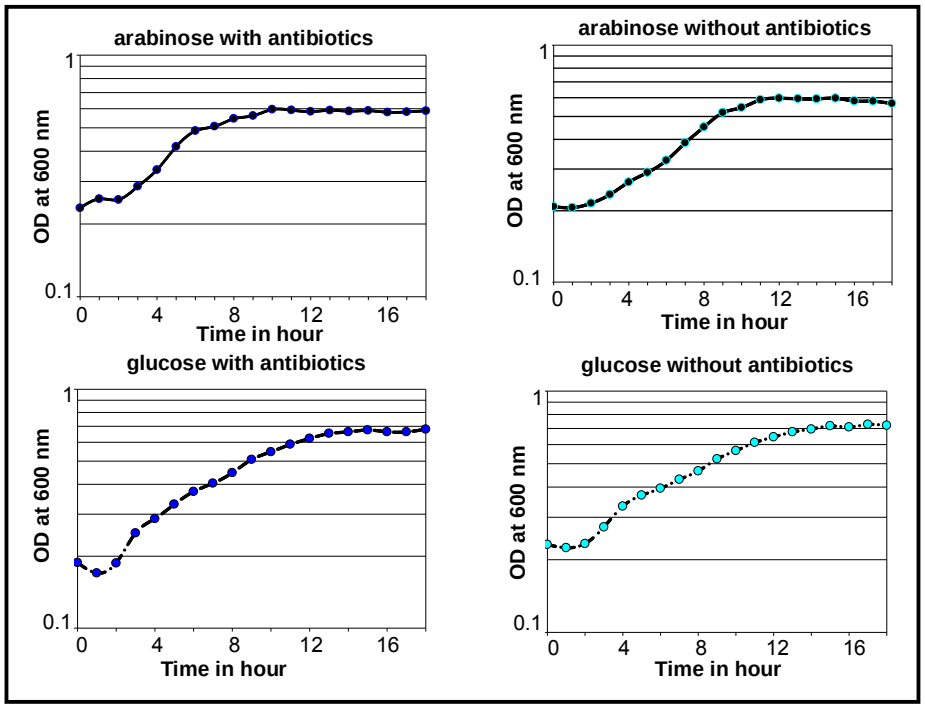


Figure 3.3.3-5 B: Confirmation of growth rate of* E. coli *(IF1⁻)/ pIF1 strain *at 37 °C in 0.2% glucose and 0.2% arabinose in growth reader instrument. OD was measured at 600nm and growth curves were plotted*

Next we wanted to employ *E. coli* (IF1⁻)/ pAraIF1 as template strain and study the expression GFP with different sets of 5' UTR region upstream of the start codon AUG of GFP.

3.3.4 Expression of dual luciferase in *E. coli* (IF1$^-$)/ pAraIF1 strain (IF1 switched on and off)

The rational of performing this experiment is to check the effect of IF1 on the expression of bicistronic mRNA carrying both the luciferase in *E.coli* (IF1-)/pAraIF1 strain. This bicistronic constructs as shown in Figure 3.3.4-1A harbours renilla luciferase (Rluc) as first cistron and firefly luciferase (Fluc) as second cistron mimicking the cistrons arrangement on polycistronic mRNA. In an experiment described in detail in Daniela Wittek's thesis, {Wittek, 2009} very interesting results were obtained with this bicistronic construct namely when oligo against first cistron was used namely Rluc, synthesis of Fluc goes down whereas the amount of Rluc remained unchanged. This observation clearly demonstrates that translation that starts on Rluc goes fairly well until it reaches the region where it is blocked by oligo. Here ribosomes are blocked and cannot continue to slide to the second cistron, therefore firefly activity drops down in comparison to renilla activity, evidencing that sliding happens within the cistrons of the reporter construct. After confirming that the translation of second cistron is dependent on the sliding 70S arriving from the first cistron, we were curious to know whether the translation initiation of Fluc by sliding 70S is dependent on IF1. In order to investigate this we expressed the dual luciferase reporter construct in our special *E.coli* (IF1-)/pAraIF1 strain and the mother MG1655 strain using the procedure described in detail in Materials and Methods section page 75-76.

The expression of Rluc and Fluc was measure for both the strains in presence of arabinose when IF1 expression of switched on and glucose when it is switched off. The result can be summarized as follows: The growth difference for *E.coli* (IF1-)/pAraIF1 in presence of glucose and arabinose as shown by pink and blue curves respectively in **Figure 3.3.4-1 B** is 1.5 times. For MG1655 there was not much difference in growth observed in the presence of either of the sugars.

When the expression pattern of luciferases was analyzed it was found that in MG1655 strain the amount of renilla and firefly luciferase are expressed in normal amounts in both glucose and arabinose as shown in **Figure 3.3.4-2 B**, but very strikingly the pattern varied in special *E.coli* (IF1-)/pAraIF1 strain.

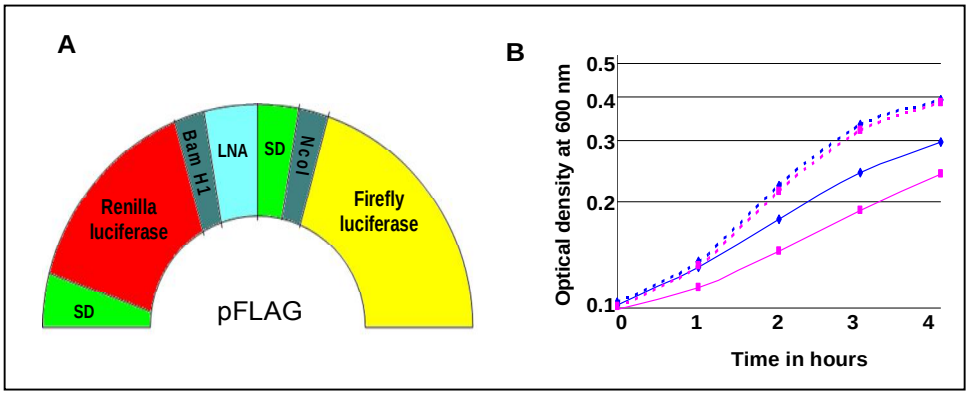


Figure 3.3.4-1: A: Scheme of Bi-cistronic dual luciferase construct; B: Growth curves for MG1655 and* E. coli *(IF1⁻)/ pAraIF1 having dual luciferase plasmid*: Cells were grown in LB glucose or LB arabinose at 30 °C. Growth was measured at 600nm and plotted automatically after every minute in growth reader machine (blue color represent sugar arabinose and pink color glucose, dotted lines represents MG1655 and solid lines* E. coli *(IF1⁻)/ pAraIF1strain)*

Here in presence of arabinose when IF1 expression is switched on, firefly and renilla luciferase are expressed in normal amounts but in the presence of glucose the expression of second cistron Fluc is effected to the highest extent, with more or less the amount of Rluc unchanged (**Figure 3.3.4-2 A**).

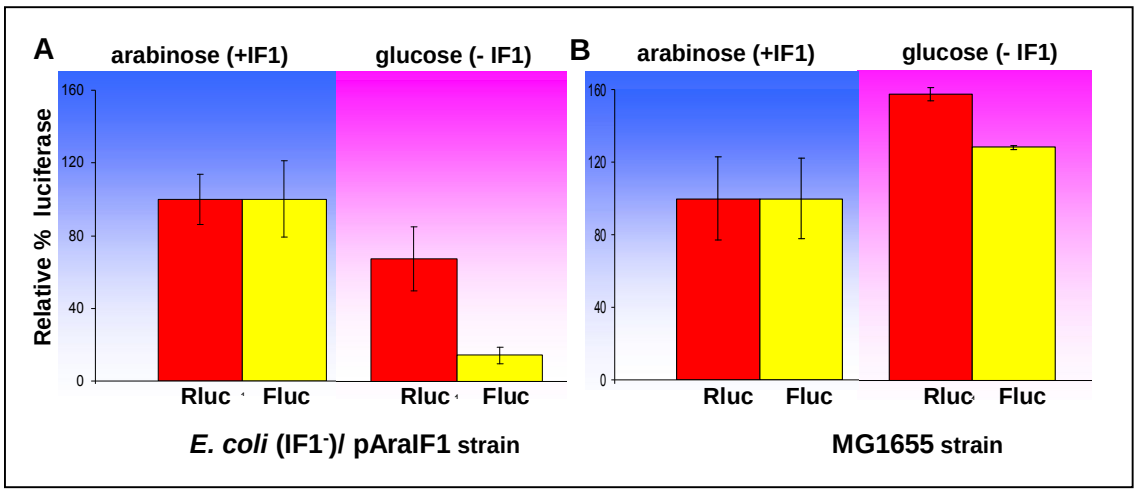
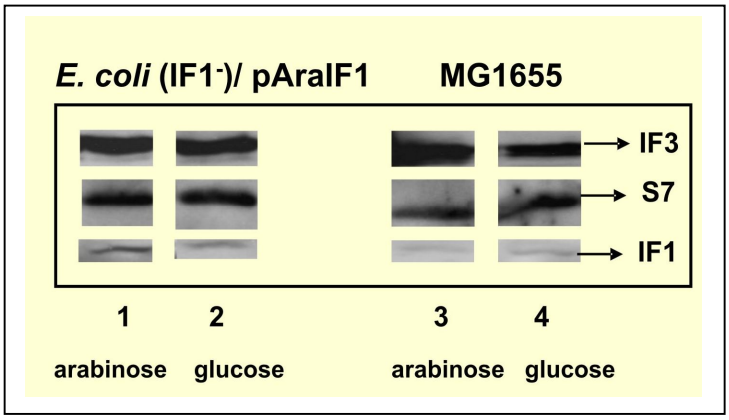


Figure 3.3.4-2*: **Amounts of renilla and firefly luciferase expressed in presence of LB glucose 0.2% and LB arabinose 0.2%. A****:* E. coli *(IF1⁻)/ pAraIF1 strain,* ***B****: MG1655 strain. Assay is performed as described in Methods section pages 75-76. The pelleted cells were lysed in 1X binding buffer with 10mg/ml lysozyme and three times freezing and thawing. Supernatant was separated by centrifugation at 10,000 rpm for 5 minutes at 4 °C and used for the measurement of luciferases for 10 seconds. Addition of first reagent I measures renilla luciferase and subsequently addition of second reagent measures firefly luciferase. The absolute values obtained in presence of arabinose for both the strains were taken as 100% and relative percentage value was calculated for both in presence of glucose and plotted in excel. Red color bar indicates renilla and yellow color firefly luciferase (same as shown in schemes), the background is either made as blue indicating presence of arabinose or pink for glucose (same color as used for growth curve plotting).*

Ratio of Fluc to Rluc was calculated in all cases and it was observed that in MG1655 strain both in presence of either glucose or arabinose the ratio is between 8-10, , which is different to the values obtained in *E.coli* (IF1-)/pAraIF1 strain. Here in case of arabinose the ratio is comparable to wild type strain namely it is approximately 8.5. but in glucose since Fluc goes down with Rluc being expressed stably, the ratio decreases to 1.8. This accounts for the relative decrease of the ratio to be a factor 4.5 in case of glucose as compared to arabinose or 22% if values in case of arabinose taken as 100%. This result is plotted and shown in **Figure 3.3.4-2 D**, where the values obtained in case of arabinose is considered 100% and relative values in glucose is then calculated. It is very interesting to notice that expression of second cistron *i.e.* Fluc is sensitive to reduced IF1 amounts with relative little effect on the expression of first cistron. This led us to infer that the sliding 70S definitely requires IF1 for the translation initiation and subsequently for the proper expression of second cistron our reporter construct.



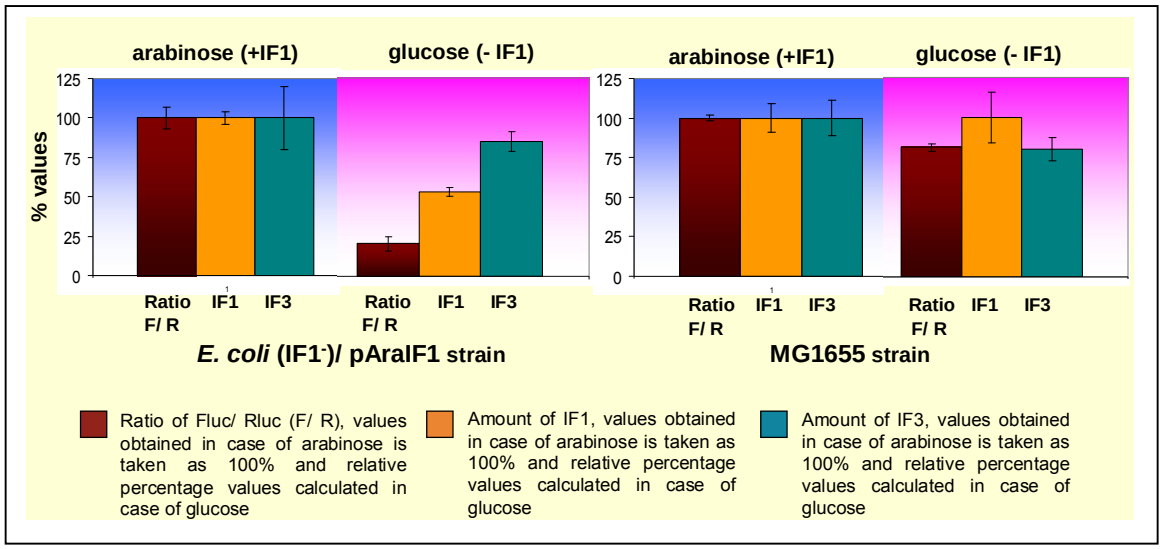


***Figure 3.3.4-2 C**: Western blotting to detect the amount of IF1 and IF3 with control S7 proteins using protein specific antibody in both the strain while expressing dual luciferase*

either in presence of arabinose or glucose. From this band quantification was made using image quantification software and the amounts of IF1 and IF3 was calculated with input amounts normalised. The values obtained for arabinose is taken as 100% and relative % value is calculated for glucose and plotted in excel. ***D****: Y axis indicates the percentage values. Ratio of Fluc/ Rluc and the amount of IF1 and IF3 in case if arabinose for both the strains is taken as 100% and relative percentage value in case of glucose is calculated and plotted in excel..*

Western blotting was additionally performed in order to quantify the reduction of IF1 amounts (**Figure 3.3.4-2 C**) when glucose is present and correlate it with the drop of the Fluc/ Rluc ratio. It was found that that amount of IF1 in *E.coli* ($IF1^-$)/pAraIF1 is about 50% in presence of glucose as compared to arabinose as shown in **Figure 3.3.4-2 D** and this reduced amount justifies the decreases in ratio of the luciferase measured in the assay.

3.3.5 Expression of GFP having different sets of upstream sequences in *E. coli* ($IF1^-$)/ pAraIF1 strain (IF1 switched on and off)

The constructs used in this experiment are as following:

A. GFP cyc3 with 5'UTR having secondary structure of -21.5 kcal/mol, upstream of Shine-Dalgarno sequence and initiator codon AUG.
B. GFP cyc3 with 5'UTR having no secondary structure upstream of Shine-Dalgarno sequence.

The logic of using these constructs are as follows, GFP with secondary structure of -21.5 kcal/mol can only be initiated by 30S binding type of initiation, which can locate internal Shine-Dalgarno sequence and start codon of GFP. In this case, 70S when bound at the 5' end will start sliding but cannot melt such a stable secondary structure, therefore in this case the expression of GFP will be exclusively due to 30S binding type of initiation. Whereas in case of GFP without secondary structure, 70S can slide after binding to the 5' end of an mRNA, can locate the internal Shine-Dalgarno sequence and the start codon. Also in this case 30S can initiate and therefore GFP expression here involves both 30S binding type and 70S scanning type of initiation. As a result, we assume that the cumulative effect of both the types of initiation would lead to increase amount of GFP synthesized in this case than the former one.

Expressions of these GFP's are checked in the condition of switching on and off IF1 in *E. coli* ($IF1^-$)/ pAraIF1 strain and therefore this assay will help us to study how different amounts of IF1 effects the expression of GFP. In turn it would let us investigate the effect of IF1 on 30S binding type of initiation (GFP with secondary

structure) and 30S binding type + 70S scanning type of initiation (GFP without secondary structure). The cloning procedure for generating these constructs is described in Methods and Materials section pages 55-57.

In **Figure 3.3.5-1**, both the GFP-mRNA's are shown with their distinct 5' UTR region (with secondary structure and without secondary structure), start is represented by green color and stop by red color.

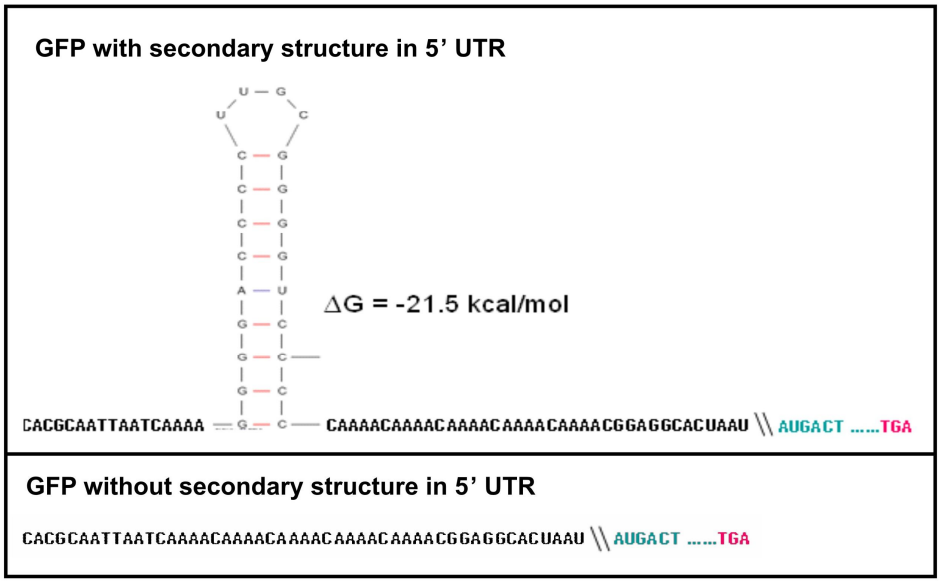


Figure 3.3.5-1: Scheme showing 5' UTR region *present upstream of AUG of different GFP-mRNA's along with the stop codon*

After transformation of these plasmids into the *E. coli* (IF1$^-$)/ pAraIF1 strain, numerous colonies were picked up and PCR was performed using gene specific primer to confirm the presence of the plasmids. As shown in **Figure 3.3.5-2** many colonies showed no amplification suggesting that there is no GFP plasmid inside the strain. The ones highlighted in a box harboured plasmid and therefore were picked up and used for the final experiment.

