Identification and Characterization of a Novel Protein Factor from *Pisum sativum* Associated with The Translation Machinery

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CERTIFICATE

This is to certify that this thesis entitled "*Identification and Characterization of a Novel Protein Factor from Pisum sativum Associated with The Translation Machinery*" comprises the work done by Ms. Sheeba Rasheedi under our guidance. This work is original and has not been submitted in part or full for any degree or diploma of any University.

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DECLARATION

I hereby declare that the work presented in this thesis, "*Identification and Characterization of a Novel Protein Factor from Pisum sativum Associated with The Translation Machinery*" has been carried out by me under the supervision of Prof. Seyed E. Hasnain (Thesis Supervisor), Centre for DNA Fingerprinting and Diagnostics, Hyderabad, India and Dr. Nasreen Z. Ehtesham (Thesis Co-supervisor), National Institute of Nutrition, Hyderabad, India. This work is original and has not been submitted in part or in full for any degree or diploma of any other University earlier.

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In the memory of Showkat

Dedicated to My Parents and Lubna

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Sheeba Rasheedi

CONTENTS

ABBREVIATIONS

APS	:	ammonium persulfate
ATP	:	adenosine triphosphate
bp	:	base pair(s)
BSA	:	bovine serum albumin
°C	:	degree celsius
cDNA	;	complementary deoxyribonucleic acid
CM	:	complete medium
cm^2	:	centimetre square
CMV	:	Cytomegalovirus
cpm	:	counts per minute
C terminal	:	carboxy terminal
Ci	:	Curie
dGTP	:	deoxyguanosine triphosphate
DNA	:	deoxyribonucleic acid
DNAse	:	deoxyribonuclease
dNTP	:	deoxynucleotide triphosphate
DTT	:	dithiothreitol
EDTA	:	ethylenediamine tetraacetic acid disodium salt
EF	:	Elongation factor
eIF5B	:	A eukaryotic translation initiation factor
EMSA	:	electrophoretic mobility shift assay
EtBr	:	ethidium bromide
FBS	:	fetal bovine serum
g	:	gram(s)
GFP	:	Green fluorescent protein
GTP	:	guanosine triphosphate

hr(s)	:	hour(s)
HEPES	:	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
h.p.i	:	hours post infection
IF	:	Initiation factor
kb	:	kilo basepairs
kDa	:	kilo Dalton(s)
lit	:	litre
LB	:	Luria Bertani
MCS	:	multiple cloning site
□Ci	:	micro Curie
□l	:	micro litre
ml	:	milli litre
M	:	molar
mA	:	milli amperes
min(s)	:	minute(s)
□M	:	micro molar
mM	:	milli molar
mm	:	milli metre
mRNA	:	messenger RNA
mV	:	milli volts
N	:	normal
ng	:	nano gram
NLS	:	Nuclear localization signal
nM	:	nano molar
nm	:	nano metre
NMD	:	Nonsense-mediated decay
NMR	:	nuclear magnetic resonance
NP-40	:	Nonidet P-40
N-terminal	:	amino terminal
OB fold	:	oligonucleotide-binding fold
ORF	:	open reading frame

PAGE	:	polyacrylamide gel electrophoresis
PBS	:	phosphate buffered saline
PCR	:	polymerase chain reaction
PMSF	:	phenylmethylsulfonyl fluoride
PTC	:	premature termination codon
RF	:	Release factor
RNA	:	ribonucleic acid
RNase	:	ribonuclease
RT	:	room temperature (ambient temperature)
rpm	:	revolutions per minute
s	:	second(s)
SDS	:	sodium dodecyl sulfate
SD		Shine-Dalgarno
TC	:	ternary complex
tRNA	:	transfer RNA
Tris	:	tris(hydroxymethyl)aminomethane
TAE	:	tris-acetate-EDTA buffer
TBE	:	tris-borate-EDTA-buffer
TE	:	10mM Tris-Cl, 1mM EDTA, pH 8.0
TEMED	:	N,N,N',N' tetramethylethylenediamine
TLC	:	Thin layer chromatography
U	:	units
UV	:	ultraviolet
V	:	volts
W	:	watts
x g	:	times (the force of) gravity

Chapter 1

REVIEW OF LITERATURE

Chapter 1: REVIEW OF LITERATURE

Contents

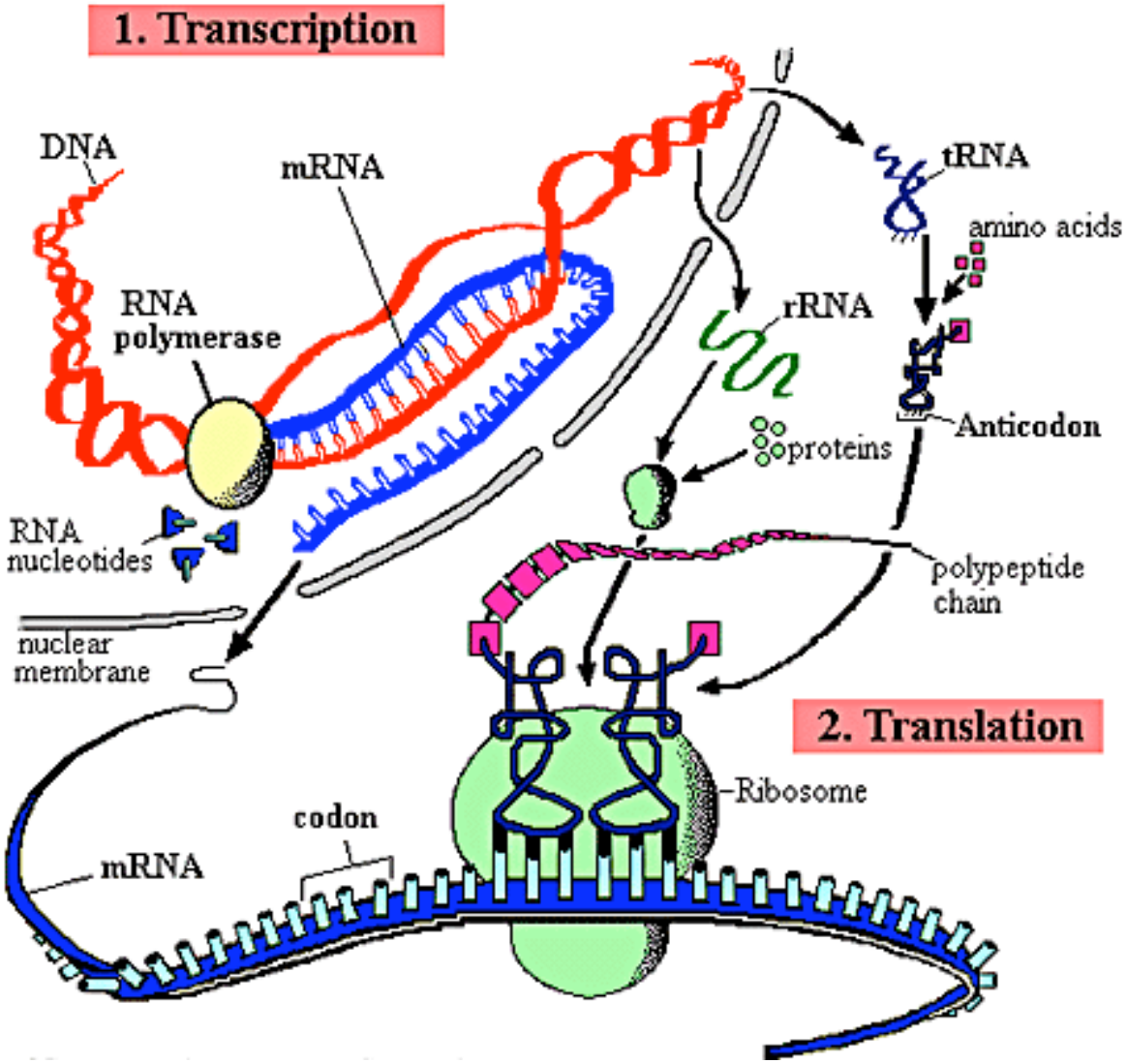
REVIEW OF LITERATURE

1.1 What is Translation

Proteins are the end products of most information pathways. Genes carry the information for the type of proteins synthesized by the cell. However, deoxyribonucleic acid (DNA) is not the direct template for the protein synthesis. Rather this information is relayed through ribonucleic acid (RNA) molecules. The process whereby DNA encodes for the production of amino acids and proteins can be divided into two parts (Figure 1.1):

(1) Transcription- Transcription is the process through which a DNA sequence is enzymatically copied by an RNA polymerase to produce a complementary RNA.
(2) Translation- Translation is the synthesis of polypeptide under the direction of messenger RNA (mRNA).

This completes the simple genetic code, the relation between the sequence of bases in DNA and the sequence of amino acids in a protein. Translation is a more complex process than replication and/or transcription that involve simple copying of bases on the basis of complementarity. The process of translation involves an intricate machinery of high complexity. At this step the nucleotide sequence of mRNA is converted into the amino acid sequence of the protein. Protein synthesis can account for upto 90% of the chemical energy used by a cell for all biosynthetic reactions. Despite the great complexity, proteins are made at exceedingly high rates. The rate at which ribosomes assemble on the mRNA is in the order of seconds, although it is specific for each mRNA. The mRNA is translated on the ribosomes at a rate of about 12 amino acids per second (Kennell and Riezman, 1977).



Source:
http://www.accessexcellence.org/RC/VL/GG/protein_synthesis.html

FIGURE 1.1*: Flow of information from DNA to RNA to protein. DNA is transcribed into RNA by RNA polymerase in the nucleus of a eukaryotic cell. The cellular translation machinery translates the genetic code carried in the mRNA into polypeptide chain in the cytoplasm.*

1.2 Translation: Prokaryotes vs Eukaryotes

The underlying principle of translation and its basic mechanism is somewhat same in prokaryotes and eukaryotes. However, eukaryotic protein synthesis involves more protein factors participating in more complex and intricate steps. Some highlights are as follows:

(1) Prokaryotic ribosomes are smaller than that in eukaryotes. Bacterial ribosomes have a sedimentation rate of 70S and a molecular weight of 2700 kDa. The 70S ribosome is composed of a large 50S and a small 30S subunit. Eukaryotic ribosomes consist of a 60S large subunit and a 40S small subunit that together form an 80S particle with a mass of 4200 kDa. The 40S subunit of eukaryotes contains an 18S RNA that is homologous to the prokaryotic 16S RNA. The 60S subunit contains three RNAs: The 5S and 28S RNAs are the counterparts of the prokaryotic 5S and and 23S molecules; its 5.8S RNA is unique to eukaryotes.
(2) In prokaryotes, the first amino acid to be incorporated is *N*-formylmethionine whereas in eukaryotes the initiating amino acid is methionine. However, in both the systems at the initiation step a special transfer RNA (tRNA) is involved known as initiator tRNA that brings the first amino acid.
(3) In prokaryotes, the initiating codon is AUG that is preceded by a purine rich sequence known as “Shine-Dalgarno sequence”, 5-7 base pairs towards the 5’side to distinguish initiator AUGs from internal ones. In eukaryotes, on the other hand, the first AUG from the 5’end of mRNA is read as the initiation codon. A eukaryotic mRNA has only one start site and hence encodes a single polypeptide whereas in prokaryotes, mRNA can have multiple start sites and can act as template for the synthesis of many proteins.
(4) In terms of the complexity of the translation machinery involved, the system is more intricate in eukaryotes than in prokaryotes. The number of protein factors employed for this task at all the steps of translation in eukaryotes is enormous.

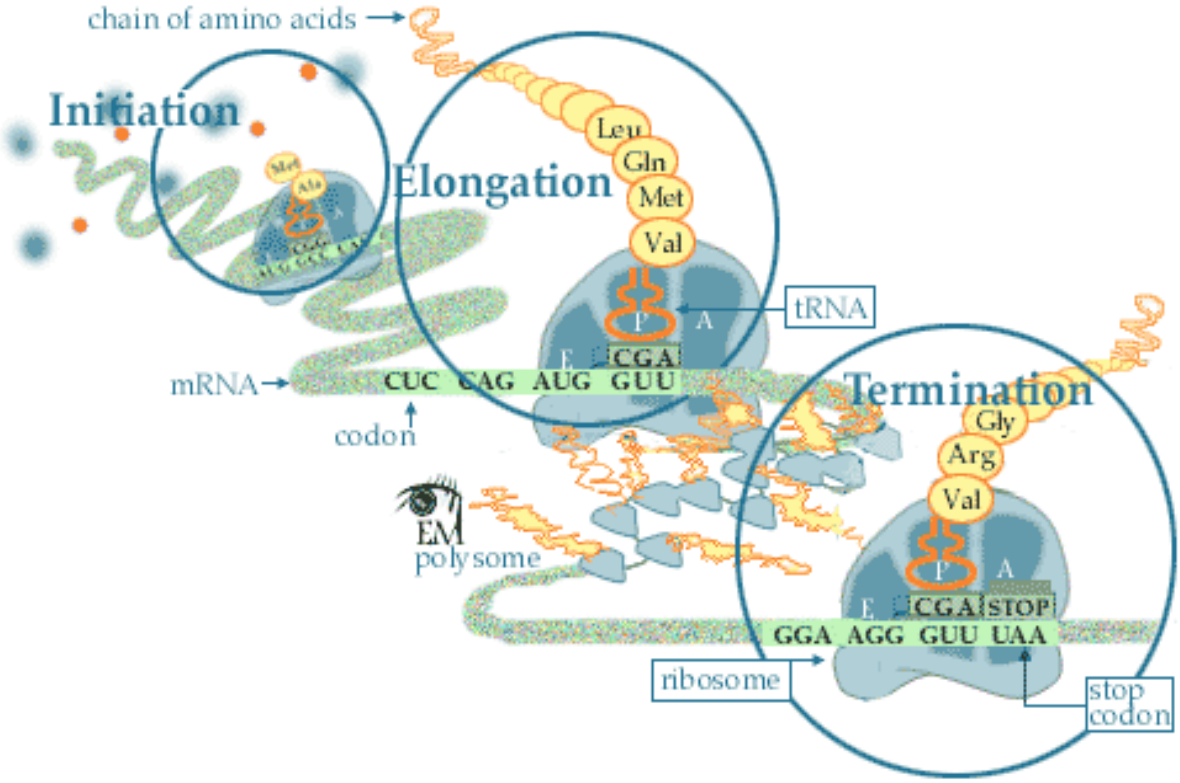
1.3 Steps in Translation

Protein synthesis takes place in three distinct stages: initiation, elongation and termination (Figure 1.2).

1.3.1 Translation Initiation

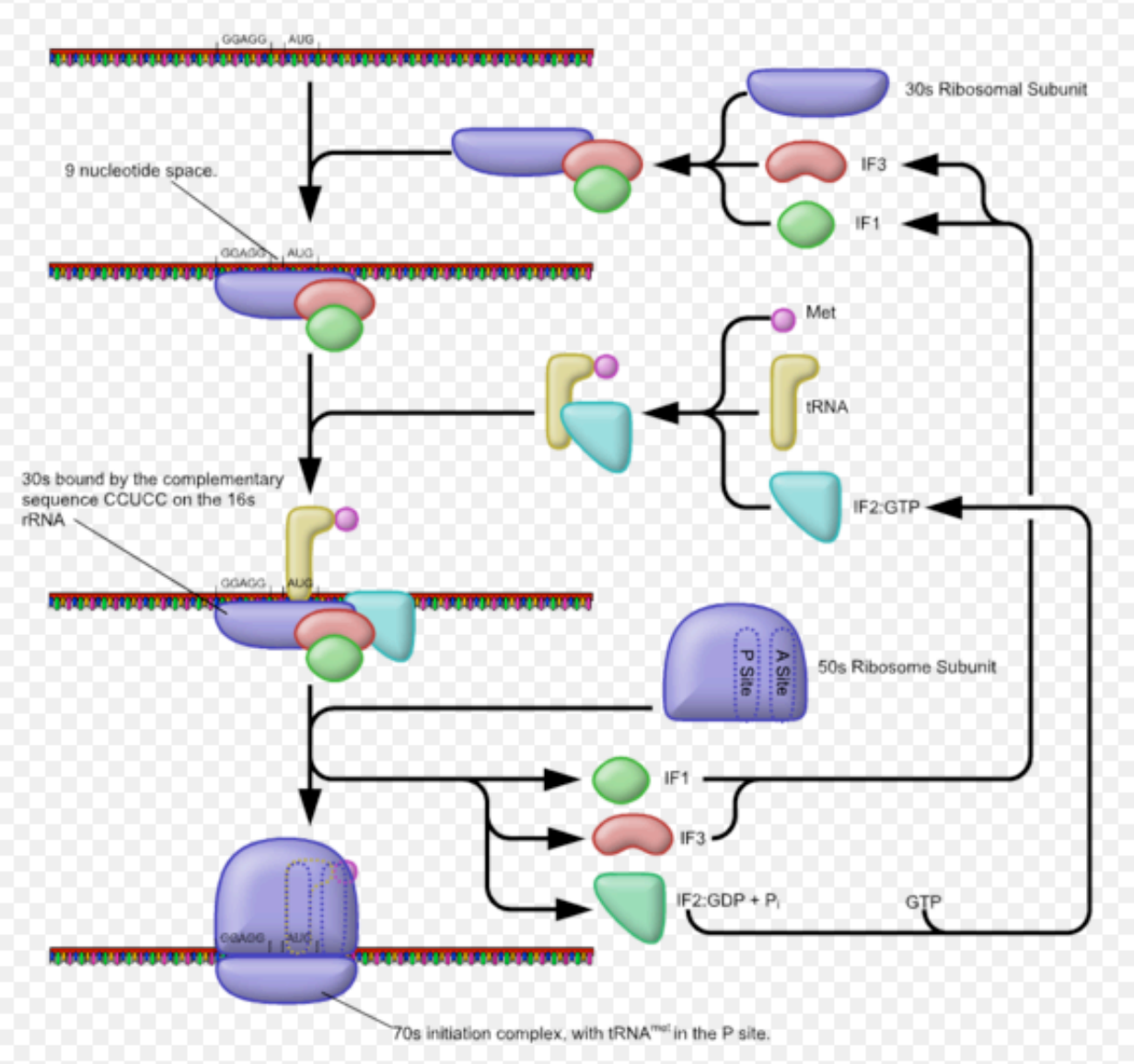
The first step in translation is initiation. During this step, initiation complex is formed where the initiator tRNA is bound to the initiator codon on the mRNA and to the P (peptidyl) site on the ribosome. Three distinct tRNA binding sites are present on ribosomes: the aminoacyl (A), peptidyl (P) and exit (E) sites. In turn, ribosome is bound to the specific initiation site on the mRNA. It is at this step that the reading frame on the mRNA is recognized by the translation machinery that is determined by the position of the initiation codon. This results in the accurate translation of mRNA into a biologically active protein. Various translation initiation factors (IFs) are involved in this step. Considerable progress has been made in recent years to expand our understanding of these factors and the roles they perform. High-resolution, well-defined three-dimensional structures of the initiation factors and other components of the translation machinery contributed largely to the study of their molecular mechanisms (Ban *et al*., 1999; Cate *et al*., 1999; Clemons *et al*., 1999). The initiation of translation can be divided into several sequential steps that show fine differences between prokaryotes and eukaryotes (Figure 1.3 and 1.4, respectively).

In prokaryotes: Translation in prokaryotes is coupled to transcription. Also the mRNAs are generally polycistronic carrying several translation initiation and termination sites. Hence, the translation complex must read the initiation signals spread along the mRNA at several different positions. The initiator AUG is differentiated from other AUGs by two mechanisms: (1) The internal AUGs are masked by the secondary structure of mRNA and hence escape interaction with 30S ribosomal subunit. As a tool for translational control, the



Source: www.il.mahidol.ac.th

FIGURE 1.2*: Translation proceeds in three phases: Initiation, elongation and termination. Initiation involves binding of the small ribosomal subunit to 5' end of mRNA with the help of initiation factors. Elongation occurs when the next charged aminoacyl-tRNA binds to the ribosome along with GTP and an elongation factor. Termination of the polypeptide is executed when the release factors recognize a stop codon in the mRNA.*



Source:
http://en.wikipedia.org/wiki/Translation_(genetics)#Basic_mechanisms

***FIGURE 1.3**: Translation initiation in prokaryotes. It results in the assembly of the ribosomal subunits at the initiation codon and binding of charged initiator tRNA to the ribosomal P site through codon-anticodon base pairing with mRNA in presence of translation initiation factors- IF1, IF2 and IF3.*

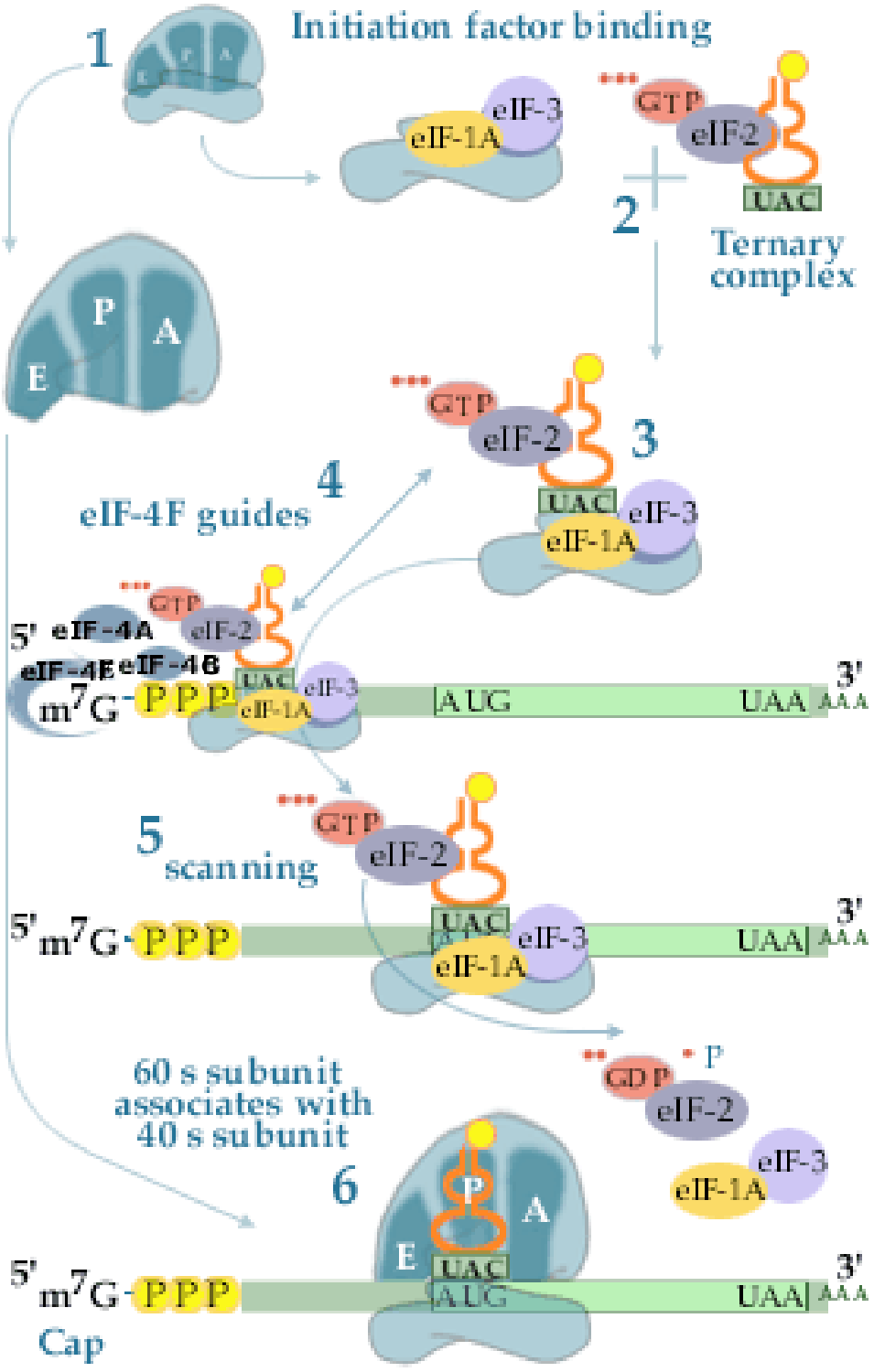


FIGURE 1.4: *Translation initiation in eukaryotes. eIF1A and eIF3 bind to 40S ribosomal subunit. GTP-bound eIF2 brings charged $tRNA_i$ in the form of ternary complex to the P site on the ribosome. eIF4F assisted scanning of the mRNA results in the docking of 40S preinitiation complex on the initiation codon. Finally all the factors are released followed by reunion of the 40S and 60S subunits.*

stabilization/destabilization of the secondary structure of mRNA near initiator AUG is used in several cases (Simons and Grunberg-Manago, 1998). (2) The sequence specific interaction of mRNA with both ribosomal RNA (rRNA) and anticodon of fMet-$tRNA_f^{Met}$. One such RNA-RNA interaction is between the purine-rich Shine-Dalgarno (SD) sequence on the mRNA and a complementary pyrimidine-rich motif at the 3' terminus of 16S rRNA known as anti-Shine-Dalgarno (ASD) sequence. This mRNA-rRNA interaction was first shown by Steitz and Jakes (1975) and later demonstrated *in vivo* (Hui and de Boer, 1987; Jacob *et al*., 1987). Initiation of translation in prokaryotes involves the assembly of the components of the translation system that include: the two ribosomal subunits (30S and 50S), the mRNA to be translated, the initiator tRNA charged with the first amino acid, energy rich GTP molecules and an array of initiation factors that help in the formation of a functional initiation complex. In prokaryotes, the formation of initiation complex follows the following steps:

1. Initiation of translation begins with the binding of IF3 to the smaller 30S ribosomal subunit at the E-site. Binding of IF3 to the 30S ribosomal subunit promotes dissociation of the ribosome into its constituent subunits (Petrelli *et al*., 2001). *E.coli* IF3, encoded by *infC* gene, is a 20.4 kDa polypeptide of 180 amino acids (Olsson *et al*., 1996; Sacerdot *et al*., 1982). IF3 is composed of two structural domains of approximately equal size. The structures of the amino- and carboxy-terminal halves of IF3 were solved by nuclear magnetic resonance (NMR) (Garcia *et al*., 1995a,b). IF3 has two globular □/□ domains joined by an □-helical linker (Biou *et al*., 1995). The IF3N domain consists of a globular □/□-fold, with helix □1 packed against a mixed five-strand □-sheet. This fold is followed by helix □2 that connects IF3N to IF3C. IF3C is composed of two-layer □/□ sandwich fold composed of a four-strand mixed □-sheet packed against two parallel □-helices □3 and □4), leading to a □□□□□□ topology. The N-terminal domain was suggested to interact with RNA while the C-terminal domain, apart from binding to the 30S ribosomal subunit, prevents the association of ribosomal subunits. IF3 has several functions to perform: (1) It binds to 30S ribosomal subunit and in turn prevents the

association of 50S subunit with 30S subunit (Grunberg-Manago *et al*., 1975; Sacerdot *et al*., 1996). (2) Checks the codon-anticodon interaction and in cases of incorrect amino-acyl tRNA (noninitiator tRNA) bound to initiation codon or initiator tRNA bound to codons other than AUG, GUG and UUG in the P-site, IF3 destabilizes the initiation complex (Hartz *et al*., 1990, 1989; Haggerty and Lovett, 1997; Meinnel *et al*, 1999; Sussman *et al*., 1996). (3) It accelerates the formation of codon-anticodon interaction at the ribosomal P-site (Gualerzi *et al*., 1977; Wintermeyer and Gualerzi, 1983). (4) Helps in the positioning of mRNA from standby site to the decoding P-site of the 30S ribosomal subunit (La Teana *et al*., 1995). (5) IF3 participates in the dissociation of deacylated tRNAs from posttermination complexes and in the dissociation of 70S ribosomes into their constituent subunits (Hirokawa *et al*., 2002; Karimi *et al*., 1999). By *in vitro* studies the IF3C domain was found to support all the functions that the full-length protein can carry out while IF3N domain modulates the thermodynamic stability of the IF3-30S complex (Petrelli *et al*., 2001). In the cell, the two subunits of the ribosome (30S and 50S) occur in equilibrium with the 70S ribosome (Godefroy-Colburn *et al*., 1975). IF3 binding to the 30S subunit prevents the association of the two. Next is the binding of IF1 to the "native" 30S subunit (Zucker and Hershey, 1986). IF1 binds specifically to the base of the A-site on the ribosome and hence directs the initiator tRNA to the ribosomal P-site by blocking the A-site for the binding of amino acid laden tRNAs during the elongation step of translation (Carter *et al*., 2001; Dahlquist and Puglisi, 2000). IF1 stimulates the activity of IF3 and hence also the dissociation of the ribosomal subunits (Gualerzi and Pon, 1990; Dottavio-Martin *et al*., 1979). IF1 is also known to enhance the association rate for 70S ribosome mainly by stimulating the activity of IF2 (Pon and Gualerzi, 1984). IF1 also favors binding of IF2 on the 30S ribosomal subunit and hence the release of IF2 is facilitated after IF1 is removed during late translation initiation step (Celano *et al*., 1988; Moreno *et al*., 1999; Stringer *et al*., 1977). IF1 also helps IF2 in its proof reading activity that restricts the entry to the P-site for only initiator tRNA and ensures that it interacts with the initiation codon of the mRNA (Canonaco *et al*., 1986; Hartz *et al*., 1990; Meinnel *et al*., 1999; Wu

and RajBhandary, 1997). IF1 is encoded by *infA* gene and is the smallest among all the three initiation factors with a molecular mass of 8.2kDa. The solution structure of IF1 was solved by NMR (Sette *et al*., 1997). It consists of a five-strand beta barrel with the loop between strands 3 and 4, capping one end of the barrel. There is structural and functional resemblance between IF1 and the cold shock proteins both belonging to the family of oligonucleotide-binding (OB) fold proteins (Weber *et al*., 2001). IF1 binds in a cleft between the 530 loop and helix 44 of 16S RNA and ribosomal protein S12. Following subunit dissociation, mRNA binds to the 30S subunit of ribosome such that the initiation codon (AUG) binds at specific position on 30S subunit guided by the SD sequence. This 4-9 purine rich sequence base-pairs with a complementary sequence near the 3' end of the 16S rRNA of the 30S subunit (Yusupova *et al*., 2001). The initiator factors (especially IF3) are responsible for adjusting the AUG codon in the P-site of the ribosome (La Teana *et al*., 1995).