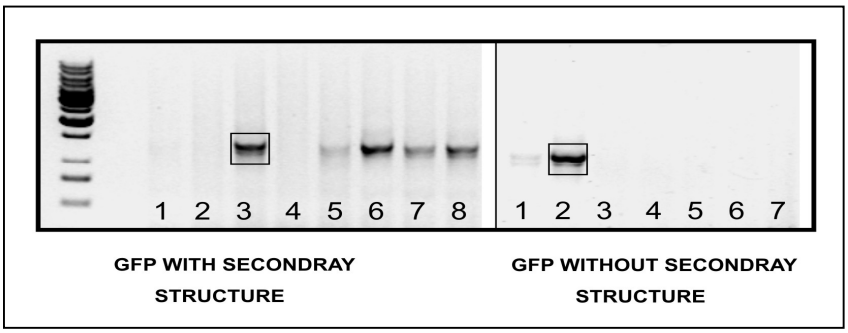


Figure 3.3.5-2: Confirmation of the presence GFP-plasmid in* E. coli *(IF1$^-$)/ pAraIF1 strain by PCR*. After transformation PCR was performed using pACY177 tac GFP forward primer and pACY177 tac GFP reverse primer. Anealing temperature used is 53 °C, to prevent unspecific bands and hence avoid false positive colonies for the experiment. Only true clones shown in blocked area were selected for the expression study in the presence of arabinose and glucose.*

Figure 3.3.5-3 shows the scheme for performing the experiment. Growth curves were plotted for both the constructs in *E. coli* (IF1$^-$)/ pAraIF1 strain as shown in **Figure 3.3.5-4, panel I,** where blue curve represents growth under arabinose and pink curve growth under glucose. We observe that in both the case, growth is enhanced 1.5 times in presence of arabinose as compared to glucose which is the property of *E. coli (*IF1$^-$)/ pAraIF1 strain.

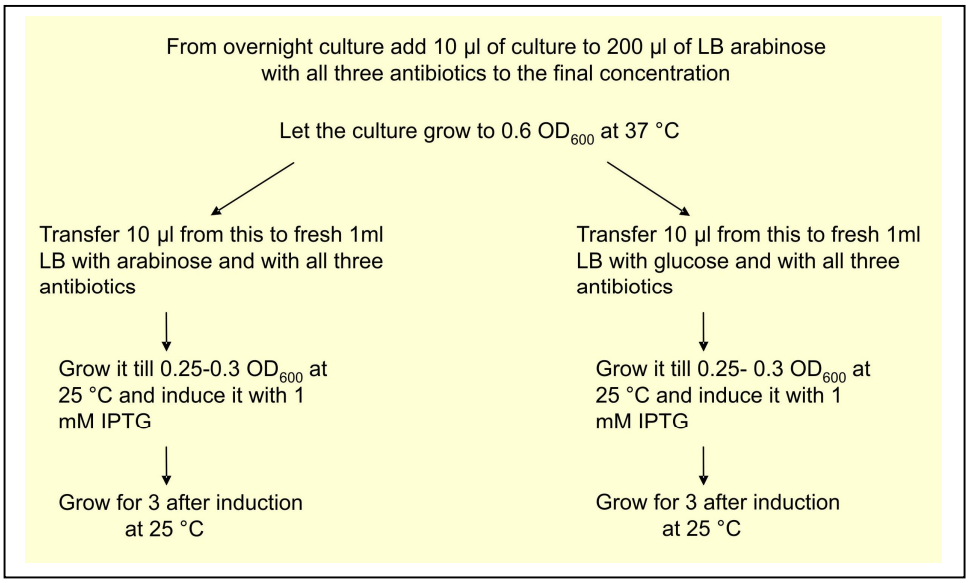
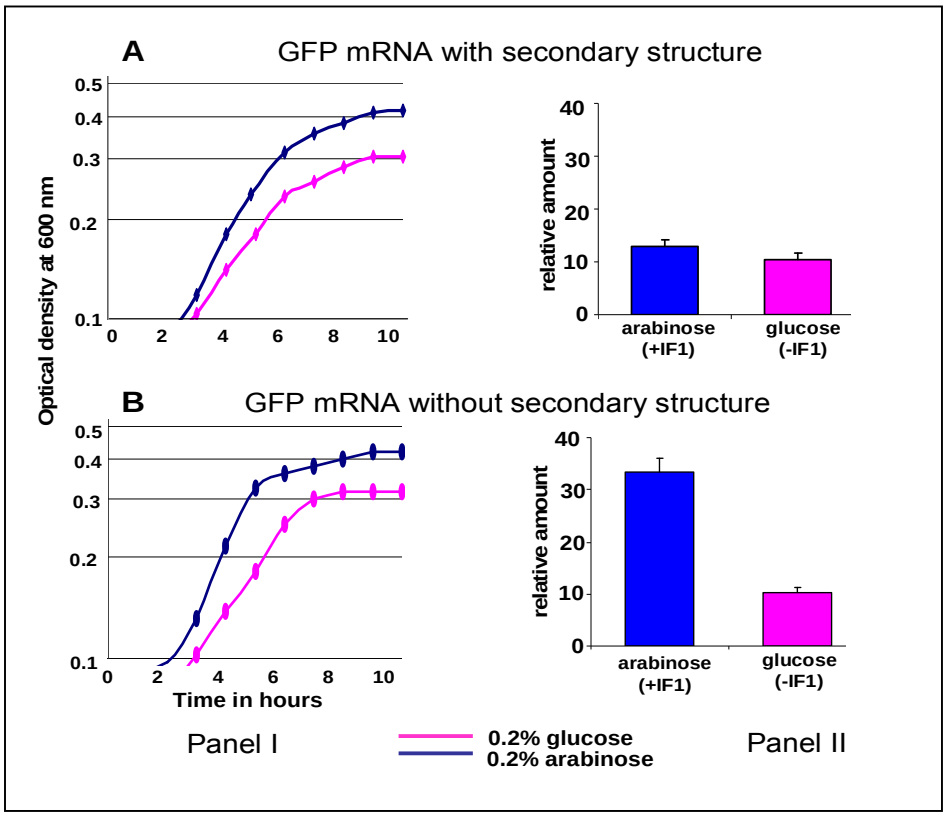


Figure 3.3.5-3: General scheme for performing experiment in Growth reader instrument*: pACY177 GFP with and without secondary structure were expressed in* E. coli *(IF1$^-$)/ pAraIF1 strain in the presence of glucose and arabinose.*

GFP expression was checked for the same sets of cells for which growth curve was plotted in presence of glucose and arabinose. Induction was done with 1 mM IPTG when the OD_{600} of the cells reached to 0.25- 0.3 and over-expression was measured after 3 hours. All the samples were processed as described in legend and the result is plotted, shown by bar diagram in panel II.



A: GFP with secondary structure; B: GFP without secondary structure

Figure 3.3.5-4 I: Growth curves for* E. coli *(IF1⁻)/ pAraIF1 having different plasmid constructs*: Cells were grown in LB glucose or LB arabinose at 25 °C. Growth was measured at 600nm and plotted automatically after every minute in growth reader machine.*
II. Amounts of GFP expressed in presence of glucose and arabinose*. Same cells were denatured in SDS loading dye, heated at 85 °C for 5 minutes and were run on 15% SDS gels. The gels were coomassie stained and the bands density was measured and normalized with control protein band. Readings were plotted in excel with un-induced amount subtracted. Relative amounts indicated on Y axis represents the pixels values*1000.*

As seen in **Figure 3.3.5-4 II**, after 3 hours of IPTG induction, GFP with secondary structure shows little increase in arabinose as compared to glucose Strikingly, GFP with no secondary structure has the maximum increase in GFP expression in presence of arabinose. Apart from having enormous difference in presence of arabinose *i.e.* IF1 present in normal amount and glucose IF1 present in minimal amount, there is also great difference in absolute amount of total GFP protein synthesized in the two constructs.

As explained earlier, GFP with no secondary structure ideally serves to promote both 30S binding type and 70S scanning type of initiation. An mRNA with strong secondary structure at the 5'-end can have only 30S binding type of initiation. This initiation type does obviously not depend on the IF1, since in the presence of arabinose (IF1 expressed) and glucose (IF1 not expressed) the same expression is observed. In

striking contrast, without secondary structure at the 5' end the expression is dramatically dependent on the IF1 expression (about 3.5 times increase with IF1). Here the 30S binding and the 70S scanning mode should be active suggesting that the strong increase is due to the additional 70S scanning type obviously working just from the 5'-end on.

After performing series of *in vivo* experiments, which indicated the involvement of IF1 in 70S scanning type of initiation, we went ahead to use *in vitro* system executing other assays testing our hypothesis. In the next sections a detailed *in vitro* analysis is described.

3.3.6 Study of the GFP constructs in an *in vitro* RTS assay system

RTS system: Reaction principle

This system is provided by Roche and incorporates components which, can perform both transcription and translation simultaneously. The transcript made by T7 RNA polymerase is translated efficiently by ribosomes present in the *E. coli* lysate. The lysate is prepared from the desired strain by the method of Zubay (De Vries, Hoge et al. 1980) and provides an optimized environment, which can enhance the expression of translated product of a test gene (Kim, Chang et al. 2000; Kim, Chang et al. 2001). It is also ensured that the strain selected for this purpose has low exonuclease activity.

To use this system the gene of interest should be under the T7 promoter, and also should harbor ribosomal binding site along with T7 terminator after the stop codon of the gene. Proteins of molecular weight from 10 to 120 kDa can be expressed, and harvested after 1-6 hours of reaction. The limitation of the system is that it cannot introduce post translational modifications such as glycosylation, phosphorylation, disulphide bond formation and signal sequence cleavage.

The optimal temperature for protein synthesis is 30 ºC and requires a reaction time of up to 6 hours. Lower temperature could be opted, if test protein tends to aggregate.

For this *in vitro* assay, we have GFP with different sets of upstream sequences before its start codon AUG. The first construct is pET-GFPcyc3**,** it has the gene under normal T7 promoter followed by Shine-Dalgarno sequence as shown in **Figure 3.3.6-1**. GFP coding region starts with methionine and the protein made has His tag at the C-terminus. In this construct initiation can be done by directly binding of 30S to SD and AUG, which is immediately following T7 promoter and hence is a representative

of 30S binding type of translation initiation. It could also be initiated by scanning 70S from 5' end.

The second construct is called pBER-GFPcyc3. This construct basically employs the concept of 70S scanning type of initiation, where we assume that the following cistrons are being initiated not by canonical 30S but rather by scanning 70S. When the cistrons have overlapping stop and start codons, then 70S ribosome does not tend to fall off but instead it performs mono-dimensional diffusion along the mRNA to the second cistron and start translation event.

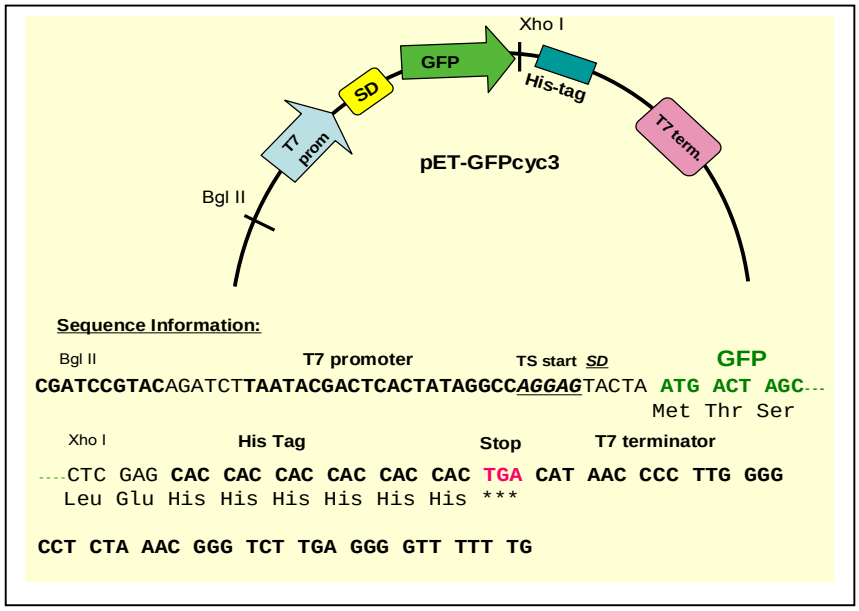


Figure 3.3.6-1: Canonical pET-GFPcyc3 plasmid map and nucleotide sequence

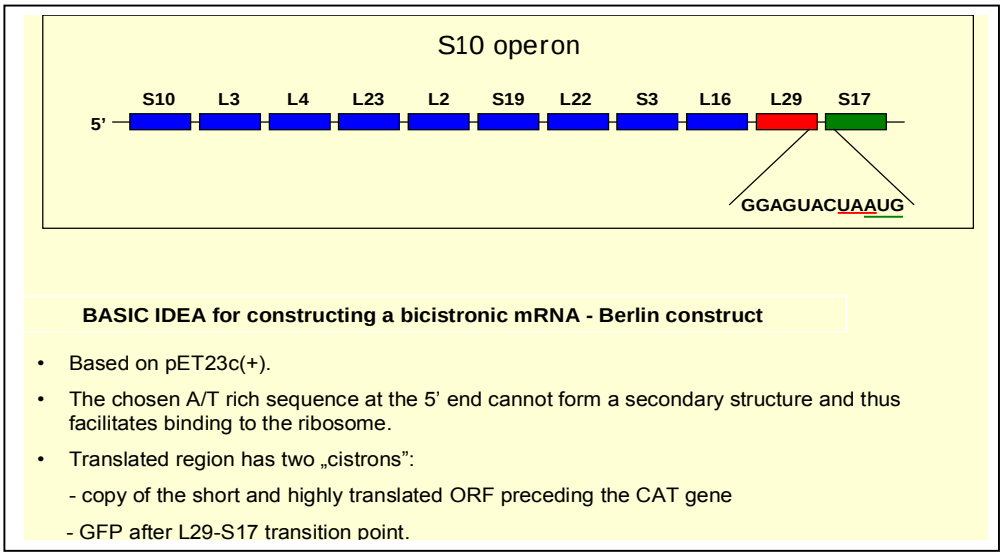


Figure 3.3.6-2: Scheme showing ribosomal proteins encoded in S10 operon *with the basic idea enlisting all the parameters that pBER-GFPcyc3 construct should have.*

In line with overlapping stop and start codon, this construct mimics the transition between the L29 to the S17 cistron of the S10 operon shown in **Figure 3.3.6-2.**

With these details kept in mind pBER-GFPcyc3 was made which ideally has

1. Here the first cistron reads a small CAT leader peptide which is then followed by second cistron GFP in -1 frame, the same way seen in L29-S17 transition case.
2. The gene is under T7 promoter followed by a long stretch of AU rich sequence which enables the RNA to have less stable secondary structure which could be melted easily by ribosome to start the initiation process. This 'AU' rich stretch is named as Berlin sequence**.**

All these features of pBER-GFPcyc3 as described above are shown in **Figure 3.3.6-3**

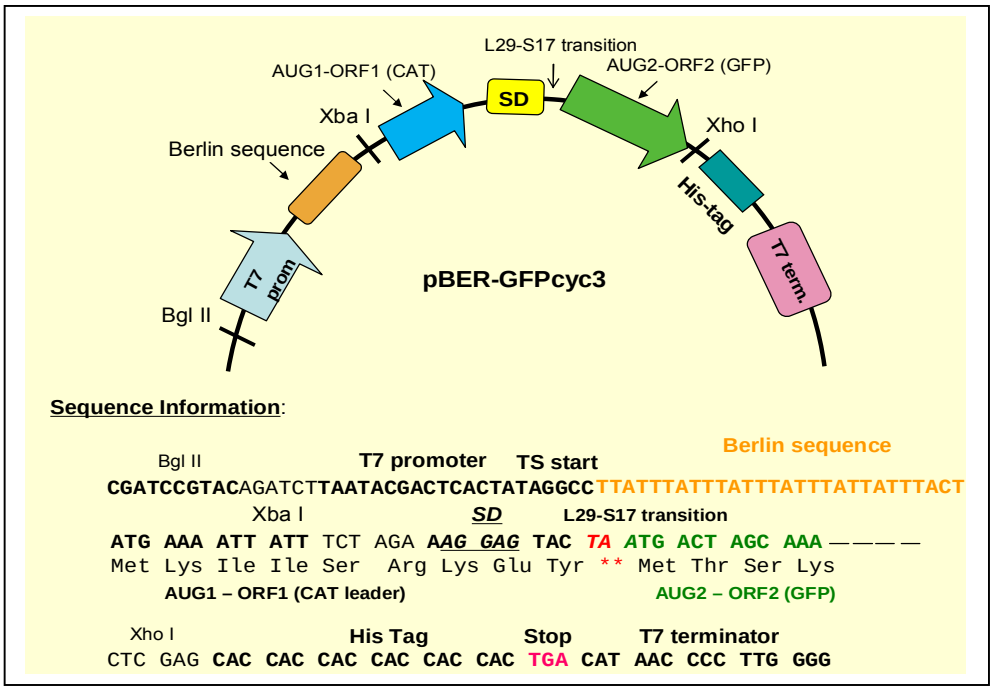


Figure 3.3.6-3: pBER-GFPcyc3 map and nucleotide sequence

In order to the test the expression of GFP's whether it is enhanced by IF1 or not, the purified protein was added in the RTS assay although the *E. coli* lysate contain already some amount of IF1.

In the first experiment purified IF1 in the ratio of 1.5 times over ribosome was added to the RTS reaction. Under this condition the increase of the expression of pBER-GFPcyc3 was observed to be higher as compared to pET-GFPcyc3 and pIVEX-GFPcyc3.

As seen in native gel lane 4 in **Figure 3.3.6-4**, the expression is increased to about 20% as compare to the reaction where it is not added lane 3 in the same figure. The native gel also shows that there is no change observed in the case of pIVEX-GFPcyc3 and pET-GFPcyc3 expression represented by lane 1, 2 and lane 5, 6 respectively.

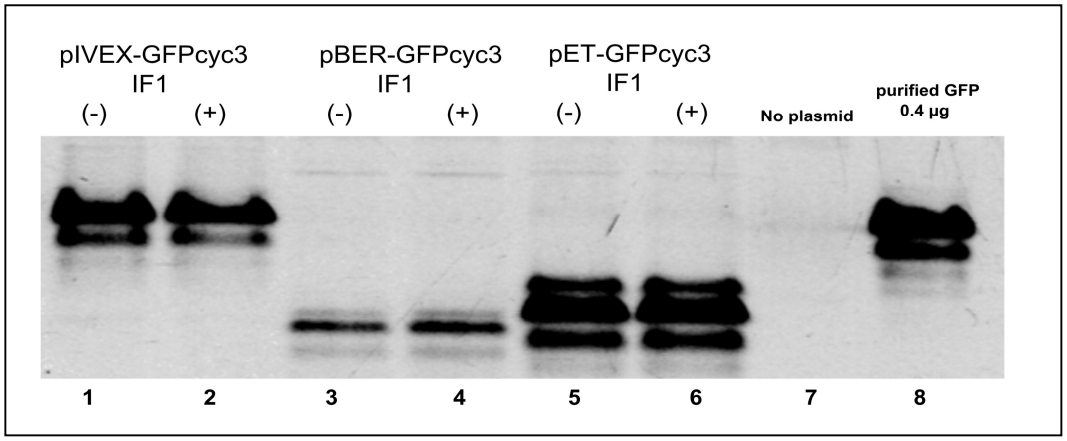


Figure 3.3.6-4: RTS performed with GFP constructs with or without additions of IF1*. RTS was performed with pET-GFPcyc3, pBER-GFPcyc3, and pIVEX-GFPcyc3 with and without IF1 with the standard pipetting scheme. GFP was then loaded on 15% native gel along with control purified protein.*

After identifying specific increase in pBER-GFPcyc3 expression due to addition of IF1 at 1.5 times excess over ribosome in the previous assay, an IF1 titration row was performed in order to find the optimum concentration at which the expression is maximal. In a broad range of chosen concentrations the expression was highest, when IF1 is added in a 7.5 times excess over ribosome as shown in lane 5 of **Figure 3.3.6-5 A.**

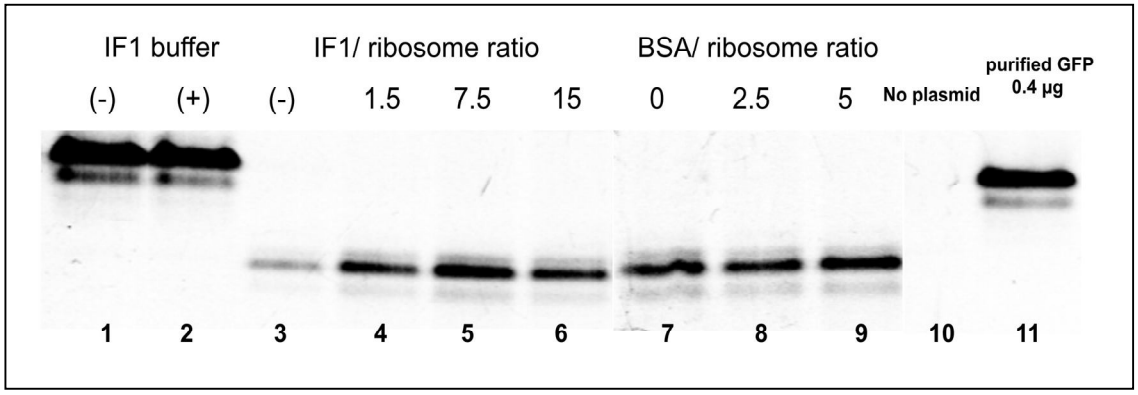


Figure 3.3.6-5 A: Test of IF1 on pBER-GFPcyc3 expression. *A: Reaction having pET-BER-GFPcyc3 with increased amount of IF1 1.5, 7.5 and 15 times excess over ribosomes, along with control BSA protein. GFP was then loaded on 15% native gel along with control purified protein.*

In order to be assured that the enhanced expression of pBER-GFPcyc3, is a specific property of the addition of IF1, BSA was used as control protein in the experiment. Lane 7, 8 and 9 where the increasing concentration of BSA (0, 2.5 and 5 times excess over ribosome) is used, expression of pBER-GFPcyc3 is not affected. This is also shown as curve plotted by quantitating the GFP band intensity and comparing it with known amount of purified GFP loaded as control in the same gel as shown in **Figure 3.3.6-5 B**.

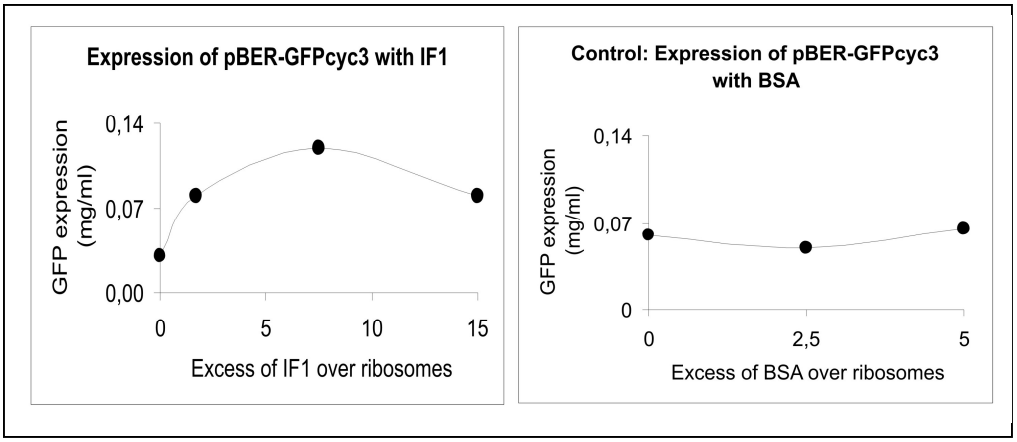


Figure 3.3.6-5 B: Increase of the pBER-GFPcyc3 expression *in the above experiment was measured* via *quantitating the band by image quantification software, comparing it with intensity of the known amount of purified GFP and then using excel to plot the amounts.*

In order to show that IF1 exclusively enhances pBER-GFPcyc3 expression and not the standard pET-GFPcyc3, identical assays were performed with pET-GFPcyc3 with the same molar excess of IF1 that was used for the previous pBER-GFPcyc3 experiment. The results show that there is no change observed in this case, as seen in **Figure 3.3.6-6 A** lane 2 to 5 with increasing concentration of IF1 over ribosome used. In **Figure 3.3.6-6 B**, the data is plotted as curve.

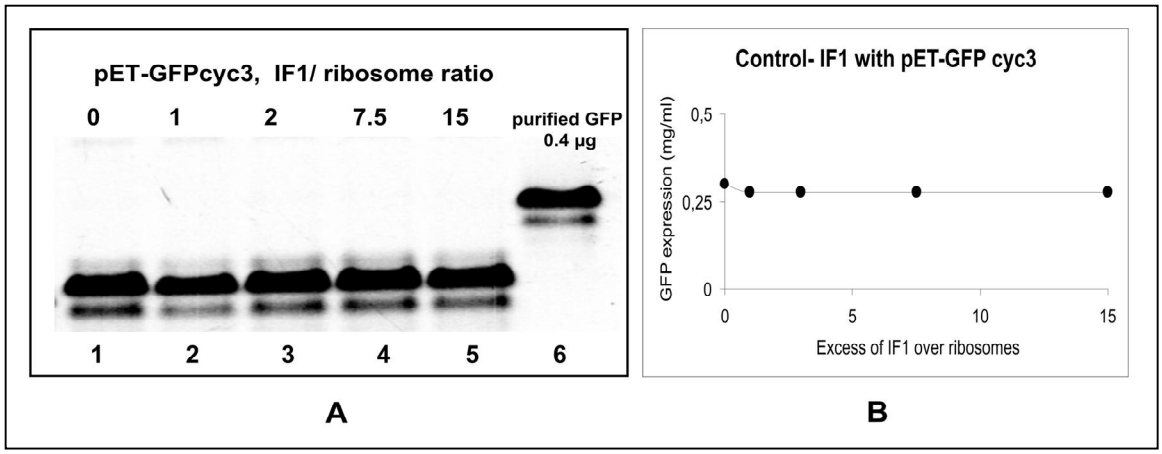


Figure 3.3.6-6: Test of IF1 on pET-GFPcyc3 expression *A: Reactions expressing pET-GFPcyc3 with increasing amounts of IF1 (1, 3, 7.5 and 15 times excess over ribosomes). B: increase was plotted as curve by quantitating the GFP band intensity by image quantification software and comparing it with intensity of the known amount of purified GFP loaded in the same gel.*

These results illustrate the requirement of IF1 for the expression pBER-GFPcyc3 but not pET-GFPcyc3. Canonical pET-GFPcyc3 employs 30S binding type of initiation whereas pBER-GFPcyc3 is initiated by scanning 70S that is present on stop codon of first cistron. It is the difference of these two modes of initiation that distinguishes the critical need of IF1 in the process on initiation which is shown in this *in vitro* assay.

3.3.7 Study of the GFP constructs in *in vivo* system

In order to perform this experiment we used canonical pET-GFPcyc3 and pBER-GFPcyc3 construct, their properties have already been described before in section 3.3.5. Besides these two, we additionally use one more construct called pBER-int-GFPcyc3. It endows all the property of pBER-GFPcyc3 except that the stop codon of the CAT leader peptide is separated by 39 nucleotides from the start codon AUG of GFP as shown in **Figure 3.3.7-1.** If here also 70S scanning would take place, the 70S have to move over 39 nucleotides.

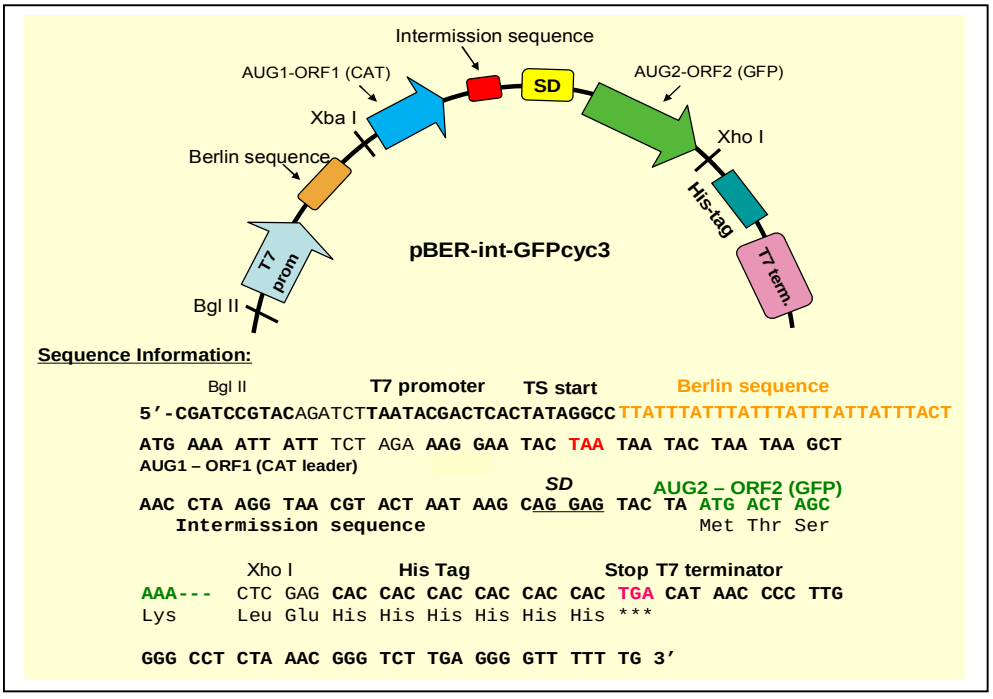


Figure 3.3.7-1: pBER-int-GFPcyc3 map and nucleotide sequence

All the three constructs were tested *in vivo*, namely their expression was checked in BL21 (DE3) strain induced by 1 mM IPTG. The three pBER-GFPcyc3 clones 3, 7, 8 shown in lanes 2, 3 and 4 in **Figure 3.3.7-2 A** were tested for the expression. Clone 7 showed highest expression and hence was selected for next experiment. In next experiment, where the expression of all the GFP's constructs were checked, it was found that pBER-GFPcyc3 showed the highest expression as compared to pET-GFPcyc3 or pBER-int-GFPcyc3 (**Figure 3.3.7-2 B,** lane 5)**.**

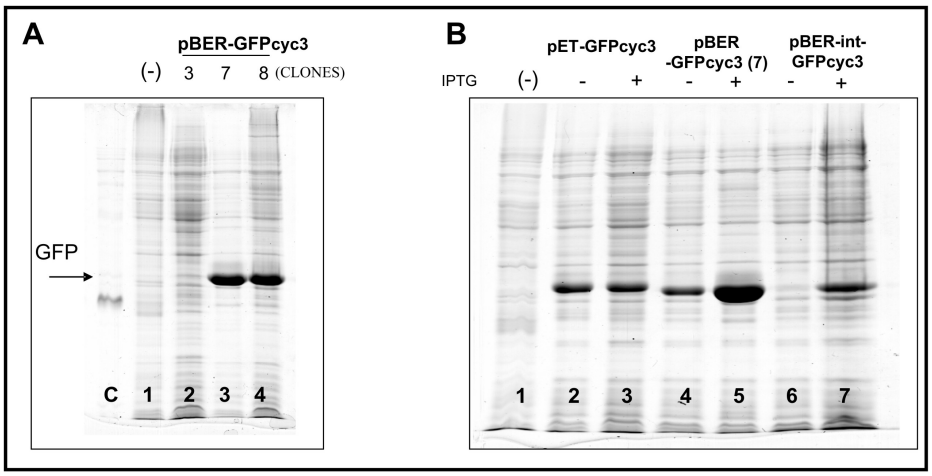


Figure 3.3.7-2:* In vivo *expression of GFP from pET-GFPcyc3, pBER-GFPcyc3 and pBER-int-GFPcyc3 in BL21 (DE3). *All the three constructs were transformed in BL21 (DE3) cells. Over-expression was performed by inducing the cells at 0.4-0.5 OD_{600} by 1 mM IPTG at 37 ºC.*

Expression was also performed at different temperatures (25 °C, 30 °C and 37 °C), and it was observed that pBER-GFPcyc3 exhibit higher level of expression compared to canonical pET-GFPcyc3 at 37 °C after IPTG induction as shown in lane 1 of **Figure 3.3.7-3 A and B.**

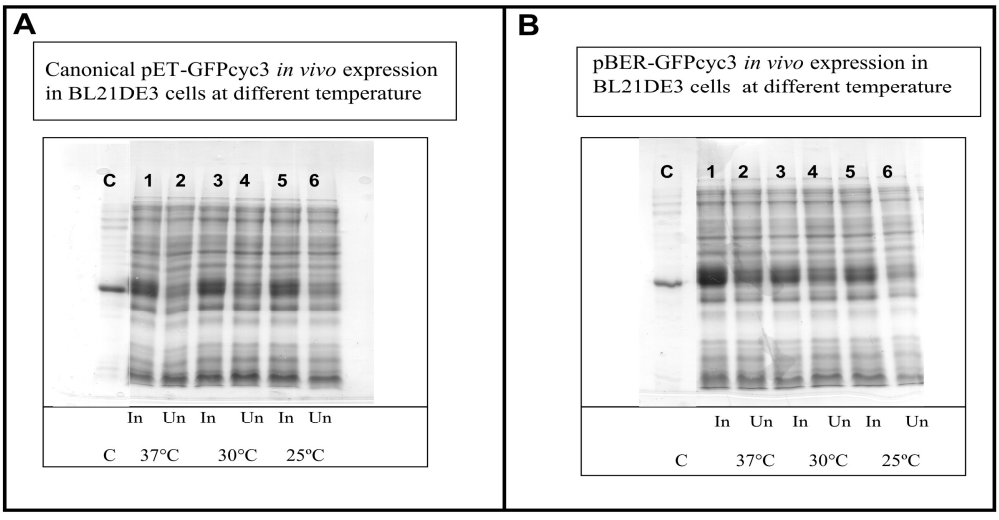


Figure 3.3.7-3: Expression of pET-GFPcyc3 and pBER-GFPcyc3* in vivo *in BL21(DE3) cells at different temperature. *Different constructs were transformed in BL21 (DE3). Over expression was performed by inducing the cells at 0.4-0.5 OD_{600} by 1 mM IPTG at 37 ºC, 30 ºC and 25 ºC. A: pET-GFPcyc3; B: pBER-GFPcyc3. (Un: uninduced; In: induced)*

3.3.8 Test of the expression of modified pET23c-BER-GFP constructs *in vivo*: Concept for designing modified pET23c-BER-GFP (in-frame and out-frame mutations)

As explained before, in case of pBER-GFPcyc3 scanning 70S might perform translation initiation. This principle of 70S scanning type of initiation might be true for most of the ribosomal protein because most of the cistrons in polycistrons are overlapping. In fact, it was suggested that 70S ribosome can scan up to over a distance of at least 40 nucleotides without leaving the mRNA (Adhin and van Duin 1990) and when correct start codon is encountered they are preferably used for the translation initiation.