2. In the second step of the initiation process, IF2, GTP and formyl-methionine charged initiator tRNA (fMet-$tRNA_f^{Met}$) bind to the pre-existing assembly of 30S ribosomal subunit, IF3, IF1 and mRNA (Gualerzi and Pon, 1990; Wu *et al*., 1996; Wu and RajBhandary, 1997). IF2, mRNA and fMet-$tRNA_f^{Met}$ associate with the 30S ribosomal subunit in an unknown and possibly random order. The initiator tRNA recognizes the mRNA through the codon-anticodon base-pairing and binds to the P site on the ribosome (Malhotra *et al*., 1998). The initiator tRNA is positioned on the 30S ribosomal subunit in three steps: codon-independent binding, codon-dependent binding and fMet-$tRNA_f^{Met}$ adjustment (Tomsic *et al.,* 2000). The binding of fMet-$tRNA_f^{Met}$ is promoted and stabilized by IF2 and IF3 (Mangroo *et al*., 1995). Furthermore, IF3 proofreads any mismatched codon-anticodon interaction (Gualerzi *et al*., 1977). The structure of full-length IF2 has not been solved due to problems of crystallization of the protein. Attempts have been made to define different functional domains of IF2 by partial protease fragmentation (Spurio *et al*., 1993; Vornlocher *et al*., 1997; Monero *et al*., 1999). Six domains are assigned: Domain I is suggested to be located between the head

and the platform in a solvent exposed region hence contributing to the high index of hydrophilicity shown by IF2 (Sacerdot *et al*., 1984). Deletion studies as well as inhibitory effect of mAb with epitopes within IF2 domain II led to the conclusion that domain II directly interacts with the 30S ribosomal subunit (Moreno *et al*., 1998). IF2 domain III acts as a bridge between Domain II and IV (Mortensen *et al*., 1998). Domain IV of IF2 is located opposite of the 30S platform that allows close contact with the 50S L7/L12 stalk. IF1 interacts with IF2 domains III-V and hence stimulates the binding of IF2 to the 30S ribosomal subunit. It is also found that domain V of IF2 interacts with the T-arm of the fMet-$tRNA_f^{Met}$ (Yusupova *et al*., 1996). The IF2 domain VI at the extreme C-terminus is involved in the interaction with fMet-$tRNA_f^{Met}$ (Gualerzi *et al*., 1991). Brock *et al*. (1998) presented a model that suggests interaction between IF1 and IF2 that resembles domains IV and V in EF2 that in turn mimics the tRNA structure. The complex of IF1-IF2 blocks the A site on the ribosome hence making this site unavailable for the initiator tRNA. Binding of IF2 to the A site of the 30S ribosome may also help to position the 50S ribosomal subunit during the initiation complex formation.

3. The assembly of 30S ribosomal subunit, the three initiation factors, and mRNA in a standby position together constitute the 30S preinitiation complex where fMet-$tRNA_f^{Met}$ is bound in a codon-independent manner. This unstable complex undergoes a conformational change that results in codon-anticodon interaction and forms the stable 30S initiation complex (Gualerzi *et al*., 1977; Pon and Gualerzi; 1984). Lastly, to complete the initiation complex, 50S subunit binds with the simultaneous release of IF1 and IF3. IF2 stimulates the association of the 50S ribosomal subunit to the complex. fMet-$tRNA_f^{Met}$ is adjusted to the correct position in the P-site. 70S ribosome catalyzes GTP hydrolysis into GDP and P_i with the release of IF2•GDP. Now this 70S initiation complex enters the elongation phase.

In eukaryotes: Initiation of protein synthesis in the cytoplasm of eukaryotes is in some ways similar to prokaryotic initiation. Both involve certain common steps:

Translation initiation starts with the dissociation of ribosomes into its subunits, the participation of a unique and specific initiator tRNA, mostly the recognition of the same initiation codon (AUG), the assembly of several translation components to form the initiation complex on the small ribosomal subunit and the participation of several translation initiation factors. However, there are many differences between the prokaryotes and eukaryotes. In eukaryotes, the protein synthesizing machinery occurs in the cytoplasm. Hence, protein synthesis is uncoupled both temporally and spatially from transcription. Before the step of translation, the mRNA is transcribed in the nucleus using DNA as the template. This is followed by the post-transcriptional modifications of the mRNA: capping, splicing, polyadenylation etc. The processed mRNA is then transported to the cytoplasm for participating as a template in the process of translation. The mature mRNA is exported into the cytoplasm through nuclear pores, where it emerges as a messenger ribonucleoprotein (mRNP) complex. It is proposed that mRNA in mRNP particles is highly ordered and structured, ruling out the possibility of recognition of the initiation sites by a similar mechanism as employed by prokaryotes. Rather, eukaryotes employ an entirely different mechanism for reading the initiation codon. Initiation of translation involves interaction of ribosomes with 5'cap (m7GpppN) followed by scanning of the 5' untranslated region (5'UTR) for an initiating AUG start codon. This scanning model is referred to as "cap-dependent translation initiation" (Kozak and Shatkin, 1978). It involves recognition of the m^7G-cap structure at the 5'terminus of the mRNA, binding of the 40S ribosomal subunit and scanning the downstream sequence for the initiation codon. However, in many eukaryotic and viral mRNAs, as opposed to ribosomal scanning an alternate mechanism exists that involves internal initiation. These mRNAs carry internal ribosomal entry sites (IRES) that are recognized by the ribosomes in a cap-independent manner. Translation starts at the very first AUG codon downstream of the IRES. Another feature of eukaryotic translation initiation is the involvement of an array of translation initiation factors. Eleven or more initiation factors are reported till date (Table 1.1). The sequence of events in eukaryotic translation initiation is as follows:

Table 1.1

Initiation Factor	Activity
eIF-1	repositioning of met-tRNA to facilitate mRNA binding
eIF-2	ternary complex formation
eIF-2A	AUG-dependent met-$tRNA_{met}^{i}$ binding to 40*S* ribosome
eIF-2B (also called GEF) guanine nucleotide exchange factor	GTP/GDP exchange during eIF-2 recycling
eIF-3 composed of ~10 subunits	ribosome subunit antiassociation, binding to 40*S* subunit
Initiation factor complex often referred to as eIF-4F composed of 3 primary subunits: eIF-4E, eIF-4A, eIF-4G and at least 2 additional factors: PABP, Mnk1 (or Mnk2)	mRNA binding to 40*S* subunit, ATPase-dependent RNA helicase activity, interaction between polyA tail and cap structure
PABP: polyA-binding protein	binds to the polyA tail of mRNAs and provides a link to eIF-4G
Mnk1 and Mnk2 eIF-4E kinases	phosphorylate eIF-4E increasing association with cap structure
eIF-4A	ATPase-dependent RNA helicase
eIF-4E	5' cap recognition
4E-BP (also called PHAS) 3 known forms	when de-phosphorylated 4E-BP binds eIF-4E and represses its' activity, phosphorylation of 4E-BP occurs in response many growth stimuli leading to release of eIF-4E and increased translational initiation to
eIF-4G	acts as a scaffold for the assembly of eIF-4E and -4A in the eIF-4F complex, interaction with PABP allows 5'-end and 3'-ends of mRNAs to interact
eIF-4B	stimulates helicase, binds simultaneously with eIF-4F
eIF-5	release of eIF-2 and eIF-3, ribosome-dependent GTPase
eIF-6	ribosome subunit antiassociation

Source:
http://web.indstate.edu/thcme/mwking/protein-synthesis.html

TABLE 1.1*: List of translation initiation factors participating in eukaryotic translation initiation process.*

(1) At the physiological level of magnesium ion in the cell (1-2mM), 80S ribosomes are the most prominent species. To initiate the process of translation, the 80S ribosome has to dissociate into its constituent subunits. This step is promoted by two initiation factors, eIF1A and eIF3. These two factors bind to the 40S subunit and prevent its association with the 60S subunit by steric hindrance (Goumans *et al*., 1980) or by inducing an allosteric effect due to a change in the structure of the 40S subunit upon eIF3 binding (Srivastava *et al*., 1992). eIF1A is a small, stable protein (17-22kDa). It is one of the most highly conserved translation initiation factors (>65%) (Dever *et al*., 1994) and is necessary for viability in yeast (Wei *et al*., 1995). Yeast eIF1A shows 21% sequence identity with *E.coli* initiation factor IF1 (Kyrpides and Woese, 1998a, b). eIF1A is also involved in the binding of initiator tRNA to 40S ribosomes and in mRNA binding and scanning (Pestova *et al.,* 1998). eIF1A interacts with eIF5B (Schreier *et al*., 1977) hence mimicking the binding of IF1 to IF2 (Boileau *et al*., 1983). The three dimensional structure of the human factor has been determined by NMR spectroscopy (Battiste *et al*., 2000). The solution structure of eIF1A reveals an oligonucleotide-binding (OB) fold and an additional domain. The proposed modular structure for eIF1A suggests an IF1-related domain that mediates ribosome binding and is flanked by C-terminal segments involved in ternary complex binding and interaction with eIF5B. The N-terminal domain contacts other initiation factors *viz*., eIF2 and eIF3 on the ribosome (Olsen *et al*., 2003). The mammalian eIF3 is a high molecular-weight complex with a molecular mass of 600 kDa, composed of eleven subunits, namely, p170, p116, p110, p66, p48, p47, p44, p40, p36, p35 and p28. It is shown to be involved in 80S ribosome dissociation, initiator tRNA and mRNA interaction with 40S ribosomal subunit and organization of initiation complex through interaction with other initiation factors viz., eIF1 (Fletcher *et al*., 1999), eIF5 (Bandyopadhyay and Maitra, 1999), eIF4B (M é thot *et al*., 1996), eIF4G (Lamphear *et al*., 1995; Mader *et al*., 1995). eIF1 was coimmunoprecipitated with p93 subunit of yeast eIF3 (Asano *et al*., 1998). After ribosomal subunit dissociation, another translation initiation factor, eIF6, then binds to the 60S

subunit and prevents its reassociation with 40S subunit (Russell and Spremulli, 1979; Raychaudhuri *et al*., 1984).

(2) A ternary complex is formed between initiator Met-tRNA$_i$, eIF2 and GTP before binding to ribosomes. Ternary complex formation requires GTP and is inhibited by GDP. Such a complex is prepared and detected *in vitro* by filtration through nitrocellulose membrane at physiological magnesium ion concentration in the absence of other components of the translation machinery. This initiator tRNA is differentiated from other tRNAs by methionyl residue and the A-U base pair at the end of acceptor stem (recognized by eIF2) and three G-C base pairs in their anti-codon stems (for ribosome binding). eIF2 is composed of three non-identical subunits: α, β and γ. It is the γ subunit that is involved in GTP- and Met-tRNA$_i$-binding. At the end of initiation, eIF2 leaves the ribosome as a binary complex with GDP. For the conversion of the inactive eIF2•GDP form to the active eIF2•GTP species eIF2B is required. The ternary complex binds to the 40S ribosomal subunit to form 40S preinitiation complex (also called 43S initiation complex). This complex is stabilized by eIF3 and eIF1A. Binding of the initiator tRNA to the 40S ribosomal subunit precedes mRNA binding. This was confirmed by the detection of 40S preinitiation complex lacking mRNA in rabbit reticulocytes (Smith and Henshaw, 1975). The regulation of Met-tRNA$_i$ binding to 40S ribosomal subunit is an important step of regulation of protein synthesis. eIF2 is reported from animals, plants, insects and eukaryotic microorganisms. It is found to be highly conserved with homologues found even in archaea but not in eubacteria. The concentration of eIF2 in HeLa cells is about 1 μM, with 0.5 molecule of eIF2 per ribosome (Duncan and Hershey, 1983). No three-dimensional structural information is available for eIF2 till date. The amino-terminal of eIF2α subunit is involved in AUG recognition while the β subunit has three lysine blocks near its amino-terminus that are implicated in binding to eIF2B and eIF5 (Asano *et al*., 1999) and also to mRNA (Flynn *et al*., 1994; Laurino *et al*., 1999). Near the carboxyl end, eIF2β subunit carries a zinc-finger motif without zinc ions that participate in the recognition of the initiation codon. eIF2γ subunit is

homologous to bacterial SelB and EF1A, as well as to other G-proteins. Inspite of similar function, the eIF2 subunits including eIF2 do not resemble bacterial IF2 except for the GTP-binding domain. eIF2 shows interaction with GTP and Met-$tRNA_i$ (Gaspar *et al*., 1994). Mutant forms of eIF2 in yeast are reported to show lesser GTP and Met-$tRNA_i$ binding (Harashima and Hinnebusch, 1986; Erickson and Hannig, 1996).

(3) The m^7G-cap structure is recognized by a heterotrimeric complex, eIF4F that consists of eIF4E, eIF4G and eIF4A. The binding of eIF4F to the m^7G-cap of mRNA commits the translational apparatus to the translation of that mRNA. eIF4F plays an important role in selecting an mRNA for translation. eIF4F along with eIF4B melts the secondary structure in the 5'-proximal region of the mRNA by their ATP-dependent RNA helicase activity (Rozen *et al*., 1990). It is a prerequisite for the binding of 40S preinitiation complex to mRNA. The helicase activity is further increased by eIF4H (Rogers *et al*., 1999). Following removal of the secondary structures from 5'-terminal region of mRNA, eIF4G interacts with eIF3 (bound to the 40S subunit) to bring the ribosome to mRNA.

(4) After the 40S preinitiation complex is bound to the m^7G-cap region of the mRNA, the ribosome binds and scans downstream along the mRNA from the 5' terminus towards the initiation codon. This scanning process consumes ATP. It is not known when eIF4F dissociates from the m^7G cap and eIF3 during scanning. Once the initiation codon is reached, the ribosome binds stably through RNA-RNA interaction between the AUG and the anticodon of the bound initiator tRNA to form the 48S complex (Kozak, 1999). Mutation of the Met-$tRNA_i$ anticodon disables the tRNA to recognize the AUG as the initiation codon and may read a new cognate codon instead (Cigan *et al*., 1988). The context of the AUG also plays a major role in defining the initiation site as AUG codon in a poor context is bypassed and scanning may continue till a better AUG is encountered. The presence of RNA secondary structure downstream from the initiation codon can also facilitate translation initiation (Kozak, 1990). The initiation factors also help

to recognize the AUG codon. Mutations in the yeast genes encoding eIF1, eIF5 or any of the three subunits of eIF2 allow ribosomes to initiate at UUG instead of AUG.

(5) The eIF5-promoted hydrolysis of GTP bound to eIF2 is the next step to follow. GTP hydrolysis results in conformational changes in the factor. The GDP-bound eIF2 has lesser affinity for the 40S ribosomal subunit and hence is ejected out. Ejection of eIF2 and associated factors such as eIF3 prepares the 48S initiation complex for the next step.

(6) After the dissociation of the bound initiation factors from 48S initiation complex, the 60S subunit can bind to the resulting 40S initiation complex. This step requires the activity of eIF5B•GTP (Pestova *et al*., 2000). eIF5B is a 150-160 kDa protein (Schreier *et al*., 1977; Benne *et al*., 1978, Merrick 1979) that acts catalytically with GTP to convert preformed 40S initiation complex into 80S initiation complex. GTP hydrolysis is not required for the association of the two subunits but to facilitate dissociation of eIF5B for its recycling. Hence, in eukaryotic translation step two GTP hydrolysis reactions are involved: one with GTP associated with eIF2; other with GTP bound to eIF5B. eIF5B may thus bind to the A-site on the ribosome, help in placing the 60S ribosomal subunit properly on 40S subunit and may also direct initiator tRNA binding to the P site.

1.3.2 Translation Elongation

Translation elongation involves binding of an aminoacyl-tRNA to the A (aminoacyl) site on the ribosome (Figure 1.5). It is followed by the formation of a peptide bond between the amino group of the incoming aminoacyl-tRNA at the A-site and the carboxyl group of the amino acid carried by the initiator tRNA at the P-site. This dipeptide laden tRNA then moves from the A-site to the P-site that is vacated by the initiator tRNA. Consequently, the initiator tRNA moves to the E (exit) site before leaving the ribosome. An aminoacyl-tRNA then binds to the

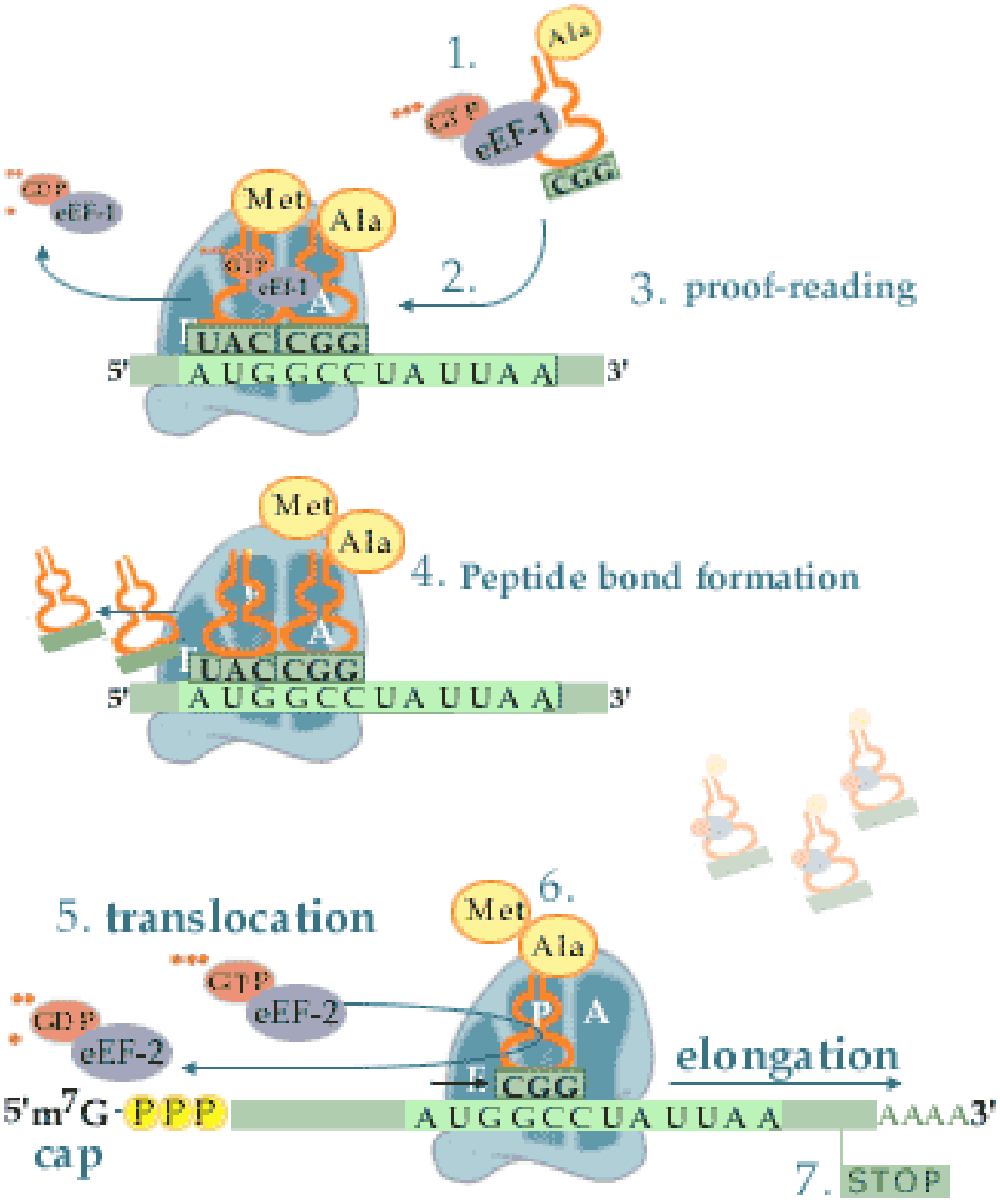


***FIGURE 1.5**: Translation elongation. Aminoacyl-tRNAs are selected and delivered to ribosomal A site by eEF1A according to the sequence of codons in mRNA and peptide bond is formed between a growing peptide and the incoming amino acid. Translocation is mediated by GTP-bound eEF2.*

empty A-site to start another round of elongation. Elongation is promoted by cytosolic proteins called elongation factors (EFs). In prokaryotes, three polypeptide elongation factors exist: EF1A, EF1B and EF2. EF1A from *E. coli* is a 393 amino acid protein. It is a GTP/GDP-binding protein with higher affinity for GDP than GTP. In prokaryotes the GDP binds more tightly than GTP by two orders of magnitude (Louie and Jurnak, 1985). GTP-bound EF1A interacts with aa-tRNA and assist in its binding to the ribosome. In *E. coli*, EF1B is a 282 amino acid protein with a molecular mass of around 30kDa. This factor catalyzes the nucleotide exchange of EF1A•GDP to EF1A•GTP. EF2 is a relatively large molecule consisting of 700 amino acids with a molecular mass in the order of 77kDa. GDP and GTP bind to this G-protein with similar affinity and hence it does not require an exchange factor. In the GTP-bound state this factor catalyzes translocation on the ribosome.

1.3.3 Translation Termination

Termination of polypeptide synthesis occurs when the translation machinery encounters a stop codon (UAA, UAG and UGA) on the mRNA that is read by a protein release factor (Craigen *et al*., 1990) (Figure 1.6). The presence of one of the three termination codons in the A site of the ribosome activates the polypeptide chain release factors (RFs) to bind and recognize the termination signal. Subsequently, the ester bond between the 3' nucleotide of the tRNA located in the P site and the growing polypeptide chain is hydrolyzed. This results in the detachment of the polypeptide from the ribosome. To carry out another round of translation, the large ribosomal subunit dissociates from the small subunit. IF3 clears the deacylated tRNA from the P site (Karimi *et al*., 1999). There are two classes of release factors: Class I RFs recognize the stop codon and facilitate the hydrolysis of ester bond between the polypeptide chain and the tRNA whereas class II RFs activate the class I RFs (Mikuni *et al*., 1994; Stansfield *et al*., 1995; Zhouravleva *et al*., 1995; Frolova *et al*., 1996). In prokaryotes there are two codon-specific class I release factors: RF1 that reads UAG and UAA stop codons

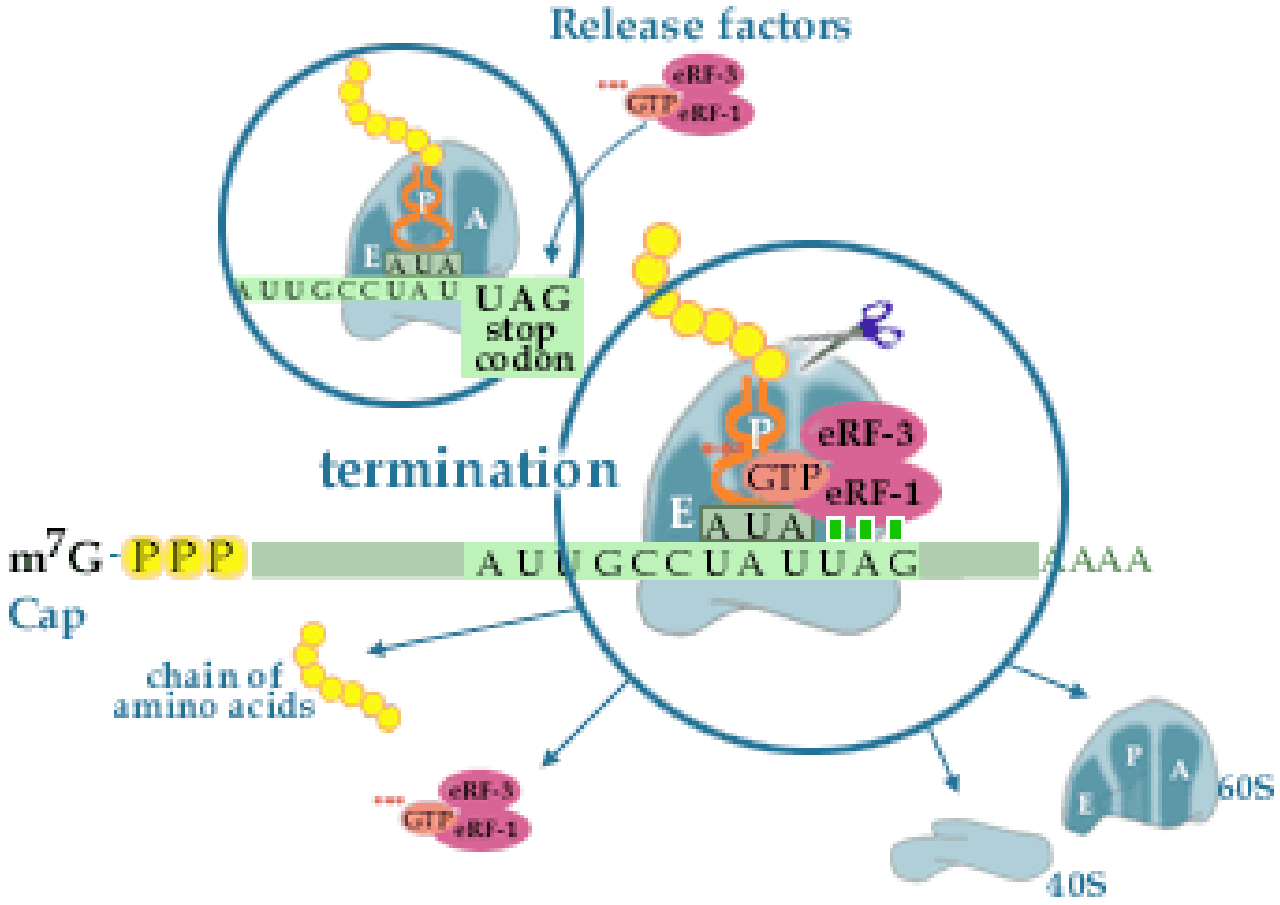


FIGURE 1.6: *Translation termination. Termination occurs when a stop codon on the mRNA is read by a protein release factor (RF). It leads to the release of the completed polypeptide chain from the ribosome. eRF1 reads the termination codons (UAG, UAA, UGA) while eRF3 stimulates the activity of eRF1.*

and RF2 that recognizes UGA and UAA codons. RF3 is a class II release factor that stimulates the termination reaction in a GTP-dependent manner (Mikuni *et al*., 1994; Freistroffer *et al*., 1997; Grentzmann *et al*., 1998). In eukaryotes, translation termination involves eRF1 that can read all three termination codons. eRF3 stimulates the activity of eRF1 in a GTP-dependent manner (Frolova *et al*., 1994; Stansfield *et al*., 1995; Zhouravleva *et al*., 1995).

1.4 Universally Conserved Translation Initiation Factors

The three major steps of the information transmission pathway of a cell, namely, replication, transcription and translation, differ from each other in the degree of evolutionary conservation they hold (Olsen and Woese, 1997; Ouzounis and Kyrpides, 1996). Greatest conservation is seen at the cellular translation process. It is evident by the vast conservation spread across the components involved from different phyla. Ribosomal RNAs and proteins, elongation factors, the tRNAs and aminoacyl-tRNA synthetases are universal in distribution (Olsen and Woese, 1997; Ouzounis and Kyrpides, 1996; Dennis, 1997). Although the basic mechanism underlying translation initiation is the same in all organisms, there are dissimilarities among the factors involved in bacteria, archaea and eukaryotes. In bacteria three single subunit factors are involved during initiation step: IF1, IF2 and IF3 whereas in eukaryotes a complex, multifactorial machinery exists. IF1 is involved in docking the charged initiator tRNA, $tRNA_f^{Met}$, to the P-site of 30S ribosomal subunit by blocking the A-site (Carter *et al*., 2001; Dahlquist *et al*., 2000). Apart from this, IF1 stimulates the activities of IF3 and hence also ribosomal subunit dissociation (Gualerzi and Pon, 1990). IF2 is a multidomain protein that is essential for protein synthesis to begin at the correct codon. It plays a central role in the formation of initiation complex that consists of 30S and 50S ribosomal subunits, GTP-associated IF2, mRNA and fMet-$tRNA_f^{Met}$. It binds to fMet-$tRNA_f^{Met}$ and 30S ribosomal subunit (Gualerzi *et al*., 2001; Boelens and Gualerzi, 2002). IF3 promotes ribosome dissociation into its subunits when bound to 30S subunit (Petrelli *et al*., 2001). Protein synthesis in eukaryotic organisms is a

rather complex process. Translation initiation process in bacteria and eukarya shows several common features but involves distinct set of factors. Eukaryotes, on the other hand, have an array of translation initiation factors (eIFs) to perform this function, many of which comprise multiple subunits. The three characterized prokaryotic translation initiation factors have their functional counterparts in eukaryotes that perform similar functions. eIF1A, like the prokaryotic IF1, stimulates the rate of ribosomal subunit dissociation as well as initiator tRNA (Met-$tRNA_i^{Met}$)-binding to the small ribosomal subunit (Thomas *et al*., 1980; Wei *et al*., 1995). Eukaryotic counterpart of IF2, eIF2, is a complex trimeric protein that too acts similarly (Pain, 1996). Finally, eukaryotic SUI1, the functional homologue of IF3, promotes the selection of the initiator tRNA and dissociation of the two subunits of ribosome (Naranda *et al.*, 1996). Although these homologues are functionally similar, they differ significantly at the sequence level. The two systems, hence, seem to have evolved separately.