In order to find out whether the expression of second cistron *i.e.* GFP is dependent on the expression of first cistron *i.e.* CAT leader peptide, the initiation codon AUG of the first ORF is changed to TTG which, is not an initiation codon (**Figure 3.3.8-1, panel A).**

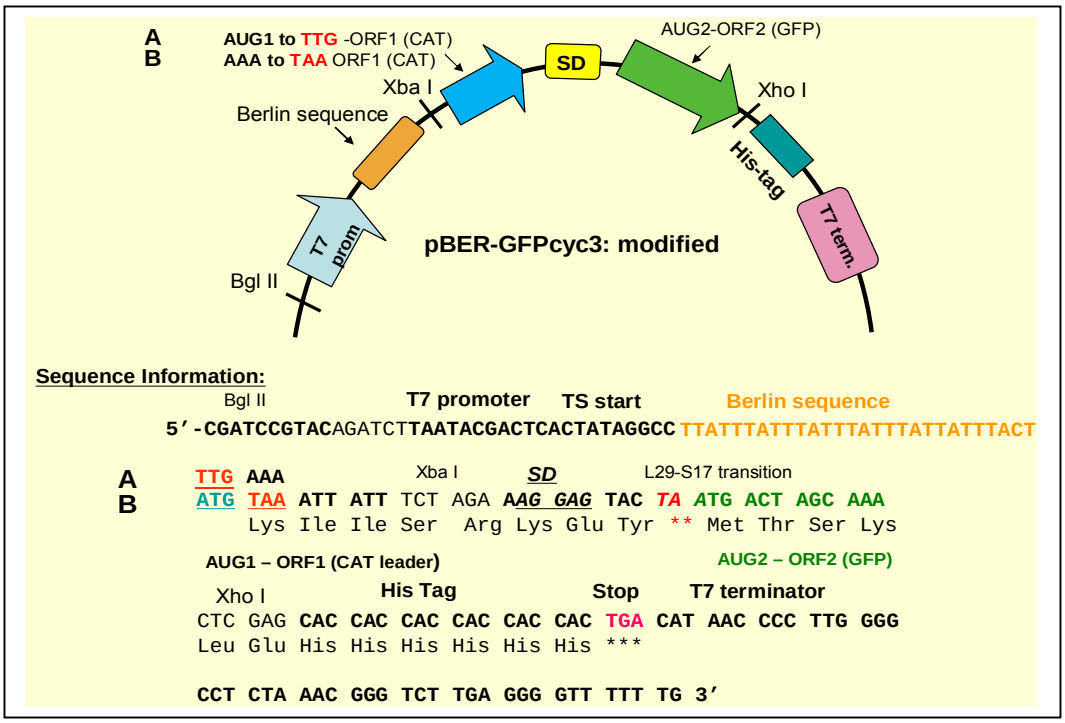


Figure 3.3.8-1: Bi-cistronic mRNA; Modified pBER-GFPcyc3 *plasmid map and nucleotide sequence: A; AUG of the first ORF mutated to TTG, B; Second codon of first ORF- Lysine mutated to TAA (Stop codon)*

In this case the first cistron will be not expressed as there is no start codon and we can assess, whether the expression of GFP (second cistron) is dependent on the translation of the first one. All other properties of this construct are identical to pBER-GFPcyc3.

In a second construct the second codon lysine of the CAT leader peptide is mutated to stop codon TAA as shown **Figure 3.3.8-1, panel B** and also shown in nucleotide sequence. This would result in no expression of first cistron and here also with GFP expression analysis we can find out if the expression of second cistron is dependent on the first one.

The third construct is made as control, where a stop codon is inserted out of frame as shown in **Figure 3.3.8-2**.

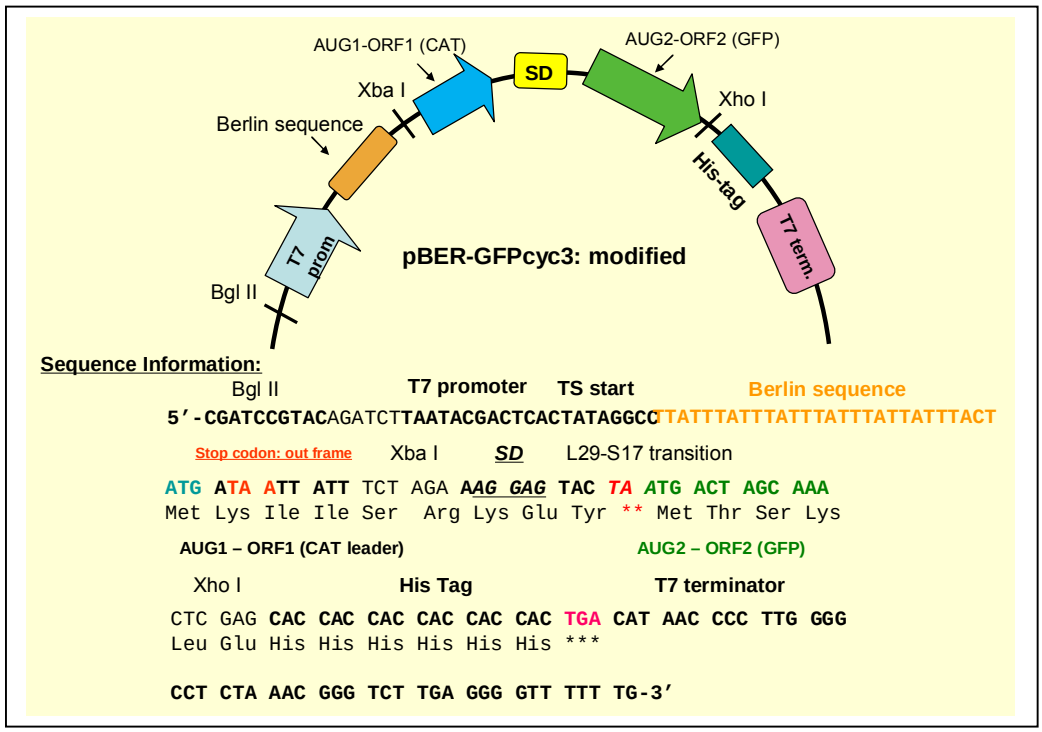


Figure 3.3.8-2: Bi-cistronic mRNA; Modified pBER-GFPcyc3 *plasmid map and nucleotide sequence: Out of frame stop codon generated by mutation as control*

These constructs were tested for the over-expression in BL21 (DE3) cells by 1 mM IPTG. In both the cases, where the change in first cistron blocks its synthesis, the expression of the GFP (second cistron) shown as bar 3 and bar 4 of **Figure 3.3.8-3 A** and **B** is unaffected.

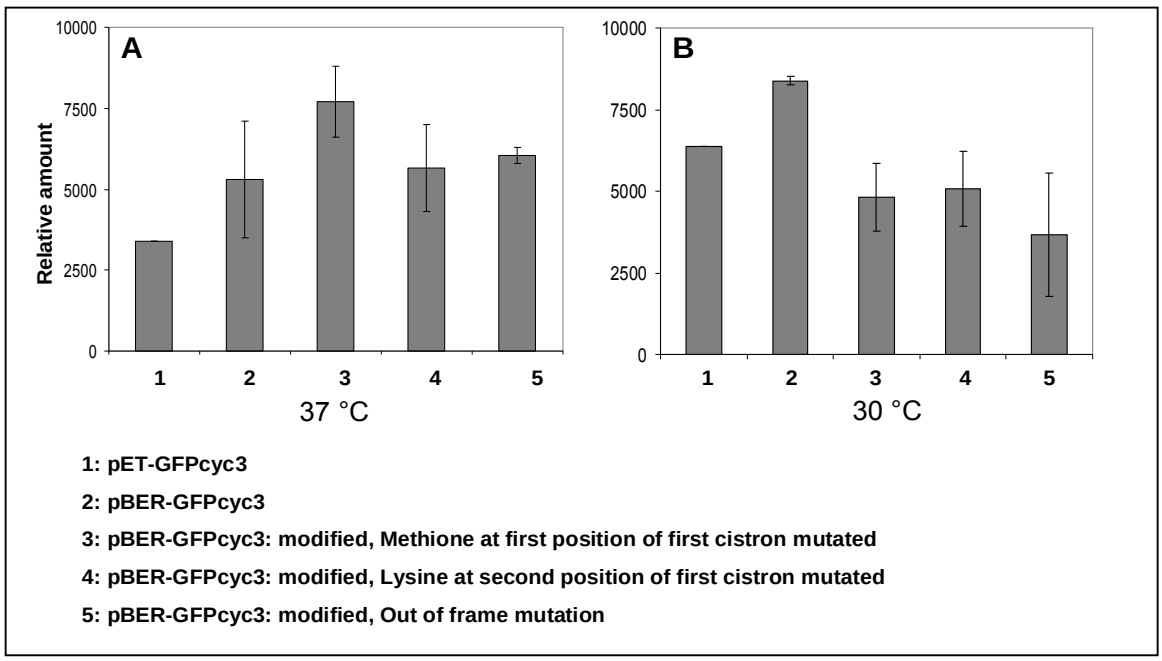


Figure 3.3.8-3:* In vivo *over-expression of modified pBER-GFPcyc3 in (BL21DE3) by IPTG. *Different constructs were transformed in BL21 (DE3). Over expression was performed by inducing the cells at 0.4-0.5 OD_{600} by 1mM IPTG both at 37 ºC (fig. A) and 30 ºC. (fig. B). Relative amount represents pixel value of the quantified band in each case.*

Expression was performed at 37 °C and 30 °C, and the result showed that the expression of GFP which is present as second cistron is not effected by the expression of the preceding CAT leader peptide, and hence unlinked.

However, as shown previously the expression of GFP could still be supported by 70S scanning mode starting at the 5'-end of the mRNA

3.3.9 Expression of pBER-GFPcyc3 in *E. coli* (IF1$^-$)/ pAraIF1 strain

In this assay we expressed pBER-GFPcyc3 in *E. coli* (IF1$^-$)/ pAraIF1 strain, where IF1 amount is altered using either arabinose or glucose. Since in earlier experiments we obtained evidence that IF1 is crucial for 70S scanning type of initiation, we now can test to which extent the expression of GFP in this construct is effected, when IF1 is switched on and off.

For this experiment the construct should have tac promoter and compatible origin of replication as explained before in section 3.3.3 and therefore a new cloning was performed described in detail in Materials and Methods section pages 55-57.

Figure 3.3.9-1 A shows the 5'UTR region of Berlin GFP constructs which is same as our standard pBER-GFPcyc3 as described before. The presence of this plasmid after transformation in *E. coli* (IF1$^-$)/ pAraIF1 strain was confirmed by PCR and only the

bacterial colony that showed the presence of GFP plasmid as marked in blocked area in **Figure 3.3.9-1 B** was selected for further experiment. Experiment procedure is same as described in scheme shown in **Figure 3.3.5-3**.

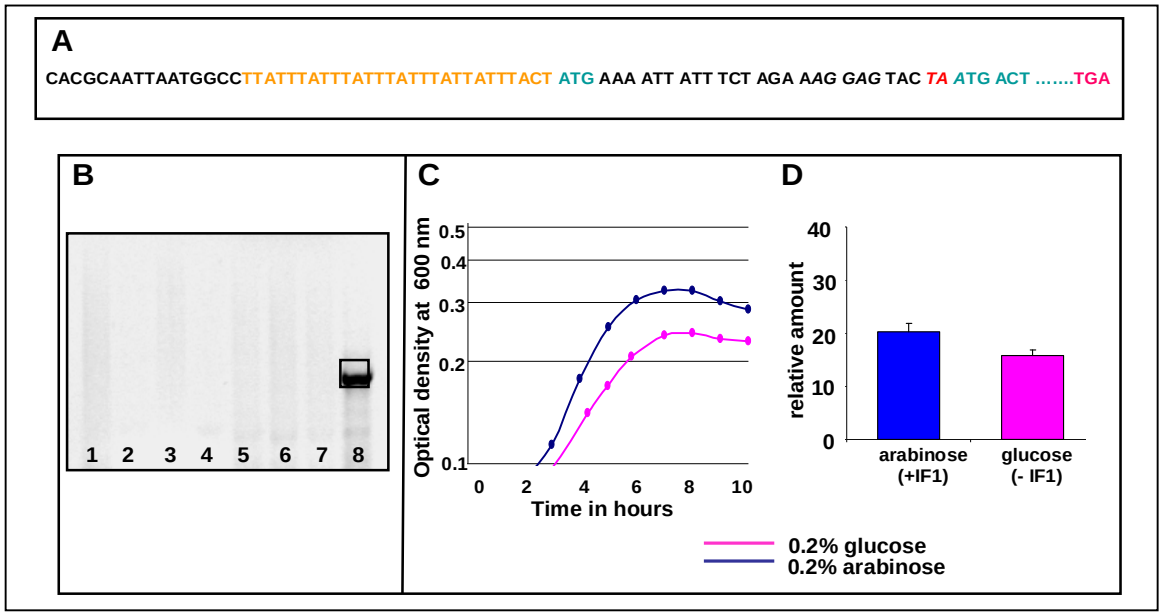


Figure 3.3.9-1: Expression of pBER-GFPcyc3 in* E. coli *(IF1⁻)/ pAraIF1 strain *A: Scheme showing 5' UTR region present in this construct, B: Many colonies were tested by PCR to confirm the presence of GFP plasmid in* E. coli *(IF1⁻)/ pAraIF1 strain, C: Growth curves for* E. coli *(IF1⁻)/ pAraIF1 strain having the GFP. Blue curve represents growth in 0.2% arabinose and pink represents growth in 0.2% glucose, D: Amounts of GFP expressed in the presence of glucose and arabinose. Same cells were denatured in SDS loading dye, heated at 85 °C for 5 minutes and were run on 15% SDS gels. The gels were coomassie stained and the bands density was measured and normalized with control protein band. Readings were plotted in excel with un-induced amount subtracted. Relative amounts indicated on Y axis represents the pixels values*1000.*

Growth curves under arabinose or glucose regime are shown in **Figure 3.3.9-1 C**. Growth is 1.5 times faster in arabinose represented by blue curve as compared to glucose represented by pink curve. When looked into the expression pattern it was observed that Berlin GFP expression is moderately increased in presence of arabinose as compared to glucose as shown **Figure 3.3.9-1 D**. This would lead to the conclusion with that Berlin GFP could be initiated equally by both 30S and 70S and therefore we see very trivial effect of the over-expression of IF1 in the presence of arabinose. Also it could be that the cumulative effect of both the types of initiation modes, namely 30S binding and 70S scanning type, happening *in vivo* lead to comparatively increased amounts of protein as seen in earlier section.

3.4 Purification of proteins and their functional assays

In order to be used for various *in vitro* assays, like RTS, poly(U) dependent poly(Phe) synthesis, translation initiation factors were purified. The functional activities were also tested in assays specific for the individual proteins.

3.4.1 Translation initiation factor 1, IF1

pET14b IF1 construct has the gene under the T7 promoter and was selected for protein over-expression and purification. The plasmid was transformed in BL21 (DE3) cells and protein was expressed and purified *via* nickel column using FPLC, as described in Materials and Methods (pages 63-64). Peak fractions were checked on SDS-PAGE as shown in **Figure 3.4.1-1 A**. After the protein was purified it was dialyzed three times for 45 minutes in 100 times volume in 1X binding buffer and rechecked on SDS gel as shown in lanes 1, 2 of **panel B**. The protein was quantified *via* Bradford method and digested with thrombin enzyme to remove the His tag of the protein. The cut protein is smaller and hence migrates faster as shown in **panel C.** The protein was also checked for a possible contamination with RNase **(panel D).**

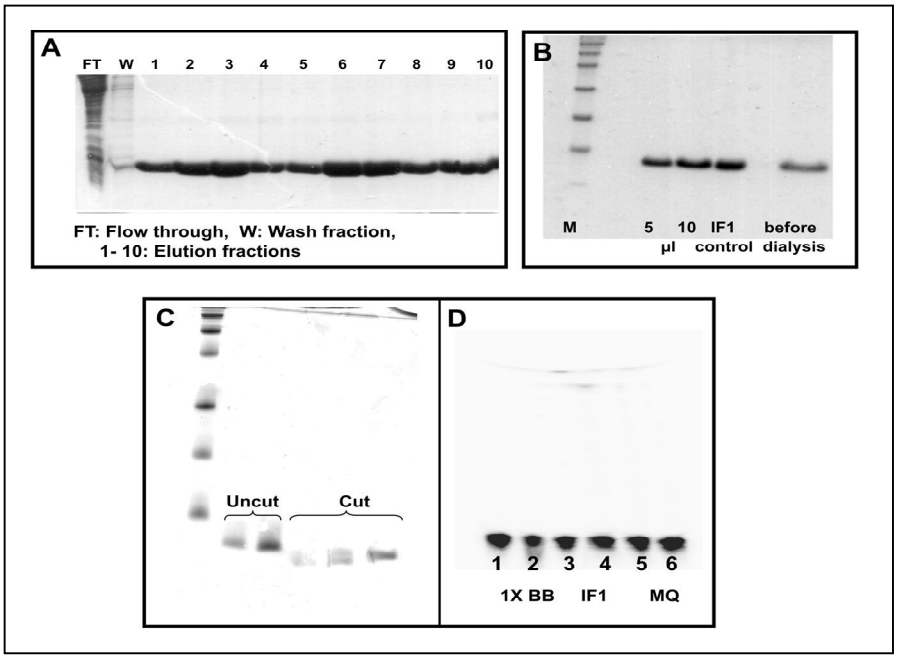


Figure 3.4.1-1: His tag IF1 protein purification via *FPLC using Ni-NTA column. A: purification of IF1 showing elution and wash fraction; B: gel check of IF1 after dialysis; C: His tag removal of IF1* via *thrombin digestion; D: Check for the RNase contamination in purified IF1 protein preparation using ^{32}P 5'-labeled MF mRNA (40 nucleotides).1X BB is our standard binding buffer also used for IF1 dialysis and MQ is milliQ water.*

RNase test was performed in a manner described in Materials and Methods section, page 67, protocol I. The result shows no RNase contamination, as there is no degradation of the labeled mRNA shown in lanes 3 and 4, **Figure 3.4.1-1 D** when purified IF1 is present. Control here used is 1X binding buffer which is actually IF1 dialysis buffer (lane 1 and 2) along with MQ water (lane 5 and 6). Poly(U) dependent poly(Phe) assay is used for checking IF1 activity and here, addition of IF1 in five-times excess over 70S ribosome reduced poly(Phe) synthesis (details provided in Daniela Wittek's thesis, {Wittek, 2009}**.**

We interpret this result that IF1 binds to the decoding centre and impairs binding of EF-Tu ternary complexes, and thus inhibits poly (Phe) synthesis, performing the role according to our hypothesis described in the Introduction.