Amino acid sequence comparisons of the translation initiation factors in prokaryotes, archaea and eukaryotes reveal that IF1/eIF1A and IF2/eIF5B form a pair of universally conserved translation initiation factors (Kyrpides and Woese, 1998a, b; Choi *et al*., 1998). Proteins with structural and functional similarities with prokaryotic IF1 are present in all phylogenetic domains. The archeal and eukaryotic homologues are named as aIF1A and eIF1A, respectively (Kyrpides and Woese, 1998a; Sørensen *et al*., 2001). Both IF1 and eIF1A show general functional similarity- both facilitate ribosomal subunit dissociation, stabilize Met-$tRNA_i^{Met}$and assist mRNA binding to the small ribosomal subunit. The sequence homology between the two is also apparent. Percent sequence identity between the factor from archaea and eukaryote is 38% whereas between the archaeal and bacterial one is 30% (Kyrpides and Woese, 1998a). Bacterial IF1 and eukaryotic eIF1A show 21% sequence identity. However within each of the three major groups, identities are greater than 50%. Similarly, homologues of IF2 found in archaeabacteria and eukaryotes are referred as aIF5B and eIF5B, respectively (Kyrpides and Woese, 1998a). Eukaryotic IF2/eIF5B was first reported in

Saccharomyces cerevisiae (Choi *et al*., 1998). Following this, eIF5B was detected in human (Lee *et al*., 1999; Wilson *et al*., 1999) and in *Drosophila melanogaster* (Carrera *et al*., 2000). The 1002 amino acid yIF2 polypeptide shows 27% identity and 48% similarity with IF2 from *E.coli*. An IF2 homolog was also identified in the archaeon *Methanococcus jannaschii* (Bult *et al*., 1996; Dennis, 1997) and this protein was found to partially substitute for the yeast protein both *in vivo* and *in vitro* (Lee *et al*., 1999). Extreme homology can be seen in the C-terminal region among the homologues (Sørensen *et al*., 2001). Overall homology among bacterial, eukaryotic and archaeabacterial IF2, eIF5B and aIF5B is highest in the G-domain area of the factor. The N-terminal regions of *E.coli* IF2 and yIF2 are dispensable for cell viability (Laalami *et al*., 1991). These homologues display similar functions like ribosome-dependent GTPase activity, promotion of ribosomal subunit association and interaction with the initiator tRNA (Choi *et al*., 1998; Pestova *et al*., 2000). Mutations in the GTP-binding domain of human eIF5B impair translation in yeast and human cells (Lee *et al*., 1999, Wilson *et al*., 1999). Although eIF5B shows sequence similarity to IF2 and shares similar role in subunit joining, eIF5B does not promote recruitment of initiator tRNA to the 30S ribosomal subunit. This function is taken up by eIF2 that has no prokaryotic homologue. Although eIF5B is not very essential for cell viability, deletion study in yeast results in severe retarded growth phenotype (Choi *et al*., 1998). Human eIF5B can complement its homologue in yeast to promote growth *in vivo* and to restore translation initiation in extracts prepared from fun12△ strains (Lee *et al*., 1999). eIF5B from *Drosophila melanogaster* is shown to interact with the DEAD box RNA helicase VAS encoded by the gene *vasa* (Carrera *et al*., 2000). However, the functional significance of this interaction is not known. There are evidences based on cross-linking experiments to show close proximity between IF1 and IF2 on the ribosome (Bioleau *et al*., 1983). The region between domains III and V of *E.coli* IF2 is involved in the interaction between the two factors (Moreno *et al*., 1999). Interactions between the eukaryotic homologues eIF5B and eIF1A have been mapped by using the yeast two-hybrid system, co-immunoprecipitation, *in vitro* binding assays and NMR spectroscopy (Choi *et al*., 2000; Marintchev *et al*.,

2003; Olsen *et al*., 2003). The C-terminal unstructured region of eIF1A, which is not present in bacterial IF1, interacts with the C-terminus of eIF5B.

1.5 Translation Fidelity and Proofreading

Proteins occupy a position high on the list of molecules important for life processes. They account for a large fraction of biological macromolecules-about 44% of the human body's dry weight (Davidson *et al*., 1973). They play major role in the reactions that sustain life. They serve several structural, transport and regulatory roles in all organisms. Hence, a large proportion of the cell's resources is diverted for the process of protein synthesis. In the bacterium *Mycoplasma genitalium*, out of a total of 480 proteins, around 101 factors are involved in the process of translation (Fraser *et al*., 1995; Hutchison *et al*., 1999). Some 127 genes in *M. genitalium* are involved in protein synthesis. Apart from such a heavy genomic investment, high proportion of cell's resources and energy is spent in the process. It involves 30-50% of the energy generated by a bacterial cell (Meisenberg and Simmons, 1998). Four high-energy bonds are consumed during the formation of one peptide bond; 25kcal/mole and this involves a very large number of tools like ribosomes, tRNAs and enzymes. A fast growing yeast cell is reported to carry 200,000 ribosomes that occupy nearly 30-40% of its cytoplasmic volume (Warner, 1999). Hence a living cell takes extra measures to monitor, regulate and fine-tune such an important and expensive biological process. The basis of translational control is that gene expression should be regulated with an efficient utilization of mRNA in specific protein synthesis without incorporating any error. Such regulation is absolutely essential for the survival of the cell. In a multistep, multifactorial pathway, regulation can be exerted at several steps. Several control points are deciphered at different levels during the translation process. One such regulatory step is at the level of translation initiation. Nevertheless, well-characterized cases do occur at other steps in the translation pathway where regulation is executed.

1.5.1 Proofreading by Aminoacyl-tRNA Synthetases

Aminoacyl-tRNA synthetases are highly selective in their recognition of both amino acid to be activated and the tRNA to be charged. tRNAs are screened on the basis of different base sequences. Most aminoacyl-tRNA synthetases contain acylation and hydrolytic sites. The acylation site selects specific amino acid on the basis of binding energy, size, surface charge distribution etc. Incorrect amino acid activated by the enzyme is hydrolyzed before being transferred to the tRNA. Hydrolysis also leaves the synthetase free to activate and transfer the correct amino acid. This ensures very high fidelity. Hydrolytic proofreading is central to the fidelity of many aminoacyl-tRNA synthetases as it is to DNA polymerases. Proofreading is considered as an expensive affair in terms of energy and time.

1.5.2 GTPase Rate of EF1A Determines the Fidelity of Protein Synthesis

The GTP-GDP cycle of EF1A plays an important role in maintaining the fidelity of protein synthesis. It depends on first checking the correct aminoacyl-tRNA brought to the ribosomal A-site before peptide bond formation. The incoming aminoacyl-tRNA is carefully scrutinized during codon-anticodon interaction. The EF1A GTPase activity acts as a barrier for the formation of a peptide bond between the growing polypeptide chain and the newly arrived aminoacylated tRNA at the ribosomal A site. This step buys time for the scrutiny. A peptide bond cannot be formed before the EF1A release from the charged tRNA; GTP hydrolysis being the prerequisite for the release of the factor.

1.5.3 Nonsense-mediate RNA Decay

Nonsense-mediated RNA Decay (NMD) is a surveillance mechanism that provides a means to down-regulate aberrant gene expression by degrading RNAs carrying premature termination codons (PTCs). It also helps to maintain a balance of normal gene expression by degrading specific, naturally occurring transcripts.

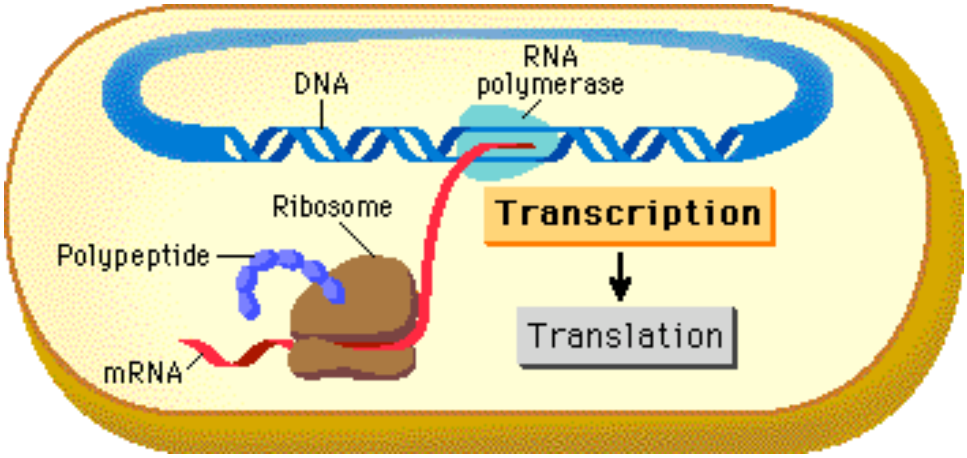
PTCs arise due to errors during routine cellular processes. Errors build up due to incomplete or inaccurate pre-mRNA splicing (Maquat, 1996). Error-prone processes include somatic rearrangements and hypermutations characterizing immunoglobulin and T-cell receptor genes which can generate a range of receptors but more frequently face gene inactivation by frameshift or nonsense mutations (Li and Wilkinson, 1998). Additionally, PTCs can occur during disease-causing translocations, deletions, insertions or point mutations within germ-line or somatic DNA (Maquat, 1996). NMD takes care of the truncated proteins that may be deleterious to cells for example the PTC-containing mRNA transcribed from Ig or TCR genes are removed by NMD (Li and Wilkinson, 1998). NMD plays a role in regulating telomeric length (Lew *et al*., 1998) and the levels of a kinetochore subunit (Dahlseid *et al*., 1998) in *S. cerevisiae* as well as certain alternatively spliced RNAs in *Caenorhabditis elegans* (Morrison *et al*., 1997). During nonsense-mediated decay the PTC carrying mRNAs are degraded during mRNA translation as in *S.cerevisiae* (Zhang *et al*., 1997). Mammalian NMD is post-transcriptional (Maquat, 1995; Li and Wilkinson, 1998; Hentze and Kulozik, 1999). In some cases NMD occurs in the cytoplasm while in others it is reported even before the mRNA have been completely transported from nuclei to cytoplasm. Cytoplasm-associated NMD is observed for β^{o}-thalassemic β-globin mRNA (Maquat *et al*., 1981; Lim *et al*., 1992), GPx1 mRNA (Moriarty *et al*., 1998) and mRNA for β-subunit of β-hexosaminidase in lymphoblasts in Tay-Sachs disease. Most mammalian mRNAs undergo NMD while still being nucleus-associated (Maquat 1995, 1996). Nucleus-associated NMD may be carried out by cytoplasmic ribosomes if mRNA-cytoplasmic ribosomal complexes were formed during nuclear pore transit or after transit but before the release into the cytoplasm (Maquat 1995, 1996; Hentze and Koluzik, 1999). A third possibility exists that NMD is carried out in the nucleus by nuclear translation machinery (Li and Wilkinson, 1998).

1.6 Nuclear Translation

In prokaryotes, it is apparent that transcription and translation of mRNAs are coupled (Mangiarotti *et al*., 1967; Miller *et al*., 1970). But, it has been assumed for a long time that in eukaryotes this coupling is truncated due to compartmentalization; transcription occurring in the nucleus while translation in the cytoplasm (Figure 1.7). In organelles like mitochondria and chloroplast, the scenario is quite different. It has been known that mitochondria and chloroplast possess their own DNA that is expressed by specific transcription and translation systems. Overall, the process of translation in these membranous organelles resembles prokaryotic protein synthesis more closely than eukaryotic translation.

Unlike these semi-autonomous organelles, for all these years, nucleus was considered to harbor the machinery involved in transcription only. This spatial separation between the nucleus and cytoplasm was supposed to be of significant importance. But, against this line of thought, some earlier studies did give the concept of nuclear translation. Frenster *et al.* (1960, 1961) isolated ribonucleoprotein particles from isolated nuclei of calf thymus lymphocytes. These isolated particles were shown to possess the ability to incorporate radioactive precursors into polypeptide in a nucleus-free system. In addition, protein synthesis was spotted within the isolated calf thymus cell nucleus. These thymus nuclear ribosomes were found to be ribonuclease sensitive while in the nucleus and required sodium rather than potassium cation environment for their metabolic activity in the nucleus. The addition of deoxyribonucleic acid of diverse sources stimulates amino acid incorporation by isolated nuclear ribosomes, but other polyanions were inhibitory. Similar active ribonucleoprotein particles were reported in rat liver nuclei (Rendi, 1960). The nuclear fractions isolated from rat liver were capable of incorporating labeled amino acids *in vitro.* Mangiarotti and Chiaberge (1997) found biologically active newly-assembled 40S and 60S ribosomal subunits in the nuclei of *Dictyostelium* cells. Furthermore, the observation that cytoplasmic ribosomal subunits containing immature RNA enter

A prokaryotic cell



An eukaryotic cell

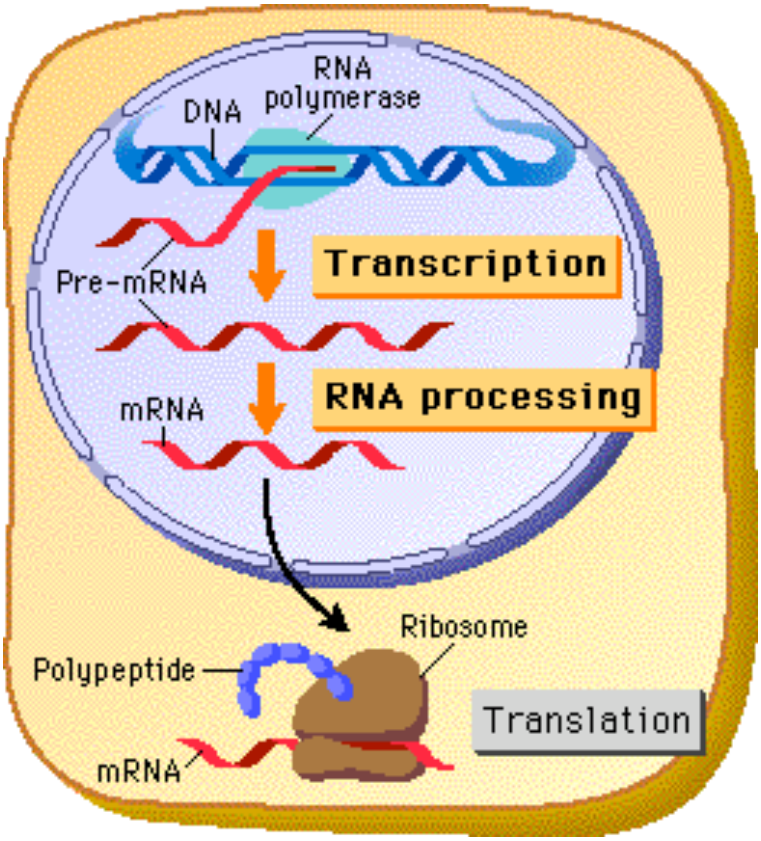
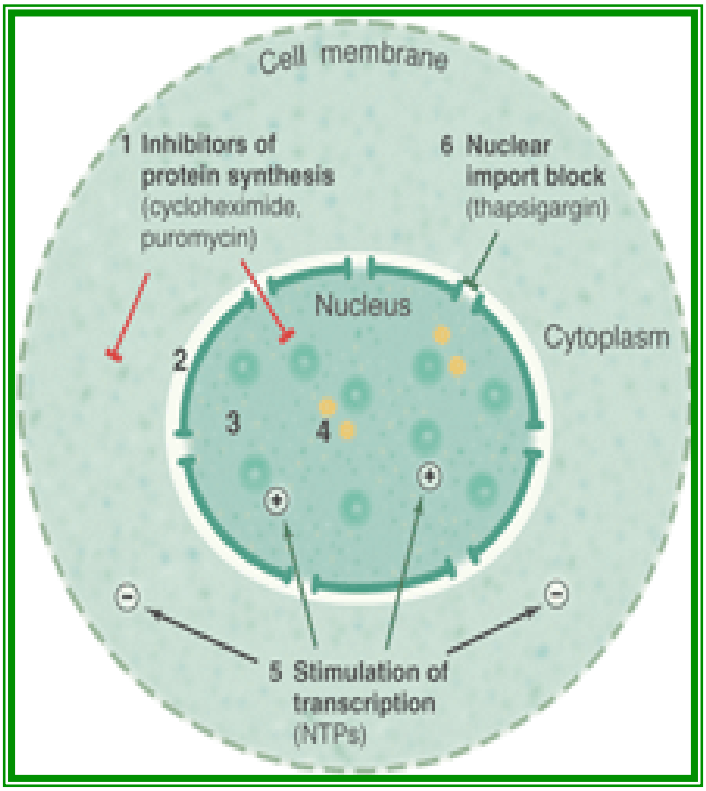