3.4.2 Translation initiation factor 3, IF3

His tag IF3 gene in pQE vector was chosen for over-expression and purification, which is described in detail in Materials and Methods, page 64. The gene is under the tac promoter and is expressed in XL1-blue strain. Induction is done when OD_{600} reaches 0.5 with 1mM IPTG and over expression is checked on 15% SDS gel.

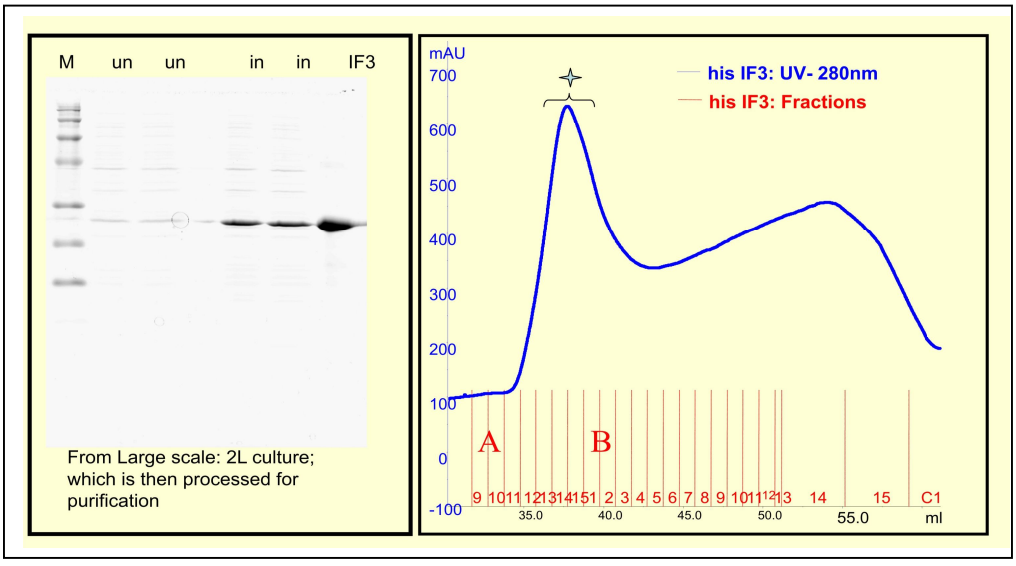


Figure 3.4.2-1: Over-expression of IF3 (in XL1-Blue strain) and FPLC purification using Ni-NTA column *A: After transformation a single colony was picked up and checked for over-expression as described in methods section on 15% SDS-PAGE. B: affinity chromatography-FPLC showing absorbance at A_{280} nm representing mAU. The arstick indicates peak fractions which were further checked on gel, pooled and dialyzed.*

After confirming over-expression as shown in **Figure 3.4.2-1A**, protein was purified *via* nickel column using FPLC. The peak eluted fractions obtained on FPLC (**Figure 3.4.2-1B)** were checked on gel as shown in **Figure 3.4.2-2 A**.

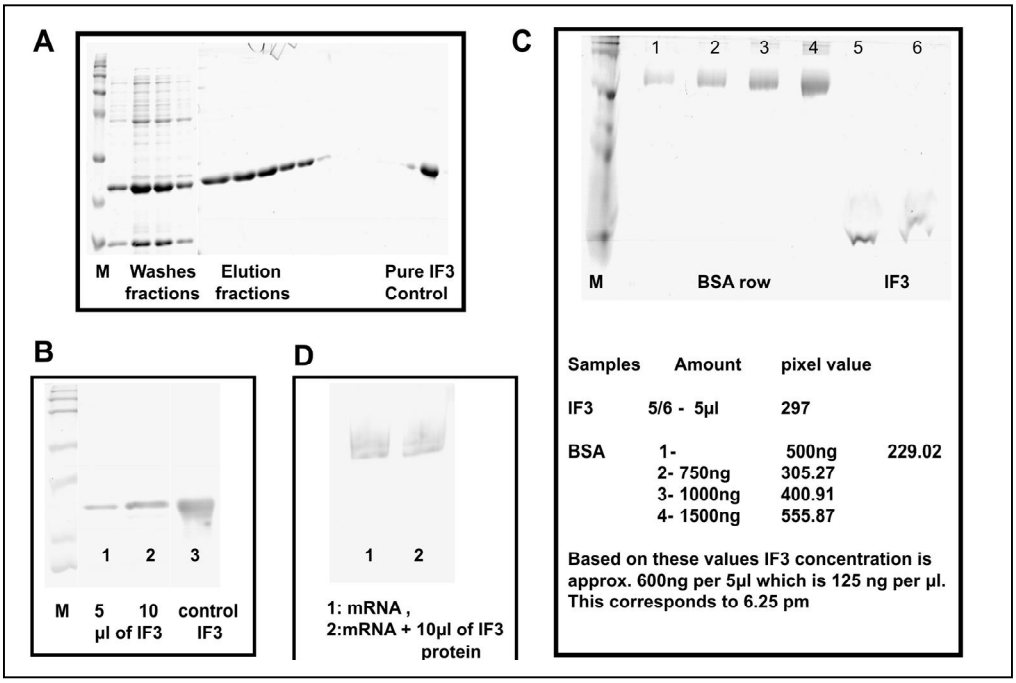


***Figure 3.4.2-2: Processing of the IF3 eluted fractions obtained from FPLC** purification. A: 15% SDS page showing eluted fractions along with wash fractions from the FPLC. B: SDS-gel check of purified IF3 protein after dialysis. C: Quantification of purified IF3 protein using BSA row, D: Checking RNase contamination in IF3 purified protein.*

After the confirmation of protein in these fraction, these were pooled and dialyzed three times for 45 minutes in 100 times volume in 1X binding buffer and were rechecked on SDS gel as shown in **Figure 3.4.2-2,** lanes 1-2 **(panel B)**. The protein was quantified by measuring its pixel intensity *via* image quantification software and the comparing it with pixel intensity of known amounts of BSA loaded in same gel shown in **Figure 3.4.2-2 panel C**. The protein IF3 was also checked for the RNase contamination in same way as described earlier and was found to be free of RNase contamination **(panel D)**.

The IF3 activity was measured *via* 70S dissociation. Increasing amount of IF3 was incubated with reassociated 70S and then loaded on sucrose gradient and checked for ribosome dissociation. A molar excess of up to 10:1 = IF3:70S practically did not induce dissociation, as shown in panel D, only at 30 to 50 times molar excess IF3, causes significant dissociation as observed in panel E and F **(Figure 3.4.2-3).**

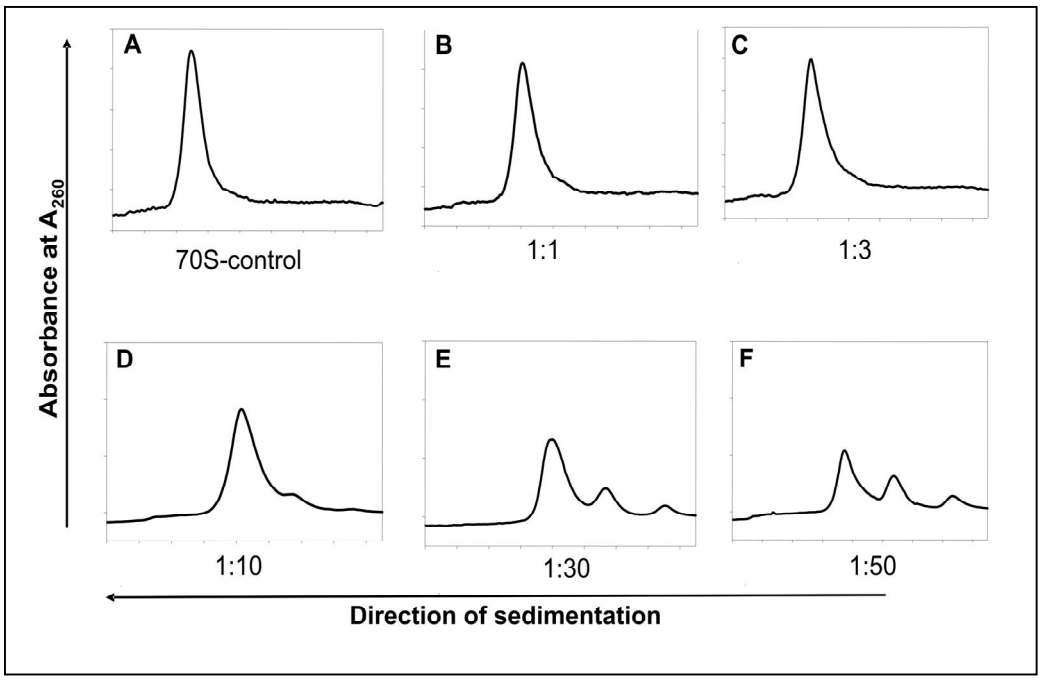


Figure 3.4.2-3: Purified IF3 activity test* via *70S dissociation. *Increasing amounts of purified IF3 over 70S is mixed and incubated at 37 °C for 15 minutes in 1X binding buffer. Sucrose gradient is done on SW60 (10-30%, 3 hours at 38,000 rpm) to obtain the ribosome pattern. The 70S: IF3 molar ratio is indicated below panels.*

This result confirms the activity of our purified IF3 to be appropriate and thus can be used in various *in vitro* experiments.

4 Discussion

4.1 Defining 30S binding type of initiation as per textbook wisdom and enlisting its limitation

According to textbook wisdom 30S binding type of initiation is thought to be the only type of initiation mode existing in bacteria {Schmeing TM, 2009}. Following this mode of initiation Translation initiation region (TIR) of mRNA is primed by 30S near the SD-AUG region with the help of all three translation initiation factors and initiator tRNA in a ternary complex fMet-tRNA•IF2•GTP and results in the formation of the 30S initiation complex. However this type of initiation does not provide satisfying answer to some important questions raised, which are key to the basic understanding in bacterial translation world.

These intriguing questions are

1: In spite of many claims about participation of IF1 in various 30S initiation steps, not one publication has pin pointed out its essential role supporting its existence in bacteria to be vital. In other words most of the functions are more of stimulatory nature namely enhancing the dissociation capability of IF3, enhancing affinity of IF2-fMet to 30S and improving the formation of 30SIC, not distinctly explaining why this smallest translation initiation factor is indispensable for cell viability. One focus of this work is to identify an exclusive IF1 specific function, which could explain lethality when this factor is absent in the cell. In bacteria an mRNA contains on average 3.3 cistrons (see Results section 3.1), and the intercistronic distances between these cistrons coding for ribosomal proteins are found to be short, (75% fall in the region of -20 to +30, Roland Krause, MPI für molekulare Genetik, personal communication). Considering the fact that both 30S and 70S cover approximately 40 nucleotides on mRNA, it is very unlikely that adjacent cistrons with short intercistronic space are initiated by binding of a new 30S, when a terminating 70S is still present on the stop codon of the previous cistron.

2: Therefore the second intriguing question is how initiation of downstream cistrons can occur in the presence of such short intercistronic distances. The short intercistronic distances exclude a simultaneous presence of a terminating 70S ribosome and an initiating 30S subunit. One has to assume that the 70S has first to leave before the downstream cistron can be initiated by a 30S subunit. But this

creates immediately another problem for the synthesis of ribosomal proteins. The translational control functions in a way, that the product of the first or second cistron of a polycistronic mRNA binds to the TIR of the first cistron and switches off the translation of all the downstream cistrons as well. The reason is that the downstream TIR's are hidden in secondary structures that have to be melted by translation of the upstream cistron. If now the terminating 70S ribosome leaves the mRNA the secondary structure will be formed again, faster than an initiating 30S subunit can bind thus compromising the stoichiometric synthesis of ribosomal proteins {Nomura, 1984}. However, stoichiometric synthesis of ribosomal proteins within narrow limits is a fact, since almost no free pools of r-proteins exist {Ulbrich, 1975}.

A solution of this dilemma would be, if the first but one cistrons are credibly initiated by same scanning 70S ribosome.

In line with answering the above put forth questions, we have proposed a 70S-scanning type of initiation. It is under this translation initiation mode, all the above problems can be solved very convincingly. The experimental strategy pursued in this direction will be discussed, which eventually provide evidence of this initiation type predominantly functioning in bacteria.

4.2 Distribution of IF1 and IF3 on ribosomes and polysomes: prompting towards their role on a 70S type of initiation

The first observation supporting our hypothesis is derived from the assay where IF1 distribution on 70S and polysomes was checked *via* Western blotting. The presence of IF1 exclusively on 70S and polysomes and not on 30S *in vivo* is shown in Results section 3.2 page 83 and is a prominent evidence of defining IF1 as a 70S factor rather than a 30S factor. In fact IF3 also localizes on 70S and polysomes besides its three-fold higher presence on 30S. This information provides the first evidence of IF1 and IF3 being involved 70S factor in contrast to what has been reported earlier in literature. Their presence of 70S ribosome is an unambiguous hint towards the function of IF1 and IF3 attributed to 70S.

Apparently the presence of IF3 on 70S ribosomes in all our experiments holds contradiction to old literature reports, according to which IF3 is an exclusive 30S factor. In particular, IF3 is thought to promote the dissociation of the 70S ribosome by antagonizing association of the 30S subunit to the 50S subunit {Grunberg-Manago, 1975} and through this activity the factor supplies the pool of free 30S subunits

required for translation initiation. However, one has to say that in the experiments various groups have demonstrated a role in dissociation an excess of 100-times higher than *in vivo* was used {Karimi, 1999; Hirokawa, 2005}, whereas under *in vivo* near conditions IF3 did not promote dissociation {Umekage, 2006}.

In order to clarify whether IF3 present in *in vivo* is exclusively performing only the above mentioned role, we checked the actual amount of IF3 that is required for the dissociation of 70S ribosomes. This shown in Results section 3.2.4 page 88 and according to this result IF3 should be present in at least a 30-50 times excess over ribosome in order to dissociate it. Our observation is supported by another publication {Petrelli, 2001} which also shows that IF3 should be present in 20-200 fold excess over ribosomes in order to dissociate them.

In a bacterial cell IF3 is present in 0.1 times excess over ribosomes (one per ten ribosomes; {Howe, 1983}. We also now know that IF3 should be present 20-50 times excess over ribosome and therefore it is to be argued that the *in vivo* concentration is far too less to dissociate 70S ribosome. It is a clear indication that with this *in vivo* amount in bacteria, it might not be actually involved in the dissociation of 70S but might stabilize 30S subunit preventing their association. Yet another publication validates our finding by emphasizing that in *in vivo* condition most of the ribosome exists as polysomes and intact 70S. IF3 can bind to 70S ribosome and loosen its structure without splitting it into the subunits. In fact the publication also demonstrates that even an excess of IF3, when incubated with 70S ribosome in a buffer mimicking *in vivo* situation, ribosomes are poorly dissociated, a very strong argument strengthening our hypothesis {Umekage, 2006}. In conclusion, rejecting dissociating function of IF3 and its presence of 70S ribosome *in vivo* along with IF1 supports strongly the role of IF1 and IF3 proposed in our 70S-scanning type of initiation model described in detail in the Introduction section.

The information obtained from our experiment namely presence of IF1 and IF3 on 70S ribosome, it was implemented in *in vitro* experiment described in detail in Daniela Wittek's thesis, {Wittek, 2009}. The highly advanced *in vitro* PURE system that has 70S ribosomes along with all the components for protein translation was used to test the effect of IF1 and IF3 on GFP expression. It was found that the GFP expression depends on the presence of IF3, whereas the addition of IF1 increases the GFP expression three-fold. In this system the GFP was the second cistron, and only re-

associated 70S ribosomes were active in contrast to 30S plus 50S, a first indication that IF1 and IF3 play a critical role for the 70S-scanning type.

4.3 Involvement of IF1 in constituting mature 50S particles *via* stochiometric synthesis of large subunit ribosomal protein

The next *in vivo* evidence convincingly supporting our hypothesis derives from an assay, which requires the generation of a special strain, where chromosomal *infA* gene encoding IF1 is knocked out and expressed in *trans* from a plasmid constitutively first from native promoter and finally conditionally from AraB promoter. The plasmid having the native promoter is called pIF1 and carries an ampicillin resistance mediating gene. The first strain having pIF1 plasmid is transformed with another plasmid called pAraIF1, which has *infA* gene under AraB promoter and that helps in conditional expression of IF1 *i.e*. the gene is switched on in presence of arabinose and off in presence of glucose. This plasmid carries kanamycin and chloramphenicol resistance genes. The mix strain which has both the plasmids is grown for over a period of months in the presence of arabinose, and antibiotics kanamycin and chloramphenicol. This led the strain to cure out pIF1 and have only pAraIF1 plasmid and hence make the strain dependent on the presence of arabinose for IF1 expression. Since IF1 is an essential gene and is required for the cell viability, any alteration in the cellular amount would be reflected on the growth of cells. Same is observed in our experiments shown in Results section 3.3.1 page 93.

In order to understand if the observed growth defect in any way is correlated to the ribosome profile of cells, we prepared S30 from both the strains where IF1 is expressed from either pIF1 or pAraIF1 in the presence of glucose and checked the ribosome profile. This is shown in Results section 3.3.3 page 98. We observe that the strain having pAraIF1 plasmid has 50S assembly defects and increased 30S peak, when IF1 expression is switched off. In contrast, the strain having pIF1 plasmid, IF1 is expressed in sufficient amounts under the same condition and the ribosome pattern appears normal. The study demonstrates strong 50S assembly defects in IF1 deficient state suggesting that it is essentially involved in the orderly synthesis of 50S ribosomal proteins but less for those of the 30S subunit. This asymmetry can be understood, when the intercistronic distances of large and small subunit ribosomal proteins were investigated. The result is summarized in **Table 4-1**.

Table 4-1: Cistron position of the ribosomal gene. *The start position of the respective genes on polycistronic mRNA is indicated. Highlighted in red colour belong to assembly proteins of 50S subunit, some of which are involved in the early assembly such as L24, L4 and L22 {Spillmann, 1977}.*

Counting the nucleotides before and after a stop codon:

-7 -4 -1+1 +4 +7

NNN NNN UAA NNN NNN NNN

Cistron position	Ribosomal genes
-4	L23
-1	L29, S17
3	L1, L30, L15
4	S18
9	L18
10	L24, L4
12	L6, L16
14	L5, S14, L22, S5
15	S9
16	S19, S11
17	L2, S3
20	L27, L33
31	L36
32	L3
33	S8, S4
40	L17

The analysis provides us the clear insight that most of the large ribosomal proteins have short intercistronic distance. Many 50S ribosomal proteins enlisted in this table are early assembly proteins of large subunit and thus are absolutely crucial for constituting 50S particles. In contrast, small subunit ribosomal proteins have longer intercistronic distances as shown in the above Table and early assembly proteins such as S4 and S8 have longer intercistronic distances of 33 nucleotides. As demonstrated in the Introduction section, 70S and 30S ribosomes span approximately 40 nucleotides on mRNA and therefore if the intercistronic distance happens to be small, the downstream cistrons will not be able to be initiated by 30S, when the upstream cistrons stop codon is still covered by terminated 70S. Here 30S binding type of initiation cannot occur as its simultaneous binding will be prevented because of steric clash. Therefore the only way these downstream cistrons can then be initiated is by the empty terminated 70S which scans the short intercistronic path on mRNA and located the correct start codon to initiate translation.

Another reason why the downstream cistrons on the polycistronic mRNA's encoding many ribosomal proteins are more likely to be initiated by 70S is because it is exclusively through this way that their precise 1:1 stochiometric synthesis of all ribosomal proteins constituting particles is achieved and free pool is prevented in the bacterial cell. If ribosomal proteins that are present on polycistrons are to be initiated independently of each other by 30S binding type of initiation than their stochiometric synthesis of 1:1 is unlikely to be achieved. But if the downstream cistrons are initiated by same 70S present of first cistron by scanning down to locate the correct initiation site then it is very plausible to have their translation coupled, allowing them to be synthesized in 1:1 stoichiometric ratio. Any hindrance in this mechanism will lead to chaotic synthesis of the proteins which will eventually be reflected as ribosomal assembly defect.

Having explained two very convincing reasons as why this scanning 70S initiation would be operating at downstream cistrons in polycistronic mRNA's, it is very effortlessly understood that since mostly 50S ribosomal proteins have very short intercistronic distance and all of them have to be synthesized in precise 1:1 stochiometry, they are the main candidates to be initiated by scanning 70S ribosomes. Ribosomal proteins constituting 30S particles have longer intercistronic distances and therefore they may be initiated also by 30S subunits. Furthermore according to our hypothesis, IF1 is considered to be essential for 70S scanning type of initiation and hence any variation in IF1 content would be interfering this process and lead to 50S ribosomal assembly defect. Indeed this is what we observe as shown Results section 3.3.3 page 98, providing a sharp argument of the role of IF1 associated with scanning 70S type of initiation. Small subunit ribosomal proteins support 30S binding type of initiation and since IF1 plays minor role here, the process of ribosomal protein synthesis is not so much affected.

4.4 Illustration of IF1 effect on the expression of bi-cistronic dual luciferase mRNA in *E. coli* (IF1$^-$)/ pAraIF1 strain (IF1 synthesis switched on and off)

In order to portray the cistrons present in polycistronic mRNA we have employed dual luciferase bi-cistronic reporter construct for the assay. In another set of experiments (described in detail in Daniela Wittek's thesis, {Wittek, 2009} it was observed that when complementary oligo DNA (anti-Rluc) against the first cistron was, used,

namely renilla luciferase (Rluc), synthesis of firefly luciferase (Fluc) goes down together with Rluc, whereas in monocistronic mRNAs Fluc is not affected by this anti-Rluc. The ribosomes are blocked in the first cistron (Rluc) and cannot slide to the second cistron, therefore firefly both activities drop down, pointing out that in the unblocked case sliding happens within the cistrons of the reporter construct. Confirming the involvement of 70S-scanning type of initiation in second cistron, which is line with our hypothesis the next agenda was to find out whether IF1 is involved in this process. The subsequent assay was performed with the bi- cistronic dual luciferase construct in a special *E. coli* ($IF1^-$)/ pAraIF1 strain, where we can modulate the expression of IF1 by supplying either arabinose (switch on) or glucose (switch off). The measure of expression of both the luciferase under these conditions were analyzed and it was found out that the first cistron-renilla expresses stably in both glucose and arabinose case, but the expression of second cistron is decreased appreciably in the presence of glucose as compared to arabinose. When Fluc to Rluc ratio was calculated we strikingly found that in case of glucose the ratio is decreased by factor of 4.5 as compared to arabinose, an observation that is supported by the Western blotting analysis quantifying the IF1 amount to be reduced to half as shown in result section 3.3.4 pages 105-106. We infer that the reduced IF1 amounts lead to a decrease in 70S scanning mode of initiation operating in downstream cistron.

After providing evidence of the involvement of IF1 in scanning 70S initiation mode operating in the downstream cistrons (here bi-cistron), next we were curious to know if this initiation mode is also a likely candidate in case of monocistronic mRNA's. We hypothesize that a 5'-UTR of a monocistronic mRNA can be initiated by both the 30S binding and the 70S scanning mode, whereas the presence of a strong secondary structure abolishes the 70S scanning in contrast to the 30S binding initiation. Therefore the next experiment was performed to understand what is happening in context with monocistronic mRNA's having characteristic 5'UTR region.

4.5 Model to canvass the IF1 effect on the distinct translation initiation modes: Expression of GFP from mRNA's with and without a secondary structure in 5' UTR when IF1 synthesis is switched on and off

The description of another assay that provides very strong evidence of the involvement of IF1 in 70S scanning type of initiation rather than 30S binding type of initiation is as follows. The experiment employs modelled GFP mRNA's having

different kinds of upstream region that would serve as the platform to study the distinct translation initiation mode. GFP with a strong secondary structure in the 5' UTR is the candidate for 30-binding type of initiation as 70S which binds at the 5' end cannot melt the secondary structure by scanning and therefore is unable to reach the next translation initiation site. The only way GFP in this context is initiated is by 30S-binding type, which can efficiently recognise the internal SD with AUG.

GFP without secondary structure ideally serves to support both kinds of translation initiation process. Here 70S which binds at the 5' end slides down very smoothly downstream on the mRNA, recognises SD with AUG and initiates the translation. Also 30S can recognise the internal SD with AUG binds to it and initiates the translation. Therefore in this construct we see the additive effect of both the types of translation initiation.

Reduced IF1 amounts affect the expression of GFP without any secondary structure the most. GFP with secondary structure is affected minimally as shown in Results section 3.3.5 page 110. As explained before GFP with secondary structure is initiated by 30S binding type of initiation and therefore reduced IF1 amount does not affect the GFP synthesis so much, clearly indicating that IF1 has a very minor role in 30S binding type of initiation. In case of GFP without secondary structure case where both 30S binding type of initiation as well as 70S scanning type of initiation occur, the effect of IF1 becomes magnanimous. It is quite imperative that the real candidate, where IF1 function is critically crucial, is 70S scanning mode of initiation and therefore the authentic function of IF1 is attributed to this mode of initiation. It is also to be argued here that GFP without secondary structure which is our model mRNA to study 70S scanning type of initiation is a mono-cistron mRNA. Considering the fact, we postulate that scanning 70S type of initiation is not only the feature of translation initiation of cistrons present in polycistrons after the first cistron, but is also a major translation initiation mechanism operating in the case of non structured monocistronic mRNA's. Taken together 70S scanning type of initiation would then account for the most prevalent type of initiation existing in bacteria being the main candidate for initiating translation of both mono-cistron having unstructured 5'UTR regions and three fourth cistrons organised in polycistrons (as 75% of them have short intercistronic region) in bacteria.

After having confirmed the IF1 function critical for 70S ribosome we were curious to know its exact role. For this we employed *in vitro* assay system such as poly (U)

dependent poly (Phe) assay and checked the effect the IF1 (details provided in Daniela Wittek's thesis, {Wittek, 2009}. It was observed that IF1 is capable of blocking the binding of aminoacyl-tRNA•EF-Tu•GTP ternary complex to 70S ribosome leading to a decrease in total Phe incorporation detected at various time intervals. This observation clearly indicates that their binding is interdependent *i.e.* the presence of IF1 would hinder the entry of aminoacyl tRNA at the A site during decoding and *vice versa*, since both have overlapping binding site at the decoding centre {Carter, 2001}.

After demonstrating the exact role of IF1 on 70S ribosome, next step would be elucidating why scanning 70S type of initiation needs IF1. In order to answer this we first need to understand the problems, which empty scanning 70S ribosomes faces, and then solve them by employing the functional property characteristic of these translation initiation factors. The first issue is that while scanning and reaching the next initiation site it encounters a problem, namely interference by aminoacyl-tRNA•EF-Tu•GTP ternary complex, which might enter the A site depending on the A-site codon in any frame leading to premature false "initiation" ultimately resulting to abort translation. Additionally, during scanning 70S ribosomes might encounter other AUG's in the inter spacer region, which are not the correct initiation codons, and if these are selected this too will abrogate the translation without having the required protein being made.

In order to tackle the above mentioned problems, the two translation initiation factors come into picture. We think that IF1 by binding to the A site of 70S ribosomes, blocks a premature binding of an aminoacyl-tRNA•EF-Tu•GTP ternary complex in any reading frame, favoring correct translation initiation of the downstream cistron on the polycistronic mRNA. The second issue of selecting the correct initiator codon is performed by IF3, which by binding to the scanning 70S ribosomes screens for the correct AUG's while sliding the intercistronic region and only at authentic SD-AUG start site promotes the binding of initiator codon. This function of IF3 is derived from the earlier reports, which state that IF3 monitors the codon-anticodon interaction by stimulating the on and off rates of "30S initiation complex" formation ensuring translational efficiency and fidelity by stimulating rapid formation of codon-anticodon interaction at the ribosomal P-site {Gualerzi C, 1977}.

The interaction of these two factors might weaken the binding of deacetylated tRNA present in the 70S post-termination state. However still the clear picture is lacking as

of whether the scanning takes place in presence or absence of deacetylated tRNA. Furthermore the time point, at which the third translation initiation factor IF2 binds to 70S ribosome is also argued. Two possibilities exist: it either acts at the ribosomal level stimulating initiator tRNA binding without prior formation of binary complex {Canonaco, 1986} as soon as the initiation site is recognised, or, alternatively, it acts as a carrier of fMet-tRNA and delivers it when the start site is located. In both the cases it is known to increase the affinity and promote stable binding of initiator tRNA to ribosomes {Canonaco, 1986}, subsequently leading to the release of IF3. Moreover its binding causes conformational change that aids the incoming of aminoacyl tRNA ternary complex to the A site consequently chasing away the IF1. It is also speculated that IF2 stimulates the release of IF1 and IF3. After the GTP hydrolysis and Pi release, IF2 gets dissociated from the ribosome because of the change in its conformation. However at present whether EF-G or any other factor contributes to 70S scanning is yet to be learned. We do not rule out the possibility of the participation of other factors or their order in participating 70S scanning process and but as per current work we confirm that IF1 and IF3 are critically required for this process. Therefore the area is open for further addition of new information strengthening the existence of this prevalent initiation mode.

Our scanning 70S initiation model can be described as follows: After encountering the stop codon of cistron n, the empty 70S ribosomes (post-termination state) do not immediately get dissociated into subunits with the help of factors. They in fact scan for some time period over short intercistronic distances eventually initiating the translation of next n+1 cistron. While doing so it encounters a problem of getting blocked by the binding of premature ternary complexes or by selection of incorrect start codons. These both problems are taken care by IF1 and IF3 in a manner described before leading to generation of highly competent 70S scanning ribosomes. This ribosome complex after locating the correct start site allows the initiator tRNA ternary complex to bind, after which the factors are released paving the path for the entry of aminoacyl-tRNA•EF-Tu•GTP ternary complex to A site resuming the protein synthesis of n+1 cistron. (**Figure 4-1**)

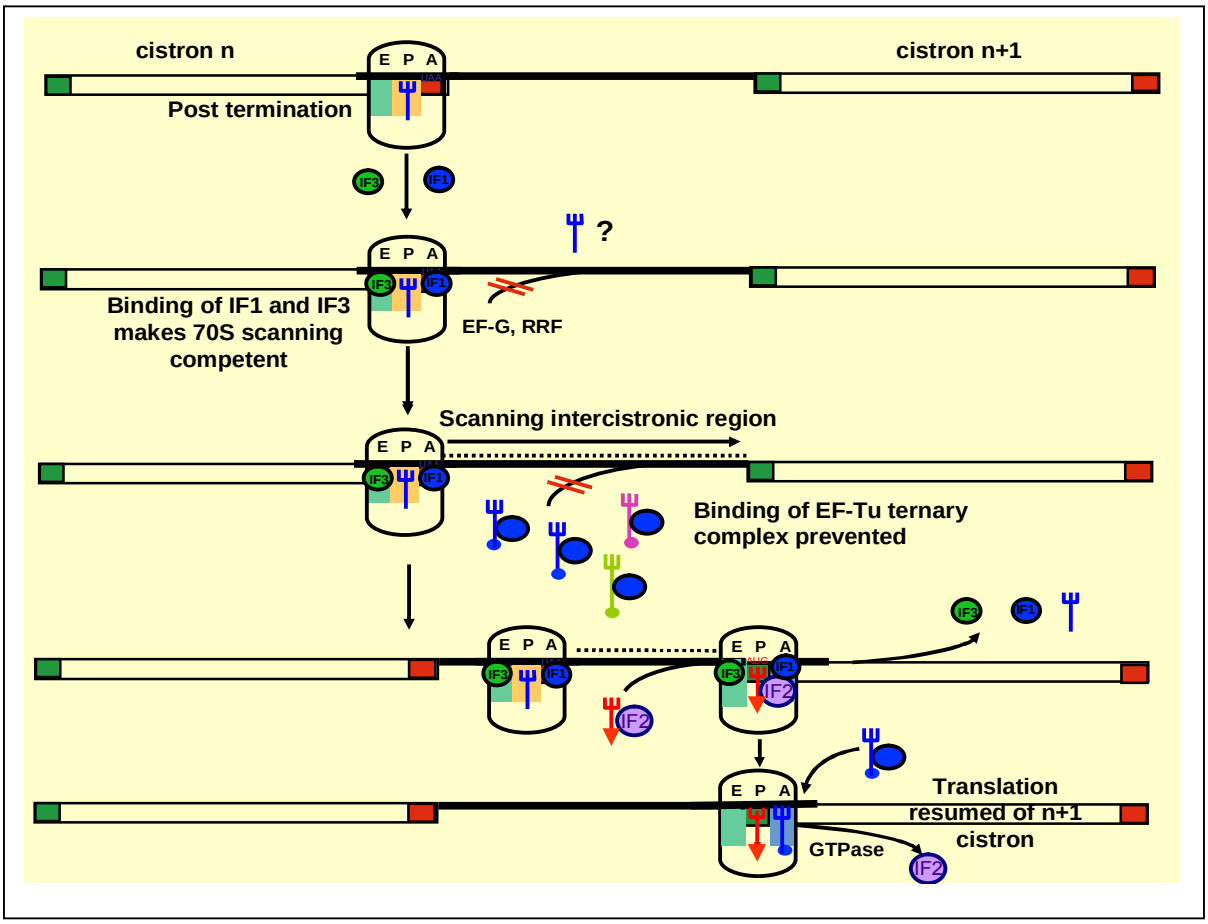


Figure 4-1: Outlining our new translation initiation model "70S-scanning type of initiation". *The two cistrons are designated as n and n+1 and their start and stop codons are indicated by green and red colour, respectively. The first step is the formation of post-termination state after encountering stop codon at the A site. The terminated 70S has deacetylated tRNA and at this moment IF1 and IF3 bind at A and E site respectively making 70S ribosome scanning competent. This interaction may weaken the binding of deacetylated tRNA to ribosomes (represented as loosened tRNA-mRNA complex) after which the scanning 70S ribosomes slides smoothly through the intercistronic region. The binding of EF-Tu ternary complex is prevented by IF1. After it encounters the correct SD-AUD site it facilitates the binding of IF2-initiator tRNA ternary complex leading to release to IF1 and IF3. IF2 is also released after GTP hydrolysis consequently leading to the entry of aminoacyl-tRNA•EF-Tu•GTP to A site resuming protein synthesis of n+1 cistron.*

In the frame of this new model the essential character of IF1 is proposed, namely IF1 being a 70S factor binds to the decoding centre of 70S after termination and before sliding starts, prevents premature ternary complex binding, which makes 70S ribosome sliding competent during the translation of polycistronic mRNA together with IF3. IF3 apart from being a 30S factor is also a 70S factor, which by binding to 70S ribosome, loosens it, without causing any dissociation and under our hypothesis making sliding ribosome scanning competent and helping in screening for the correct start site. Lack of IF1 hampers sliding because of getting blocked by the binding of

premature ternary complexes to the empty ribosomes which leads to disruption of the equimolar synthesis of ribosomal proteins (particularly L-proteins), leading to 50S assembly defect. This defect finally is reflected on global translation of bacterial cell consequently leading to the cell death, reasonably justifying why IF1 is indispensable for cell viability.