FIGURE 1.7*: Transcription and translation are coupled in prokaryotes while in eukaryotes the two processes are compartmentalized.*

polyribosomes with the same efficiency as mature ribosomal subunits, suggests the probability of coupled transcription and translation even in the nuclei (Mangiarotti *et al*., 1997). Later evidence of coupled transcription-translation in eukaryotes was provided by Mangiarotti (1999) in *Dictyostelium discoideum*. It was shown that biochemically active polyribosomes occur in the nuclei of *Dictyostelium* cells. The presence of active nuclear polyribosomes is a prerequisite for the coupling of transcription and translation. mRNA molecules enter nuclear polyribosomes while they are being synthesized. They show that nonsense-mediated decay occurs while the mRNAs are still bound to newly formed nuclear ribosomes. It is seen that RNA-RNA duplexes occur within the cell nucleus that are involved in antisense inhibition of ribosome translation. Goidl (1978) presented biochemical evidences that hint towards protein synthesis within the nucleus. Hence, it has been accepted that most of the components of translation machinery are present inside the nucleus.

And at last, breakthrough into this concept came in when Iborra *et al*. (2001) conclusively proved coupled transcription and translation within the nucleus of mammalian cells (Figure 1.8). They localized distinct translation sites within mammalian nuclei. This nuclear translation and transcription by RNA polymerase II was found to be concurrent. They visualized sites for nuclear translation in permeabilized mammalian cells using fluorescent lysine. They put forward several concrete evidences in support of incorporation of fluorescent amino acids into polypeptide by translation inside nucleus. They kept such experimental conditions that would allow the synthesis of only few polypeptide chains. This would keep a check on the number of complete polypeptide chains being transported to the nucleus. Also there was no extranuclear or perinuclear fluorescence detected in purified nuclei. Also the efficiency of polypeptide synthesis in purified nuclei was equivalent to that in permeabilized cells supporting the idea that there was no import of proteins from cytoplasm. It was found that the sites for transcription and translation overlapped. It was also noted that if transcription rate was enhanced by increasing the nucleotide concentration, it affected the nuclear fluorescence in the



Source: Iborra, *et al*. (2001) Science 293, 1139-1142

FIGURE 1.8*: Coupled transcription-translation in Hela cell nuclei. In the permeabilized mammalian cells sites for nuclear translation were traced by fluorescent lysine.*

forward direction whereas there was no effect on cytoplasmic fluorescence. Finally nuclear translation was well observed in the presence of a nuclear-import inhibitor, thapsigargin. Nuclear translation was estimated to be 10-15% of the total cellular protein synthesis. Hence, despite several controversies against the emerging concept of "nuclear translation", stronger evidences are continuously building up in support of it.

1.7 Objectives of Present Research Work

The present study carried out for the fulfillment of the degree of "Doctor of Philosophy" focuses on basic questions related to the translation process. While full-length eIF5B protein sequences have been characterized from *Saccharomyces cerevisiae* (Choi *et al*., 1998)*, Homo sapiens* (Lee *et al*., 1999; Wilson *et al*., 1999) and *Drosophila melanogaster* (Carrera *et al*., 2000), no eIF5B-like factor has been characterized from plant system. Hence this is the first report of the presence of eIF5B from plant system. The association of this factor with nuclear translation is presented. The idea of nuclear translation refined through the past few decades can have tremendous implications for research on nonsense-mediated decay, the process by which flawed messenger RNAs are identified and degraded. As far as the nature of nuclear translation is concerned, it is suggested that transcription and nuclear translation are coupled as in prokaryotes. Most components of translation initiation machinery, for example, ribosomal subunits, various translation initiation and elongation factors, aminoacyl-tRNA synthetases etc have been shown to be present in the cell nucleus (Frenster *et al.,* 1960, 1961; Rendi, 1960; Mangiarotti and Chiaberge, 1997; Lejbkowicz *et al*., 1992; Lang *et al*., 1994; Chudinova *et al*., 2004). There is however no evidence of translation in the nucleus of higher plant cells. The work presented in this thesis reports the characterization of a novel translation initiation factor from *Pisum sativum* that seems to form part of nuclear translation machinery. It provides one more evidence for nuclear translation process. The objectives of my thesis were the following:

1- Screening of cDNA library from *Pisum sativum* and *in silico* analysis of PeIF5B.

2- Cloning, expression, purification and structural characterization of recombinant PeIF5B.

3- Functional characterization of PeIF5B.

Chapter 2

SCREENING OF cDNA LIBRARY FROM *Pisum sativum* AND *In silico* ANALYSIS OF PeIF5B

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2.1 Rationale

Protein synthesis is the final step of gene expression and a key point for regulation. Several evidences indicate that the expression of a number of genes is controlled at the level of translation initiation. During translation initiation, ribosomes loaded with initiator tRNA position themselves at the start codon on the messenger RNA in the presence of several initiation factors and start the process of polypeptide chain synthesis. It is becoming increasingly apparent that translation initiation involves several factors that work in coordination with each other. Several newly reported translation initiation factors have been described including their biochemical features and crystal structures. Clearly a comprehensive dissection of the mechanism of action of initiation factors will be important for our understanding of the control of translation initiation in prokaryotic and eukaryotic systems.

In prokaryotes mainly three initiation factors are involved, namely, IF1, IF2 and IF3. IF1 directs the initiator tRNA, $tRNA_f^{Met}$, to the P-site of the small ribosomal subunit by blocking the A-site (Carter *et al*., 2001; Dahlquist *et al*., 2000). IF2 is a multidomain, GTPase that is required for charged $tRNA_f^{Met}$ binding and ribosomal subunit joining (Gualerzi *et al*., 2001; Boelens and Gualerzi, 2002). IF3 promotes

ribosome dissociation into its subunits when bound to 30S subunit (Petrelli *et al*., 2001). It checks the fidelity of the interaction between the charged initiator tRNA and mRNA. IF3 is also involved in the ribosome recycling (Laursen *et al*., 2005). Protein synthesis in eukaryotic organisms is significantly more complex process. Translation initiates with the recruitement of the ternary complex of eIF2•GTP•Met-tRNA$_i^{Met}$ to the 40S ribosomal subunit with the help of eIF1, eIF1A and eIF3. This 43S initiation complex then recognizes the mRNA to be translated through 5' 7-methylguanosine cap. The AUG start codon is then located by scanning the mRNA by several translation initiation factors. When initiation codon is reached, eIF2-bound GTP is hydrolyzed in the presence of eIF5 and eIF2•GDP is released. At this point, the 60S ribosomal subunit joins the 40S initiation complex in the presence of eIF5B (Pestova *et al*., 2000). After subunit joining, eIF5B hydrolyzes its bound GTP. This results in reduced affinity of eIF5B for the 80S ribosome and hence dissociates the translationally competent 80S initiation complex (Lee *et al*., 2002; Shin *et al*., 2002).

Translation initiation process in bacteria and eukarya shows several common features but involves distinct set of factors. Eukaryotes, on the other hand, have an array of translation initiation factors (eIFs) to perform this function, many of which comprise multiple subunits. The translation initiation step in prokaryotes and eukaryotes is evolutionarily linked by two common initiation factors. Amino acid sequence comparisons of the translation initiation factors in prokaryotes, archaea and eukaryotes reveal that IF1/eIF1A and IF2/eIF5B form a pair of

universally conserved translation initiation factors (Kyrpides and Woese, 1998a, b; Choi *et al*., 1998). eIF1A is one of the most highly conserved translation initiation factors with more than 65% identity (Dever *et al*., 1994) and is necessary for viability in yeast (Wei *et al*., 1995). eIF1A, along with eIF3, binds to the 40S ribosomal subunit and prevents its association with the 60S subunit (Gaumans *et al*., 1980). eIF1A is also involved in the binding of initiator tRNA to 40S ribosomal subunit and in mRNA binding and scanning (Pestova *et al.,* 1998). IF2, on the other hand, has its sequence homologue in eukaryotes (eIF5B) and archaea (aIF5B) (Choi *et al*., 2000; Lee *et al*., 1999). Deletion of the *FUN12* gene, encoding eIF5B factor in yeast, is reported to hamper the growth that can be complemented by recombinant yeast eIF5B (Choi *et al*., 1998). Pestova *et al*. (2000) reported that eIF5B is necessary for ribosome subunit joining. It also harbors ribosome-dependent GTPase activity (Merrick *et al*., 1975). GTP hydrolysis is not required for the association of the two subunits but to facilitate dissociation of eIF5B for its recycling.

Till date, among eukaryotes, full-length eIF5B protein sequences have been characterized from *Saccharomyces cerevisiae* (Choi *et al*., 1998)*, Homo sapiens* (Lee *et al*., 1999; Wilson *et al*., 1999) and *Drosophila melanogaster* (Carrera *et al*., 2000). No eIF5B-like factor has been reported from plant system so far. In the present study a cDNA clone was picked up from a cDNA expression library from *Pisum sativum* and characterized. We isolated a novel gene from *Pisum sativum* that was predicted to code for a protein of 861 amino acids with a theoretical

molecular mass of 96kDa. It was characterized as an eIF5B-like factor from *Pisum sativum* based on *in silico* analysis. It is likely that *in vivo* this factor, PeIF5B, may participate in the process of translation initiation.

2.2 Materials and Methods

2.2.1 Generation of polyclonal antibodies: Polyclonal antiserum against a 9-mer synthetic peptide (Sequence: 5'YGDTDSVMC3'), carrying conserved motif for eukaryotic DNA polymerase □, was raised in rabbit in moderately high titers. The chemically synthesized peptide was coupled to keyhole limpet hemocyanin (KLH). 0.4 mg of the conjugated peptide in Freund's complete adjuvant was injected in rabbit intraperitoneally and subcutaneously on day 0. The rabbit was boosted by additional injections on day 8, 15 and 22. Rabbit was bled and serum stored at 4^{o}C. Later the serum was passed through CNBr-activated sepharose column tagged with total *E.coli* proteins to remove all the contaminating anti-bacterial antibodies. Finally the titer of the polyclonal antibodies was checked. Peptide-antisera were used as the sources of primary antibodies.

2.2.2 Immunoscreening of cDNA library of Pisum sativum: The cDNA library was prepared from total RNA isolated from seven days old pea plants using UniZap cDNA synthesis kit from Stratagene as earlier reported (Ehtesham *et al*., 1999). The phage library had a titer of 10^{9} pfu/ml. The expression library was screened with polyclonal antibodies raised against synthetic polypeptides as described above. Out of a total of about one million plaques screened, only one positive

plaque was obtained which was further plaque purified after secondary and tertiary screening. Both the strands of the cDNA insert, ~3kb long, from the clone termed as pP1, were sequenced and clone was found to be devoid of sequences from the 5'end of the open reading frame.

2.2.3 Nucleotide screening of the library: In order to generate a full-length clone, the cDNA library was screened using a radiolabeled (Amersham Nick Translation Kit) 500bp *Eco*RI-*Hin*dIII fragment corresponding to the 5'terminus of pP1 insert. A few positive plaques were picked up after primary screening. Size of the inserts was analyzed by PCR using T3 and T7 universal primers in each pool of primary screen. Primary screen pool containing the largest insert (3.5kb) was further plaque purified using secondary and tertiary screening. One such pure plaque was selected. The 3.5kb insert was excised *in vivo* using helper plasmid and both the strands were sequenced (Sanger *et al*., 1977). The clone carrying the complete open reading frame was named pPeIF5B.

2.2.4 Databases: The nonredundant protein sequence database at the National Center of Biotechnology Information (NCBI) was used for all sequence similarity searches. Database searches were performed with BLAST (Altchul *et al.*, 1990) program, by using the BLOSUM62 substitution matrix and default parameters, at http://www.ncbi.nlm.nih.gov/BLAST/.

2.2.5 Sequence Analysis: 'Blast 2 sequences' tool (Tatusova and Madden, 1999) at NCBI was used for producing the local alignment of two given sequences. Multiple sequence alignments and phylogenetic tree analysis were performed by CLUSTALW version1.74 (Thompson *et al*., 1994) and Genchek^{TM} sequence analysis software package (Ocimum Biosolutions, India). Hydrophilicity and antigenicity plots were drawn using Protean software program. Motif identification searches were performed with the FUGUE search tool (Shi *et al*., 2001) (at http://www-cryst.bioc.cam.ac.uk/fugue/) and pfam (Finn *et al*., 2006) (at http://www.sanger.ac.uk/Software/Pfam/).

2.2.6 Northern blot hybridization: Total RNA was isolated from seven days old pea plants by guanidinium isothiocyanate method. Northern blot hybridization was performed as described in Sambrook *et al.* (1989). RNA preparation was electrophoresed in a denaturing gel and transferred to Hybond Nylone membrane (Amersham). Radiolabeled 3.5kb *Eco*RI-*Xho*I fragment from pPeIF5B was used as probe and hybridization was carried out at 65^{o}C. The blot was washed at high stringency (0.1X SSC and 0.1% SDS) at 65^{o}C and autoradiographed.

2.2.7 In vitro translation: The 3.5kb *Eco*RI-*Xho*I fragment from pPeIF5B was cloned in pSGI vector (Jameel *et al*., 1996) in the *Eco*RI and *Xho*I sites to construct pSGPeIF5B. Transcription of the gene was carried out *in vitro* using T7 RNA polymerase followed by *in vitro* translation of the transcript in the rabbit reticulocyte lysate system for 90 minutes at 30^{o}C in the presence of [^{35}S]-

methionine using TNT kit from Promega. The synthesized polypeptide was resolved on 10% SDS-PAGE followed by autoradiography.