5 Appendix

5.1 Thin layer chromatography of oligo-peptides

Thin layer chromatography (TLC) is a chromatography technique used to separate mixtures of compounds, here oligo-peptides. Thin layer chromatography is performed on a sheet of glass, plastic, or aluminium foil, which is coated with the a thin layer of adsorbent material, usually silica gel, aluminium oxide, or cellulose and cellulose derivatives. This layer of adsorbent is known as the stationary phase {Bhushan R, 1989}.

After the sample has been applied on the plate, a solvent or solvent mixture (known as the mobile phase) is drawn up the plate either *via* electrophoresis or capillary action. Because different analytes ascend the TLC plate at different rates, separation is achieved. TLC has greater resolving power, greater speed of separation and relatively wide choice of adsorbents as compared to paper chromatography.

The separation of compounds by chromatography depends on several factors:

1. Affinity of a solute for a moving solvent phase and a stationary aqueous phase.
2. Ion exchange effect: any ionized impurities in the support medium will tend to bind or attract oppositely charged ions (solutes) and will therefore reduce the mobility of these solutes, and therefore these are preferred to be inert like silica or cellulose
3. Temperature: Since temperature can affect the solubility of the solute, a given solvent temperature is also an important factor.
4. The composition of the solvent: since some compounds are more soluble in one solvent than in the other, the mixture of solvents used will affect the separation of compounds.
5. The molecular weight of a solute also affects the solubility and hence chromatographic performance.

General protocol:

1. Preparation of samples:

Amino acid Lysine, Methionine, Phenylalanine, Glutamic acid were dissolved in water to the stock concentration of 100 mM. Di-peptides were made either in water (fMet-Lys) or 100% DMSO (fMet-Phe) and had higher stock concentration.

To make the mixture of amino acid *e.g.* Methionine and Lysine, from each 40 µl of 100 mM stock concentration was mixed. Same was done to prepare Phenylalanine and Methionine mixture. When loaded 1 µl from the 100 mM stock mixture, it will have the final concentration of 100 nm.

2. Spotting of samples:

From the each of the sample first 0.5 µl is spotted on cellulose paper with 10 µl pipette having the distance of at least 2 cm between two spots. Mark the direction of loading. Let the spots dry and again load at the same place 0.5 µl. again. Let the spots completely dry. Then spread electrophoresis buffer with the help of pipette as near as possible to the spot. Then by diffusion the buffer from the two sides will merge, spreading the buffer on entire plate. It is then placed in chamber.

Make the chamber ready by pouring electrophoresis buffer to the marked point on both sides. Then the cellulose membrane is placed in the chamber after which stodder solvent is poured on the top till it covers the top of the TLC plate. Make sure that plate is covered entirely with stodder solvent. Run TLC as per required voltage for specific amount of time. After run is done the cellulose plate is taken out and dried at 55-60 ºC for 20-25 minutes.

3. Developing the TLC

After the cellulose plate is dried, spray ninhydrin with the help of sprayer in closed hood. After spraying properly on the entire plate, incubate it at 55-60 ºC till the spots appear. It requires 2 hours for the spots to appear, after which the membrane is stored in plastic cover

In our experimental setup we wanted to separate two different di-peptides formed on mRNA transcript. In order to do so, we have to standardize the method suitable for our use.

Standardization of TLC

A. Standardization of the amount of the amino acids and peptides to be detected *via* a ninhydrin-spray.

Several dilutions of samples were prepared. We started with single amino acid such as Lysine and Phenylalanine. From each dilution, spots were applied at the middle of the cellulose plates.

The result shows that the coloured spots increase in size and become clearer with the increase in the concentration. Based on this observation we decided to choose the optimum of 25 nm for each of the single amino acid.

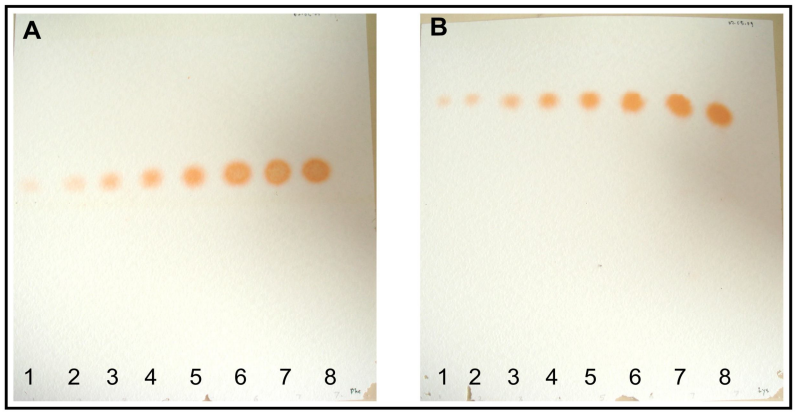


Figure 5.1-1: Concentration optimization of amino acid; *A: Phenylalanine, B: Lysine 1; 1*10*^{3} pm, 2; 2*10*^{3} pm, 3; 5*10*^{3} pm, 4; 10*10*^{3} pm, 5; 20*10*^{3} pm, 6; 50*10*^{3} pm, 7; 100*10*^{3} pm, 8; 100*10*^{3} pm. Run conditions: Lysine - 200 V at room temperature for 1.5 hour; Phenylalanine - 200 V for 2 hours (Lysine migrates faster than Phenylalanine).*

B. Standardization of place of spotting

Since positively charged Lysine moves faster than the hydrophobic Phenylalanine, it was difficult to perform chromatography for both together, when spotted in the middle of same TLC plate. The spotting was therefore changed to a lower position as shown in **Figure 3.5-2**. In this case spotting was shifted from 10 cm from base (middle) to a position 6 cm from the bottom of the plate.

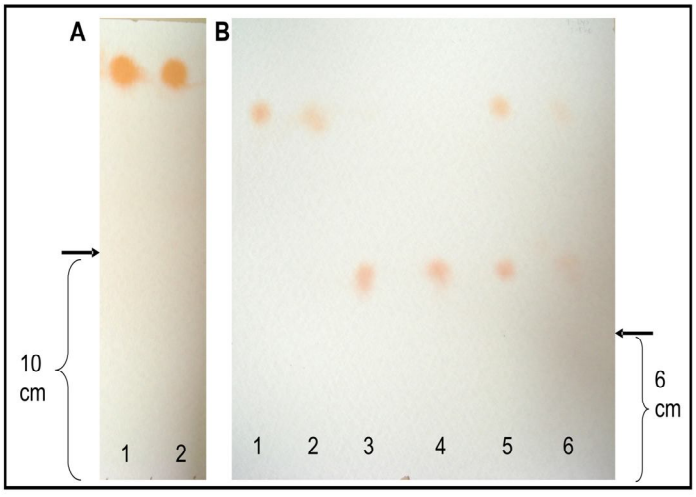


Figure 5.1-2: Optimization of spotting position;
A: Spotted in middle i.e. *10 cm from the bottom of TLC plate, 1, 2; 100*10*^{3} pm of Lysine*
B: Spotted towards the lower position i.e. *6 cm from the lower edge, 1, 2; 15*10*^{3} pm of Lysine, 3, 4; 15*10*^{3} pm of Phenylalanine, 5, 6; Mixture of 15*10*^{3} pm of Lysine and 15*10*^{3} pm of Phenylalanine. Arrows indicate position of spotting.*

C. Standardisation of running time and temperature

Initial experiments were conducted in the cold room at 4 ºC, running condition was 200 V for 4 hours. Since the organic solvent has very irritating and toxic fumes, it had to be conducted in a fume hood at room temperature. Under this condition the

chromatography was carried out at 200 V for 2 hours. High temperature increases the mobility of solutes and therefore separation requires less time.

D. Visualization of a radioactive spot by phosphor imaging along with ninhydrin-spray method; calculation of the Rf (Retention factor) value.

Retention factor or Rf is defined as the distance migrated by the compound from the spotted position relative to a reference. We use two methods to calculate it; the first one is phosphor imaging, which is more sensitive than the second one using ninhydrin. For this assay a ^{14}C-labelled amino acid is mixed with the unlabelled counterpart and is spotted, the final amounts are summarized in the table. The spotting is done at 6 cm from the base and run at 200 V for 1.5 hours at room temperature. The TLC plate if then dried and exposed to screen for 48 hours and developed. The same TLC plate is now sprayed with ninhydrin to see the coloured spots. It is marked at the three ends with a radioactive ink in-order to align the position of the respective samples.

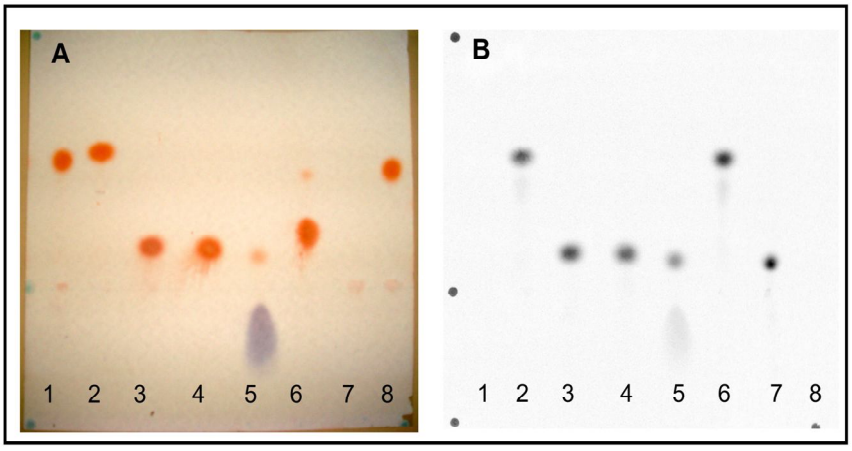


Figure 5.1-3: Analysis and comparison of radio-labelled amino acid with unlabelled one. *1; Mixture of Lysine + fMet- Phe, 2; ^{14}C Lysine + Lysine, 3; ^{14}C Phenylalanine + Phenylalanine, 4; ^{14}C Methionine + Methionine, 5; ^{14}C fMethionine + fMethionine, 6; ^{14}C Lysine + fMet- Lys, 7; ^{14}C Phenylalanine + fMet- Phe, 8; Mixture of Lysine + fMet- Phe* ***A: Ninhydrin spray method; B: Phosphor imaging method.***

Table 3.5-1: Calculation of Rf values by Ninhydrin method

Components	**Amount (*10*^{3} pm)**	**Distance from the base, where it is spotted**	**Rf = migrated distance of a compound/ migrated distance of Lysine**
Lysine	50	5.8 cm	1

Phenylalanine	50	1.9 cm	0.327
Methionine	50	1.6 cm	0.275
Formyl-methionine	1000	1.3 cm	0.224
fMet-lys (Dipeptide)	500	2.4 cm	0.413
fMet-Phe (Dipeptide)	1000	0 cm (does not migrate)	0

Table 3.5-2: Calculation of Rf values by Phospho imaging/ Radioisotope imaging method

Components	**Added amount (cpm)**	**Distance from the base where it is spotted**	**Rf = distance of compound/ distance of Lysine**
^{14}C-Lysine	4,000	5.8 cm	1
^{14}C-Phenylalanine	4,000	1.8 cm	0.31
^{14}C-Methionine	4,000	1.6 cm	0.275
^{14}C f-Methionine	2,000	1.4 cm	0.241

The distance of each spot is measured from the spotting position to the centre at the final position. Rf values are then calculated by dividing the migrated distance of a compound by the migrated distance of the reference Lysine (fastest migrating component). These Rf values will be unaffected by changing some of the chromatography conditions such as voltage or temperature, provided it is applied to all components.

Bibliography

Adhin, M. R. and J. van Duin (1990). "Scanning model for translational reinitiation in eubacteria." J. Mol. Biol. 213: 811-818.

Allen, G., A. Zavialov, et al. (2005). "The cryo-EM structure of a translation initiation complex from Escherichia coli." Cell 121(5): 703-712.

Antoun, A., M. Y. Pavlov, et al. (2006). "How initiation factors maximize the accuracy of tRNA selection in initiation of bacterial protein synthesis." Mol. Cell 23(2): 183-193.

Baan, R. A., J. J. Duijfjes, et al. (1976). "Specific in situ cleavage of 16S rRNA of E coli interferes with the function of IF-1." Proc. Natl. Acad. Sci. USA 73(3): 702-706.

Battiste, J. L., T. V. Pestova, et al. (2000). "The eIF1A solution structure reveals a large RNA-binding surface important for scanning function." Mol. Cell 5(1): 109-119.

Benne, R., N. Naaktgeboren, et al. (1973). "Recycling of initiation factors IF-1, IF-2 and IF-3." Eur J Biochem. 32(2): 372-380.

Bhushan R, M. V., Mallikharjun PV. (1989). "Thin layer chromatography of peptides and proteins: a review." Biomedical Chromatography 3(3): 95-104.

Binns N, M. M. (2002). "Expression of the Escherichia coli pcnB gene is translationally limited using an inefficient start codon: a second chromosomal example of translation initiated at AUU." Mol Microbiol. 44(5): 1287-98.

Biou, V., F. Shu, et al. (1995). "X-ray crystallography shows that translational initiation factor IF3 consists of two compact alpha/beta domains linked by an alpha-helix." EMBO J. 14: 4056-4064.

Blattner FR, P. G. r., Bloch CA, Perna NT, Burland V, Riley M, Collado-Vides J, Glasner JD, Rode CK, Mayhew GF, Gregor J, Davis NW, Kirkpatrick HA, Goeden MA, Rose DJ, Mau B, Shao Y. (1997). "The complete genome sequence of Escherichia coli K-12." Science 277(5331): 1453-62.

Boelens, R. and C. O. Gualerzi (2002). "Structure and function of bacterial initiation factors." Curr. Protein Pept. Sci. 3: 107-119.

Boileau, G., P. Butler, et al. (1983). "Direct crosslinks between IFs 1, 2, and 3 and ribosomal proteins promoted by 2-iminothiolane." Biochemistry 22: 3162-3170.

Bouadloun, F., D. Donner, et al. (1983). "Codon-specific missense errors *in vivo*." EMBO J. 2: 1351-1356.

Bremer, H. and P. P. Dennis (1996). Modulation of chemical composition and other parameters of the cell by growth rate. *Escherichia coli* and *Salmonella*. F. C. Neidhardt, R. C. III, J. L. Ingrahamet al. Washington D.C., ASM Press. 2: 1553-1569.

Brock, S., K. Szkaradkiewicz, et al. (1998). "Initiation factors of protein biosynthesis in bacteria and their structural relationship to elongation and termination factors." Mol. Microbiol. 29(2): 409-417.

Bycroft, M., T. J. P. Hubbard, et al. (1997). "The solution structure of the S1 RNA binding domain: A member of an ancient nucleic acid-binding fold." Cell 88(2): 235-242.

Canonaco, M. A., R. A. Calogero, et al. (1986). Mechanism of translational initiation in prokaryotes. Evidence for a direct effect of IF2 on the activity of the 30 S ribosomal subunit. Febs Lett. 207: 198-204.

Carter, A. P., W. M. Clemons, Jr., et al. (2001). "Crystal structure of an initiation factor bound to the 30S ribosomal subunit." Science 291(5503): 498-501.

Cech, T. (2000). "Structural biology. The ribosome is a ribozyme." Science 289(5481): 878-879.

Celano, B., R. T. Pawlik, et al. (1988). "Interaction of Escherichia coli, translation-initiation factor IF-1 with ribosomes." Eur. J. Biochem. 178(2): 351-5.

Chiaruttini, C., M. Milet, et al. (1996). "A long-range RNA-RNA interaction forms a pseudoknot required for translational control of the IF3-L35-L20 ribosomal protein operon in Escherichia coli." EMBO J 15(16): 4402-4413.

Choi, S. K., D. S. Olsen, et al. (2000). "Physical and functional interaction between the eukaryotic orthologs of prokaryotic translation initiation factors IF1 and IF2." Mol Cell Biol 20(19): 7183-7191.

Cole, J. R., C. L. Olsson, et al. (1987). "Feedback regulation of rRNA synthesis in Escherichia coli. Requirement for initiation factor IF2." J. Mol. Biol. 198(3): 383-392.

Craigen, W. J., R. G. Cook, et al. (1985). "Bacterial peptide chain release factors: conserved primary structure and possible frameshift regulation of release factor 2." Proc. Natl. Acad. Sci. USA 82: 3616-3620.

Croitoru, V. V., M. Bucheli-Witschel, et al. (2005). "In vivo involvement of mutated initiation factor IF1 in gene expression control at the translational level." FEBS Lett. 579(5): 995-1000.

Cummings, H. S. and J. W. B. Hershey (1994). "Translation initiation factor IF1 is essential for cell viability in *Escherichia coli*." J. Bacteriol. 176(1): 198-205.

Cummings, H. S., J. F. Sands, et al. (1991). "Structure and Expression of the infA Operon Encoding Translational Initiation Factor-IF1 - Transcriptional Control by Growth Rate." J Biol Chem 266: 16491-16498.

Dahlquist, K. D. and J. D. Puglisi (2000). "Interaction of translation initiation factor IF1 with the *E. coli* ribosomal A site." J. Mol. Biol. 299(1): 1-15.

Dallas, A. and H. F. Noller (2001). "Interaction of translation initiation factor 3 with the 30S ribosomal subunit." Mol. Cell 8(4): 855-864.

De Vries, O. M. H., J. H. C. Hoge, et al. (1980). "Translation of RNA from schizophyllum commune in a wheatgerm and rabbit reticulocyte cell-free system. Comparison of in vitro and in vivo products after 2-d gel electrophoresis. (bba report, bba 91493)." Biochim. Biophys. Acta 607(2): 373-378.

Dottavio-Martin, D., D. P. Suttle, et al. (1979). "The effects of initiation factors IF-1 and IF-3 on the dissociation of E coli 70S ribosomes." FEBS Lett. 97(1): 105-110.

Ehresmann, C., F. Baudin, et al. (1987). "Probing the structure of RNAs in solution." Nucleic Acids Res. 15(22): 9109-9128.

Gaur, R., D. Grasso, et al. (2008). "A single mammalian mitochondrial translation initiation factor functionally replaces two bacterial factors." Mol. Cell 29(2): 180-190.

Gold, L. (1988). "Posttranscriptional regulatory mechanisms in *Escherichia coli*." Ann. Rev. Biochem. 57: 199-233.

Gribskov, M. (1992). "Translational Initiation Factor-IF-1 and Factor-eIF-2alpha Share an RNA-Binding Motif with Prokaryotic Ribosomal Protein-S1 and Polynucleotide Phosphorylase." Gene 119.

Grigoriadou, C., S. Marzi, et al. (2007). "The translational fidelity function of IF3 during transition from the 30 S initiation complex to the 70 S initiation complex." J. Mol. Biol. 373(3): 551-561.

Grunberg-Manago, M., P. Dessen, et al. (1975). "Light-scattering studies showing the effect of initiation factors on the reversible dissociation of *Escherichia coli* ribosomes." J. Mol. Biol. 94(3): 461-478.

Gualerzi, C. O., L. Brandi, et al. (2001). "Initiation factors in the early events of mRNA translation in bacteria." Cold Spring Harb Symp Quant Biol 66: 363-76.

Gualerzi, C. O. and C. L. Pon (1990). "Initiation of messenger-RNA translation in prokaryotes." Biochemistry 29(25): 5881-5889.

Gualerzi C, R. G., Pon CL. (1977). "Initial rate kinetic analysis of the mechanism of initiation complex formation and the role of initiation factor IF-3." Biochemistry 16(8): 1684-9.

Hartz, D., J. Binkley, et al. (1990). "Domains of initiator transfer RNA and initiation codon crucial for initiator transfer RNA selection by *Escherichia coli* IF3." Gene Dev. 4: 1790-1800.

Hartz, D., D. S. McPheeters, et al. (1989). "Selection of the initiator tRNA by *Escherichia coli* initiation factors." Genes Dev. 3: 1899-1912.

Hirokawa, G., R. Nijman, et al. (2005). "The role of ribosome recycling factor in dissociation of 70S ribosomes into subunits." RNA 11(8): 1317-1128.

Howe, J. G. and J. W. B. Hershey (1983). "Initiation factor and ribosome levels are coordinately controlled in *Escherichia coli* growing at different rates." J. Biol. Chem. 258(3): 1954-1959.

Ikemura, T. and H. Ozeki (1977). "Gross map location of Escherichia coli transfer RNA genes." J. Mol. Biol 117(2): 419-446.

JA., S. (1969). "Polypeptide chain initiation: nucleotide sequences of the three ribosomal binding sites in bacteriophage R17 RNA." Nature 224(5223): 957-64.

Janosi, L., S. Mottagui-Tabar, et al. (1998). "Evidence for *in vivo* ribosome recycling, the fourth step in protein biosynthesis." EMBO J. 17(4): 1141-1151.

Janosi, L., R. Ricker, et al. (1996). "Dual functions of ribosome recycling factor in protein biosynthesis: Disassembling the termination complex and preventing translational errors." Biochimie 78(11-12): 959-969.

Kapralou, S., A. Fabbretti, et al. (2008). "Translation initiation factor IF1 of Bacillus stearothermophilus and Thermus thermophilus substitute for Escherichia coli IF1 in vivo and in vitro without a direct IF1-IF2 interaction." Mol. Microbiol. 70(6): 1368-1377.

Karimi, R., M. Pavlov, et al. (1999). "Novel roles for classical factors at the interface between translation termination and initiation." Mol. Cell 3: 601-609.

Karimi, R., M. Y. Pavlov, et al. (1998). "Initiation factors IF1 and IF2 synergistically remove peptidyl-tRNAs with short polypeptides from the P-site of translating Escherichia coli ribosomes." J. Mol. Biol. 281(2): 241-252.

Kim, C. J., Y. K. Chang, et al. (2000). "Enhancement of kasugamycin production by pH shock in batch cultures of Streptomyces kasugaensis." Biotechnol Prog 16(4): 548-52.

Kim, C. J., Y. K. Chang, et al. (2001). "Continuous culture of immobilized streptomyces cells for kasugamycin production." Biotechnol Prog 17(3): 453-61.

Ko, J. H., S. J. Lee, et al. (2006). "Differential promoter usage of infA in response to cold shock in Escherichia coli." FEBS Lett. 580(2): 539-544.

Kozak, M. (1999). "Initiation of translation in prokaryotes and eukaryotes." Gene 234(2): 187-208.

Kurland, C. G. (1992). "Translational Accuracy and the Fitness of Bacteria." Annu Rev Genet 26: 29-50.

Kyrpides, N. C. and C. R. Woese (1998). "Universally Conserved Translation Initiation Factors." Proc.Nat. Acad. Sci. USA 95(1): 224-228.

La Teana, A., C. O. Gualerzi, et al. (2001). "Initiation factor IF 2 binds to the alpha-sarcin loop and helix 89 of *Escherichia coli* 23S ribosomal RNA." RNA 7(8): 1173-1179.

La Teana, A., C. L. Pon, et al. (1996). "Late events in translation initiation. Adjustment of fMet-tRNA in the ribosomal P-site." J. Mol. Biol. 256(4): 667-675.

Laursen, B. S., A. S. S. A. de, et al. (2002). "Structural requirements of the mRNA for intracistronic translation initiation of the enterobacterial *infB* gene." Genes Cells 7(9): 901-910.

Laursen, B. S., H. P. Sorensen, et al. (2005). "Initiation of protein synthesis in bacteria." Microbiol. Mol. Biol. Rev. 69(1): 101-123.

Mccarthy, J. E. G. and R. Brimacombe (1994). "Prokaryotic translation: The interactive pathway leading to initiation." Trends Genet. 10(11): 402-407.

McCutcheon, J. P., R. K. Agrawal, et al. (1999). "Location of translational initiation factor IF3 on the small ribosomal subunit." Proc. Natl Acad. Sci. USA 96(8): 4301-4306.

Milon, P., A. L. Konevega, et al. (2008). "Kinetic checkpoint at a late step in translation initiation." Mol. Cell 30(6): 712-720.

Mitchell, S. F. and J. R. Lorsch (2008). "Should I stay or should I go? Eukaryotic translation initiation factors 1 and 1A control start codon recognition." J. Biol. Chem. 283(41): 27345-27349.

Moazed, D. and H. F. Noller (1990). "Binding of tRNA to the ribosomal A and P sites protects two distinct sets of nucleotides in the 16S rRNA." J. Mol. Biol. 211: 135-145.

Moazed, D., R. R. Samaha, et al. (1995). "Specific protection of 16 S rRNA by translational initiation factors." J. Mol. Biol. 248: 207-210.

Moreau, M., E. deCock, et al. (1997). "Heteronuclear NMR studies of E-coli translation initiation factor IF3. Evidence that the inter-domain region is disordered in solution." J Mol Biol 266(1): 15-22.

Mortensen, K. K., E. Hajnsdorf, et al. (1995). "Improved recombinant tandem expression of translation initiation factor IF2 in RNase E deficient E-coli cells." Biochem Biophys Res Commun 214(3): 1254-1259.

Mortensen, K. K., J. Kildsgaard, et al. (1998). "A six-domain structural model for *Escherichia coli* translation initiation factor IF2. Characterisation of twelve surface epitopes." Biochem. Mol. Biol. Int. 46(5): 1027-1041.

Nakamura, Y., K. Ito, et al. (1996). "Emerging understanding of translation termination." Cell 87(2): 147-150.

Nissen, P., J. Hansen, et al. (2000). "The structural basis of ribosome activity in peptide bond synthesis." Science 289(5481): 920-930.

Nissen, P., M. Kjeldgaard, et al. (1995). "Crystal structure of the ternary complex of Phe-tRNAPhe, EF-Tu, and a GTP analog." Science 270(5241): 1464-1472.

Noller, H. F. and e. al. (1981). "Secondary structure model for 23S ribosmal RNA." Nucleic Acids Res. 9: 6167-6189.

Noller, H. F., M. M. Yusupov, et al. (2002). "Translocation of tRNA during protein synthesis." FEBS Letter 514: 11-16.

Nomura, M., R. Gourse, et al. (1984). "Regulation of the synthesis of ribosomes and ribosomal components." Ann. Rev. Biochem. 53: 75-117.

Nyengaard, N. R., K. K. Mortensen, et al. (1991). "Tandem translation of *E. coli* initiation factor IF2 beta: purification and characterization *in vitro* of two active forms." Biochem. Biophys. Res.Commun. 181(3): 1572-1579.

Ogle, J. M., D. E. Brodersen, et al. (2001). "Recognition of cognate transfer RNA by the 30S ribosomal subunit." Science 292(5518): 897-902.

Ogle, J. M., F. V. Murphy, et al. (2002). "Selection of tRNA by the ribosome requires a transition from an open to a closed form." Cell 111(5): 721-732.

Ogle, J. M. and V. Ramakrishnan (2005). "Structural insights into translational fidelity." Annu. Rev. Biochem. 74: 129-177.

Olsson, C. L., M. Graffe, et al. (1996). "Physiological effects of translation initiation factor IF3 and ribosomal protein L20 limitation in *Escherichia coli*." Mol. Gen. Genet. 250(6): 705-714.

Passmore, L. A., T. M. Schmeing, et al. (2007). "The eukaryotic translation initiation factors eIF1 and eIF1A induce an open conformation of the 40S ribosome." Mol. Cell 26(1): 41-50.

Pavlov, M. Y., A. Antoun, et al. (2008). "Complementary roles of initiation factor 1 and ribosome recycling factor in 70S ribosome splitting." EMBO J. 27(12): 1706-1717.

Pestova, T. V., S. I. Borukhov, et al. (1998). "Eukaryotic Ribosomes Require Initiation Factors 1 and 1a to Locate Initiation Codons." Nature. 394(6696): 854-859.

Pestova, T. V. and C. U. Hellen (2001). "Functions of eukaryotic factors in initiation of translation." Cold Spring Harb. Symp. Quant. Biol. 66: 389-396.

Petrelli, D., A. LaTeana, et al. (2001). "Translation initiation factor IF3: two domains, five functions, one mechanism?" EMBO J. 20(16): 4560-4569.

Pioletti, M., F. Schlünzen, et al. (2001). "Crystal structures of complexes of the small ribosomal subunit with tetracycline, edeine and IF3." EMBO J. 20(8): 1829-1839.

Pon, C. L. and C. O. Gualerzi (1984). "Mechanism of protein biosynthesis in prokaryotic cells. Effect of initiation factor IF1 on the initial rate of 30 S initiation complex formation." FEBS Lett. 175(2): 203-207.

Pon CL, W.-L. B., Gualerzi C. (1979). "Structure--function relationships in Escherichia coli initiation factors. II. Elucidation of the primary structure of initiation factor IF-1." FEBS Lett. 101(1): 157-160.

RajBhandary, U. L. (1994). "Initiator Transfer RNAs." J Bacteriol 176(3): 547-552.

Ramakrishnan, V. (2002). "Ribosome structure and the mechanism of translation." Cell 108(4): 557-572.

Ramakrishnan, V. and P. B. Moore (2001). "Atomic structures at last: the ribosome in 2000." Curr. Opin. Struct. Biol. 11(2): 144-154.

Ruff M, K. S., Boeglin M, Poterszman A, Mitschler A, Podjarny A, Rees B, Thierry JC, Moras D. (1991). "Class II aminoacyl transfer RNA synthetases: crystal structure of yeast aspartyl-tRNA synthetase complexed with tRNA(Asp)." Science 252(5013): 1682-9.

Sacerdot, C., C. Chiaruttini, et al. (1996). "The role of the AUU initiation codon in the negative feedback regulation of the gene for translation initiation factor IF3 in Escherichia coli." Mol Microbiol 21(2): 331-346.

Sacerdot, C., G. Fayat, et al. (1982). "Sequence of a 1.26-kb DNA fragment containing the structural gene for *E. coli* initiation factor IF3: presence of an AUU initiator codon." EMBO J. 1(3): 311-315.

Sands, J. F., H. S. Cummings, et al. (1987). "Cloning and mapping of infA , the gene for protein synthesis initiation factor IF1." Nucl. Acids Res. 15(13): 5157-5167.

Schluenzen, F., A. Tocilj, et al. (2000). "Structure of functionally activated small ribosomal subunit at 3.3 A resolution." Cell 102: 615-623.

Schmeing TM, R. V. (2009). "What recent ribosome structures have revealed about the mechanism of translation." Nature 461(7268): 1234-42.

Schweisguth, D. C. and P. B. Moore (1997). "On the conformation of the anticodon loops of initiator and elongator methionine tRNAs." J Mol Biol 267(3): 505-519.

Sette, M., R. Spurio, et al. (1999). "Identification of the ribosome binding sites of translation initiation factor IF3 by multidimensional heteronuclear NMR spectroscopy." Rna-A Publication of the Rna Society. 5(1): 82-92.

Sette, M., P. vanTilborg, et al. (1997). "The structure of the translational initiation factor IF1 from E-coli contains an oligomer-binding motif." EMBO J 16(6): 1436-1443.

Sharma, D., A. R. Cukras, et al. (2007). "Mutational analysis of S12 protein and implications for the accuracy of decoding by the ribosome." J. Mol. Biol. 374(4): 1065-1076.

Shine, J. and L. Dalgarno (1974). "The 3'-terminal sequence of *E. coli* 16S rRNA: Complementarity to nonsense triplets and ribosome binding sites." Proc. Natl Acad. Sci. USA 71(4): 1342-1346.

Singh, N., G. Das, et al. (2005). "Evidence for a role of initiation factor 3 in recycling of ribosomal complexes stalled on mRNAs in Escherichia coli." Nucleic Acids Res. 33(17): 5591-5601.

Sommerville, J. (1999). "Activities of cold-shock domain proteins in translation control." Bioessays 21(4): 319-325.

Spahn, C. M. T. and K. H. Nierhaus (1998). "Models of the elongation cycle: An evaluation." Biol. Chem. 379: 753-772.

Spillmann, S., F. Dohme, et al. (1977). "Assembly *in vitro* of the 50S subunit from *Escherichia coli* ribosomes: Proteins essential for the first heat dependent conformational change." J. Mol. Biol. 115: 513-523.

Steitz, T. and P. Moore (2003). "RNA, the first macromolecular catalyst: the ribosome is a ribozyme." Trends Biochem Sci. 28(8): 411-418.

Stringer, E. A., P. Sarkar, et al. (1977). "Function of initiation factor 1 in the binding and release of initiation factor 2 from ribosomal initiation complexes in Escherichia coli." J. Biol. Chem. 252(5): 1739-44.

Sundari, R. M., E. A. Stringer, et al. (1976). "Interaction of bacterial initiation factor 2 with initiator tRNA." J. Biol. Chem. 251(11): 3338-3345.

Thanedar, S., N. V. Kumar, et al. (2000). "The fate of the initiator tRNAs is sensitive to the critical balance between interacting proteins." J. Biol. Chem. 275(27): 20361-20367.

Ulbrich, B. and K. H. Nierhaus (1975). "Pools of ribosomal proteins in *Escherichia coli*. Studies on the exchange of proteins between pools and ribosomes." Eur. J. Biochem. 57(1): 49-54.

Umekage, S. and T. Ueda (2006). "Spermidine inhibits transient and stable ribosome subunit dissociation." FEBS Lett. 580(5): 1222-1226.

Varshney, U., C. P. Lee, et al. (1993). "From Elongator Transfer RNA to Initiator Transfer RNA." Proc Natl Acad Sci USA 90(6): 2305-2309.

Weber, M. H., C. L. Beckering, et al. (2001). "Complementation of cold shock proteins by translation initiation factor IF1 in vivo." J Bacteriol 183(24): 7381-6.

Wilson, D. N. and K. H. Nierhaus (2003). "The ribosome through the looking glass." Angew. Chem. Int. Ed. Engl. 42(30): 3464-3486.

Wimberly, B. T., D. E. Brodersen, et al. (2000). "Structure of the 30S ribosomal subunit." Nature 407: 327-339.

Wittek, D. (2009). The bacterial ribosome: Testing a new concept for translational initiation. Biology Department, Berlin, FU Berlin.

Youngman, E. M., L. Cochella, et al. (2006). "Two distinct conformations of the conserved RNA-rich decoding center of the small ribosomal subunit are recognized by tRNAs and release factors." Cold Spring Harb Symp Quant Biol 71: 545-549.

Zavialov, A. V., R. H. Buckingham, et al. (2001). "A posttermination ribosomal complex is the guanine nucleotide exchange factor for peptide release factor RF3." Cell 107(1): 115-124.

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For reasons of data protection, the curriculum vitae is not published in the online version

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- *Mondal T, Ray U, Manna AK,* ***Gupta R****, Roy S, Das S (2008): Structural determinant of human La protein critical for internal initiation of translation of hepatitis C virus RNA.* Journal of Virology*.; 11927-38, Vol.82, No, 23*