2.3 Results

2.3.1 cDNA library screening: The cDNA expression library of *Pisum sativum* was prepared from the apical shoots of seven days old seedlings. High titer polyclonal antibodies were raised against conserved motifs found in DNA polymerase sequence. Pea cDNA library screening using peptide antibody revealed one positive clone with a 3kb insert. Sequencing of this clone revealed that it contains a 26bp poly(A) tail, 532bp 3'UTR but deletion at 5'end. To further identify and isolate the complete ORF, pea cDNA library was screened using a 500bp *Eco*RI-*Hin*dIII fragment obtained from this partial clone. A clone containing 3.5kb insert was identified, isolated and sequenced (GenBank accession no. **AF499740**). The deduced nucleotide sequence of the full-length clone carrying 3.5kb insert showed the presence of 2586bp open reading frame along with 381bp and 532bp 5' and 3' UTRs, respectively. The putative AUG initiation codon was located at 382-384bp position in the pPeIF5B clone and the UGA termination codon positioned at 2965-2967bp thereby generating an open reading frame of 2586 nucleotides coding for a protein of 861 amino acids. The gene sequence is AT rich with an average AT content of 58.74%.

2.3.2 Protein sequence of the gene: As deduced from *in silico*-translated DNA sequence (using DNASTAR's LASERGENE software), this pea gene encodes a putative protein 'PeIF5B' of 861 amino acids with a calculated molecular mass of 96354.41 daltons. This high molecular weight protein has a calculated isoelectric point (pI) of 5.76 with a net charge of –14.57 at neutral pH. It is rich in aromatic amino acids with five tryptophan, fourteen tyrosine and nineteen phenylalanine residues. It is predominantly rich (291 in all) in hydrophobic amino acids.

2.3.3 NCBI BLAST results: NCBI BLAST results predicted this protein to be a translation initiation factor. It showed highest level of identity at the protein level (82% similarity; 72% identity) to the putative translation initiation factor IF2 from *Arabidopsis thaliana* (GenBank accession no. **NP 177807**) followed by its counterpart from *Oryza sativa* (GenBank accession no. **AAT44302**) (80% similarity; 66% identity).

The PeIF5B polypeptide sequence was aligned with corresponding sequences of other translation initiation factors using 'Blast 2 sequences' tool at NCBI (Table 2.1). At the amino acid sequence level, about 46% and 55% identity was evident with the eukaryotic homologue of bacterial IF2 (eIF5B) from human and mouse, respectively. Similarly, PeIF5B showed high percent identity to eIF5Bs from *Saccharomyces cerevisiae* (46%) and *Drosophila melanogaster* (56%) and aIF5B from *Methanobacterium thermoautotrophicum* (36%). PeIF5B also showed significant sequence similarity to its bacterial counterpart. It showed 24% identity

Table 2.1

Organism	**Similarity**	**Identity**
eIF5B *H. sapiens*	64	46
eIF5B M. musculus	73	55
eIF5B S. cerevisiae	63	46
eIF5B D. melanogaster	73	56
aIF5B M. thermoautotrophicum	57	36
eIF5B *A. thaliana*	82	72
eIF5B *O. sativa*	80	66
IF2 E. coli	42	24
IF2 H. sapiens (mitochondria)	43	30
IF2 S. cerevisae (mitochondria)	42	23

TABLE 2.1: *Percent similarity and identity of PeIF5B with other eIF5Bs and IF2s using 'Blast 2 sequences' tool at NCBI*

to *E.coli* translation initiation factor IF2, 30% identity to human mitochondrial IF2 and 23% identity to yeast mitochondrial IF2. However, no similarity could be detected with eukaryotic eIF2□, eIF2□ or eIF2□. Hence, these results clearly indicate that this novel factor from pea shows extreme relatedness to eIF5B translation initiation factor.

2.3.4 Domain search: Pfam is a collection of several functional/structural protein domains. Pfam was therefore used to identify specific domains present in PeIF5B and to understand their organization on the polypeptide. A GTP-binding domain was predicted to lie in the central portion of the polypeptide sequence encompassing 269-484 amino acids (Figure 2.1). It was observed that PeIF5B polypeptide carries three motifs characteristic of GTP-binding proteins (G-I, G-II and G-III) (Bourne *et al*., 1991). Second major domain called elongation factor-Tu-domain 2 (EF-Tu-D2) that is involved in the interaction with tRNAs, was predicted to be located towards the C-terminal end of the polypeptide between 508-555 amino acids. These results clearly indicate that PeIF5B is a putative translation initiation factor. Multiple sequence alignment of PeIF5B with eIF5B and IF2 translation initiation factors from several different organisms showed distinct relatedness (Figure 2.1). PeIF5B protein from *Pisum sativum* displayed extensive similarity with the central and C-terminal regions of other IF2-like proteins. Relatively less similarity in the N-terminal sequence was observed. The similarity is greater in the GTP-binding domain where PeIF5B shows 69.7% identity to *H.sapiens* eIF5B and 72.1% to eIF5B from *S.cerevisiae* and 52.5% to

Figure 2.1


G-I
G-II
G-III
EF-Tu-D2
EF-Tu-D2
PelF5B
A.thaliana
S.cerevisiae
H.sapiens
D.melanogaster
M.themoautotrophicum
E.coli

FIGURE 2.1: *Multiple sequence alignment of PeIF5B with its eukaryotic, archaeal and prokaryotic homologues. The conserved GTP-binding motifs G-I, G-II and G-III are enclosed within red boxes while the EF-Tu-D2 domain is shown in black box. The percent identity among different sequences was evaluated using CLUSTALW version1.74 and Genchek™ sequence analysis software package (Ocimum Biosolutions, India). The identity levels and corresponding colours are: >80%=green, >60%=yellow, >40%=blue and <40%=no colour.*

aIF5B from *M.thermoautotrophicum* (Figure 2.2). Similarity is also strong in the C-terminal regions of the proteins. The N-terminal region shows minimal sequence similarity.

2.3.5 Hydrophilicity-antigenicity index: E.coli IF2 is composed of six domains (Mortensen *et al*., 1998). Domains IV-VI form the C-terminal domain that is conserved across prokaryotes. A less conserved N-terminal region harbors domains I-III (Sørensen *et al*., 2001). The N-terminal region of IF2 has a flexible and significantly accessible conformation. The structure of domain I (in the N-terminal region) named as IF2N of *E.coli* IF2 has been determined using NMR (Laursen *et al*., 2003). This IF2N domain is connected to the C-terminal region by a flexible linker (Laursen *et al*., 2004). The highly exposed nature of residues in the N-terminal region is verified by the presence of several epitopes responsible for its high antigenicity. On the other hand, C-terminal region of native IF2 has only few epitopes (Mortensen *et al*., 1998) and is more stable against proteolytic degradation due to more compact structure. Similar property is observed in the case of PeIF5B where it shows a high index of hydrophilicity and antigenicity towards the N-terminal region (Figure 2.3).

2.3.6 Phylogenetic analysis: The phylogenetic analysis (Figure 2.4) of the full-length IF2-like sequences from prokaryotes, archaea and eukaryotes places PeIF5B close to eukaryotic eIF5B factors from yeast, human, mouse and

Figure 2.2

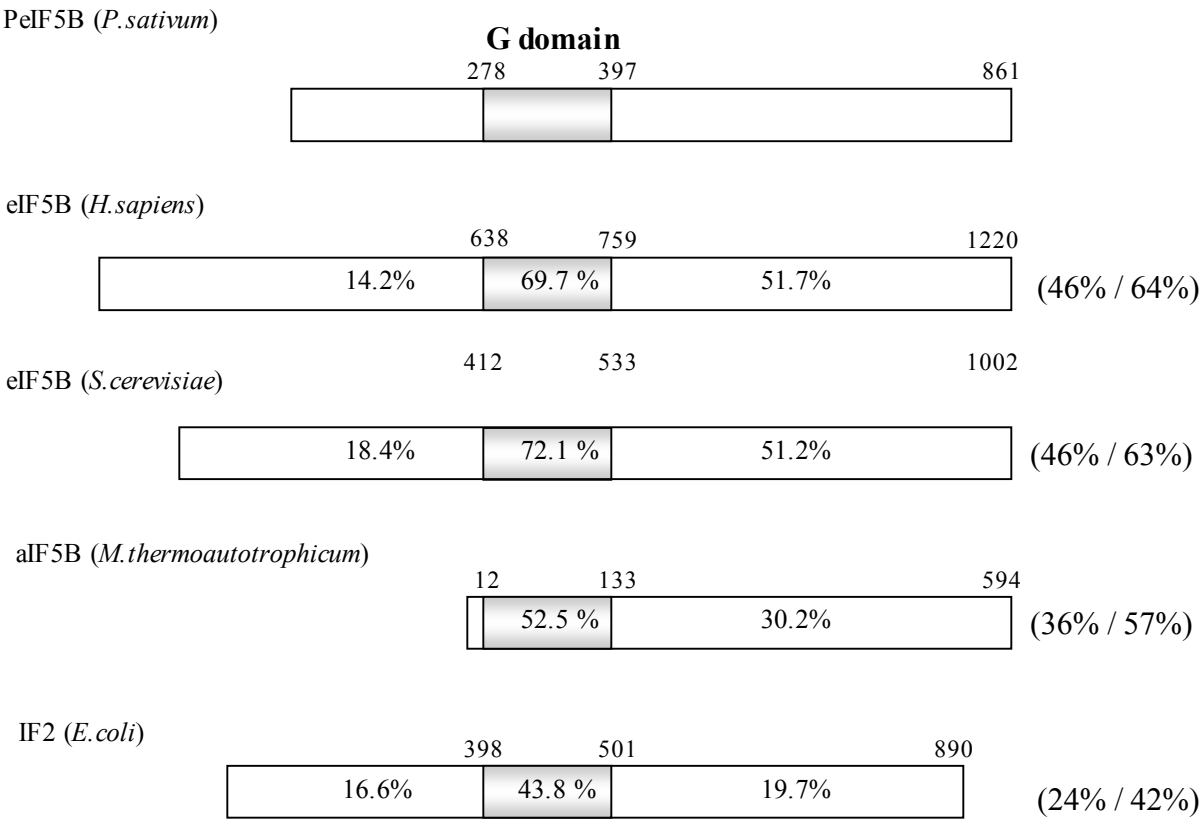


FIGURE 2.2: *Amino acid sequence conservation in the central, N- and C-terminal regions across eukaryotic and archaeal e/aIF5Bs and bacterial IF2. Domain-wise amino acid sequence identities of eIF5Bs from H.sapiens and S.cerevisiae, aIF5B from M.thermoautotrophicum and IF2 from E.coli with respect to PeIF5B were determined using LALIGN (http://www.ch.embnet.org/software/LALIGN_form.html) and are displayed on the cartoon. Full-length amino acid sequence identity/similarity of different proteins compared with PeIF5B were calculated using 'Blast 2 sequences' tool at NCBI and are shown in brackets. The GTP-binding domain is shown by shaded box. Numbers refer to amino acid residues.*

Figure 2.3

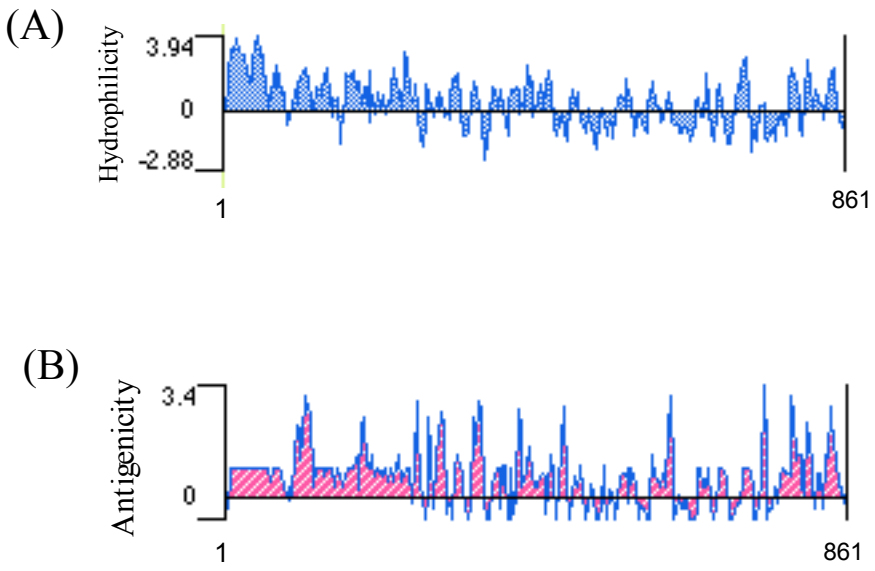


FIGURE 2.3: *Hydrophilicity* ***(A)*** *and antigenicity* ***(B)*** *plots of full-length PeIF5B protein (861 amino acids) drawn using Protean software program. Hydrophilicity was calculated according to Kyte-Doolittle algorithm. Antigenicity plot was obtained by measuring the antigenic index by Jameson-Wolf method. Numbers in the figure refer to amino acid residues.*

Figure 2.4

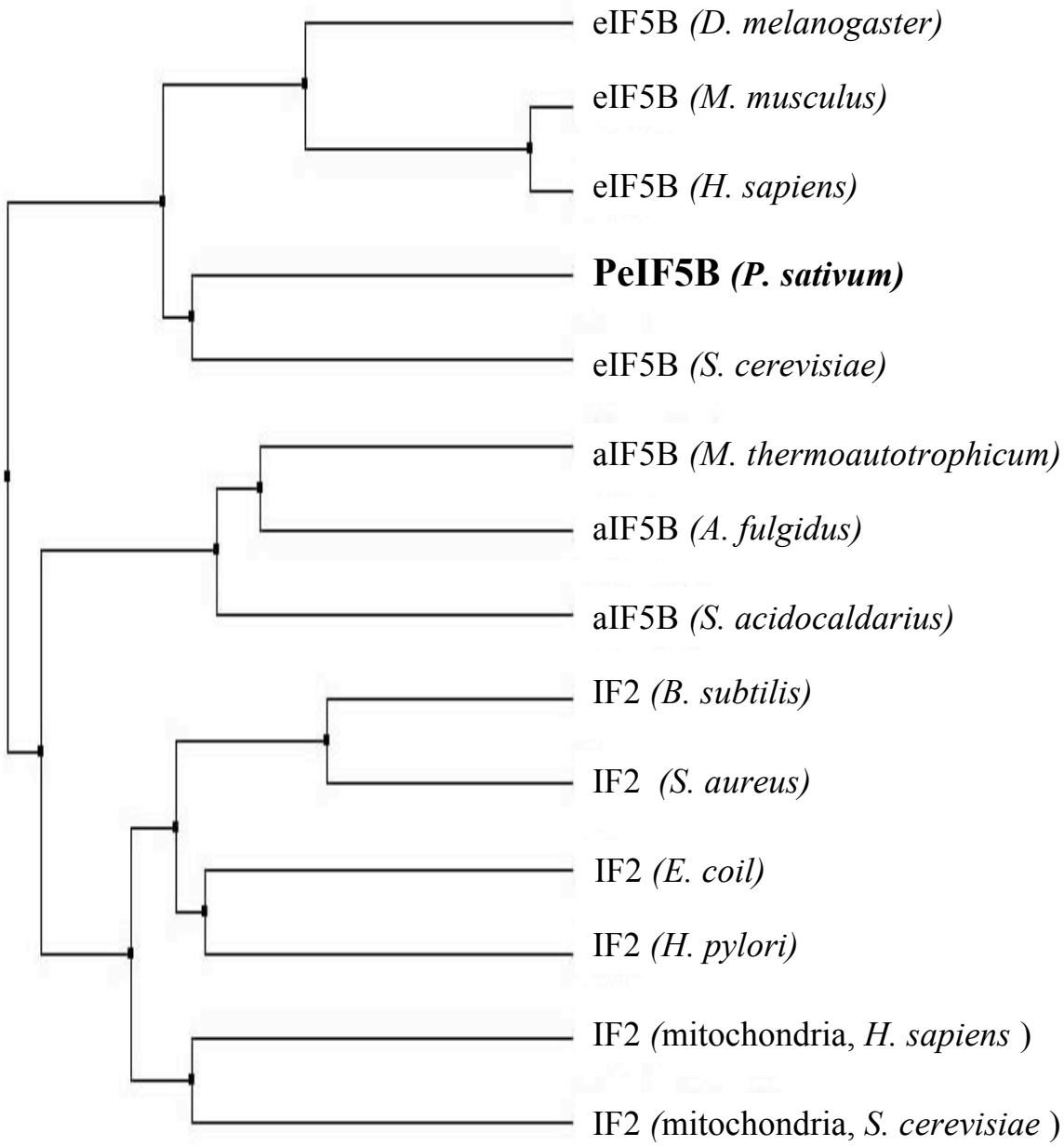


FIGURE 2.4: *Phylogenetic tree of eukaryotic, archaeal, prokaryotic and organelle IF2-like proteins. The full-length protein sequences are aligned and an average distance tree drawn using CLUSTALW version 1.74 and Genchek™ sequence analysis software package (Ocimum Biosolutions, India).*

Drosophila while archaeal aIF5Bs form a separate branch. The mitochondrial IF2s are grouped with bacterial IF2s.

2.3.7 Fugue results: FUGUE aligns a query protein with a database of proteins whose 3D-structures are solved and provides structure-based similarity score. It employs the details of the local environment of each amino acid residue before grouping the homologues together. FUGUE search for PeIF5B grouped it with hs1g7sa family that is represented by translation initiation factor aIF5B from *Methanobacterium thermoautotrophicum*. Interestingly, *E.coli* IF2, IF2 from *Arabidopsis thaliana* and IF2 from *Oryza sativa* are also grouped in the hs1g7sa family whereas eIF2□, eIF2□ and eIF2□ belong to a different class. This clearly suggests that PeIF5B is similar to eIF5Bs from eukaryotes, archaeal aIF5B and *E.coli* IF2.

2.3.8 Northern blot analysis and in vitro translation: Northern blot analysis was carried out to determine the presence of *PeIF5B* transcript *in vivo*. Hybridization using total pea RNA revealed a single transcript when probed with the 3.5kb *Eco*RI-*Xho*I fragment from pPeIF5B (Figure 2.5). Having shown the *in vivo* presence of the transcript of *PeIF5B* gene, *in vitro* transcription-translation of the gene was carried out using pSGPeIF5B to check for the translated product. A single band for the *in vitro* synthesized PeIF5B protein from pSGPeIF5B using TNT kit (Promega, USA) was observed at 116kDa (Figure 2.6). These results

Figure 2.5

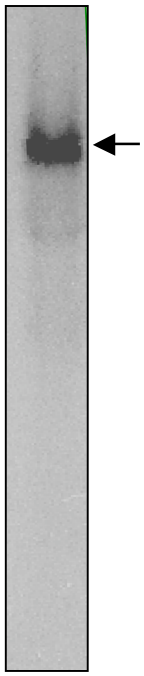


FIGURE 2.5: *Northern blot hybridization of total RNA from Pisum sativum using the 3.5kb EcoRI-XhoI fragment from pPeIF5B as probe.*

Figure 2.6

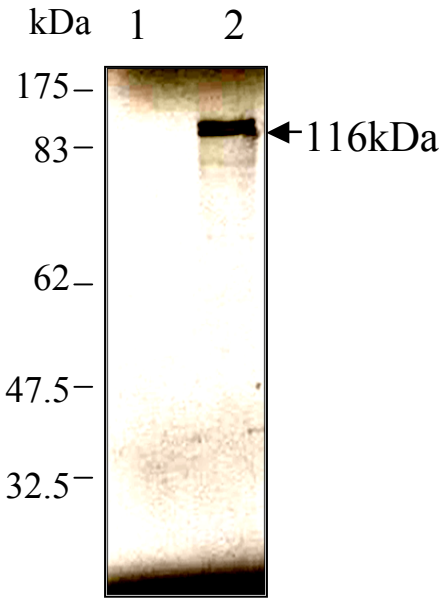


FIGURE 2.6: *In vitro transcription and translation of PeIF5B gene using rabbit reticulocyte lysate. Lane 2 shows 116kDa translated PeIF5B protein from pSGPeIF5B construct. Lane 1 is the pSGI vector control.*

clearly suggest that PeIF5B is transcribed *in vivo* and is translated into a single polypeptide chain.

2.4 Discussion

The discovery of this novel gene, *PeIF5B,* is the first report of a full-length eIF5B-like factor from plant system. The present study was originally initiated with the intention to isolate the gene coding for DNA polymerase from *Pisum sativum* genome. Around one hundred million plaques were immunoscreened to finally obtain one single clone carrying 3kb insert (pP1). It was a partial clone lacking 5'end of the ORF. With the help of this truncated clone, the cDNA library was rescreened to obtain the full-length clone. A 500bp fragment cut from *Eco*RI-*Hin*dIII sites near the 5'end of the pP1 partial clone was used as a radiolabeled probe to screen the library. Finally a clone carrying 3.5kb insert was obtained carrying complete ORF of 2.6kb (pPeIF5B). The deduced protein sequence of this gene indicated that it shows sequence similarity to eukaryotic translation initiation factor eIF5B. It is to be noted that although this clone was identified using antibodies raised against DNA polymerase conserved motifs, it showed no similarity to any DNA polymerase from any source. This cross-reactivity can be attributed to the presence of some similar conserved motifs in DNA polymerases and translation initiation factors. Till date eIF5B has been characterized in *Saccharomyces cerevisiae, Homo sapiens* and *Drosophila melanogaster*. It was earlier shown that percent similarity between yeast and human eIF5B is 64% while

that between yeast and archaea is around 59%. Going well with earlier examples, PeIF5B shows high degree of similarity with the corresponding genes from yeast, human and archaea, that is, 63%, 64% and 57%, respectively. Being an IF2 homologue, PeIF5B displays 42% similarity to *E. coli* IF2.

Apart from sequence similarity to eIF5Bs from other systems, several major domains were predicted in PeIF5B that strengthened the idea of it being a translation initiation factor. It carries a GTP-binding domain that consists of G-I, G-II and G-III motifs that are known to be important for GDP/GTP exchange, GTP-induced conformational changes and GTP hydrolysis. The consensus amino acid sequences of G-I and G-II motifs are $GX_4GK(S/T)$ and DX_2G, respectively while G-III motif is characterized by four apolar amino acids followed by (N/T)(K/Q)XD. This P-loop motif-containing G-domain is a characteristic feature of elongation factor-Tu and several members of G-protein superfamily. Other Pfam predicted domain was EF-TU-D2 domain. This EF-Tu-domain 2 possesses β-barrel structure and is known to interact with charged transfer ribonucleic acids (tRNAs) (Nissen *et al*., 1995). Apart from elongation factor EF-Tu, this domain is also observed in elongation factor EF-G. Similar G-domain and EF-TU-D2 domain are also predicted for *E.coli* IF2.

The structure of archaeal homologue of *E. coli* IF2 (aIF5B) from *Methanobacterium thermoautotrophicum* has been reported (Roll-Mecak *et al*., 2000). The full-length archaeal aIF5B covers only the conserved central and C-

terminal segments that are found conserved across all eukaryotic and archaeal e/aIF5Bs and bacterial IF2s (Sander and Schneider, 1991). Regions of translation initiation factor, IF2, that are involved in fMet-$tRNA_f^{Met}$ binding and nucleotide binding and hydrolysis are found to be highly conserved. The highest sequence conservation among IF2 factors is found in the G domain (pairwise identities 43%-70%). N-terminal domain of IF2 is reported to be responsible for its interaction with the 30S and 50S ribosomal subunits (Moreno *et al*., 1999). In agreement with earlier reports on several translation initiation factors, PeIF5B shares similar functional domains such as GTP-binding domain and EF-Tu-D2 domain with other eIF5Bs. Like other IF2 and e/aIF5B factors, the central and C-terminal regions of PeIF5B were found to be highly conserved.

Northern blot analysis showed that *PeIF5B* gene is transcribed *in vivo*. In the *in vitro* transcribed-translated *PeIF5B* gene, the single polypeptide could be seen at a size of 116kDa rather than at an expected size of 96kDa. This size discrepancy between the predicted size of the protein (96kDa) and that observed after *in vitro* synthesis of the polypeptide (116kDa) may be explained by the presence of highly charged N-terminus of PeIF5B that may contribute to its aberrant movement in SDS-PAGE. Similar observation has been reported for eIF5B from *Homo sapiens* where the polypeptide with the predicted size of 139kDa moved as a band corresponding to 175kDa on SDS-PAGE (Lee *et al*., 1999).

The *in-silico* analysis suggests that PeIF5B probably plays a role in the cellular translation process. This novel factor is phylogenetically related to other known e/aIF5Bs from eukaryotes and archaea and prokaryotic IF2.

Chapter 3

CLONING, EXPRESSION, PURIFICATION AND STRUCTURAL CHARACTERIZATION OF RECOMBINANT PeIF5B

Chapter 3: CLONING, EXPRESSION, PURIFICATION AND STRUCTURAL CHARACTERIZATION OF RECOMBINANT PeIF5B

Contents

CLONING, EXPRESSION, PURIFICATION AND STRUCTURAL CHARACTERIZATION OF RECOMBINANT PeIF5B

3.1 Rationale

Synthesis of polypeptides in a cell involves an array of various translation factors, ribosomal subunits, nucleotides and transfer RNAs (tRNAs). The initiation of translation is an important regulatory step that is marked by the formation of a stable initiation complex between 30S ribosomal subunit, initiator tRNA and mRNA (Kozak, 1999). A very major step during translation initiation is the formation of a ternary complex between initiation factor 2 (IF2), GTP and charged initiator tRNA. IF2 is one of the three factors required for the initiation of protein biosynthesis in bacteria. IF2 promotes the GTP-dependent binding of the tRNA to the small subunit of the ribosome (Gualerzi and Pon, 1990; Kozak, 1999; Wu *et al.*, 1996). IF2 is a protein of about 70 to 95kDa that contains a central GTP-binding domain flanked by a highly variable N-terminal domain and a more conserved C-terminal domain (Laalami *et al.*, 1991; Spurio *et al.*, 2000). Bacterial IF2 is structurally and functionally related to eukaryotic mitochondrial IF2 (IF2mt) (Ma and Spremulli, 1995; Ma and Spremulli, 1996; Vambutas *et al*., 1991) as well as to algal and plants chloroplast IF2 (IF2cp) (Ma and Spremulli, 1990; Stern *et al*., 1997; Gillham *et al*., 1994). Sequence homologues of IF2 in archaea and eukaryotes are aIF5B and eIF5B, respectively (Kyrpides and Woese, 1998). eIF5B is a conserved, monomeric G-protein that plays a major role in protein synthesis in all living organisms. The observation that eIF5B knockout yeast strain shows

retarded growth clearly suggests that this factor is important for the survival of the organism but nonessential (Choi *et al*., 1998). Similar to eubacterial IF2, eIF5B helps in ribosomal subunit joining (Pestova *et al*., 2000; Unbehaun *et al*., 2004) and plays role in the binding of Met-tRNA$_i^{Met}$ to the small ribosomal subunit (Choi *et al*., 1998). It also shows GTP-hydrolyzing activity required for the release of the factor after ribosomal subunit association (Lee *et al*., 2002; Pestova *et al*., 2000; Shin *et al*., 2002).

The *PeIF5B* gene isolated from cDNA library prepared from seven-days old pea plants shows sequence similarity to other eukaryotic and archaeal IF2 homologues as well as bacterial IF2. *In silico* study shows promising evidences that it may form part of the cellular translation initiation machinery. It codes for an 861-amino acid polypeptide with a calculated molecular mass of 96kDa. BLAST results of PeIF5B showed its relatedness to yeast and human eIF5B (identity 46%; similarity 64%) and prokaryotic translation initiation factor IF2 (identity 24%; similarly 42%). It suggests their structural, functional and evolutionary similarity. To study this interesting factor in detail, the gene was cloned, expressed and recombinant protein was purified to homogeneity from *E.coli*. A study elaborating upon the structural properties of this novel protein was done to validate all the *in silico* predictions. Fluorescence spectroscopy and circular dichroism were employed to study the structural details of the novel translation initiation factor eIF5B from *Pisum sativum.* Analysis of the biophysical events following ligand-binding was performed. This study gives preliminary information regarding the mechanism of

action of this translation initiation factor. Stability studies were also performed on PeIF5B by measuring the structural changes in the protein after guanidine hydrochloride (GnHCl)- and temperature- induced denaturation.

3.2 Materials and Methods

3.2.1 Materials: 8-Anilino-1-naphthalene-sulfonic acid (ANS), methionine, *E.coli* aminoacyl tRNA synthetase and formylmethioinine specific *E.coli* tRNA were purchased from Sigma-Aldrich, USA. A stock solution of ANS was prepared in methanol and used further. Concentration of ANS was determined using an extinction coefficient of ε=5000 $M^{-1}cm^{-1}$ at 350nm (Khurana and Udgaonkar, 1994). GnHCl was obtained from USB. Monoclonal anti-His as well as HRP-linked anti-mouse antibodies were purchased from Santa Cruz Biotechnology. All other reagents used were of analytical grade. All solutions were prepared in double distilled water.

3.2.2 Cloning, expression and western blot analysis of PeIF5B: The gene coding for PeIF5B was amplified from pPeIF5B clone by PCR using degenerate primers carrying restriction sites for *Eco*RI and *Xho*I in the forward and reverse primers, respectively. The sequence of forward and reverse primers was: GGAATTCATGCAAGAATTACTAGCTCGAAGA and TCTCGAGTTGTATCTTGAAAAGACTCTTCAATTTC. PCR was initiated by adding 50ng of pPeIF5B as template to a 50μl of total reaction containing 100ng

each of forward and reverse primers, 0.2mM dNTPs (Amersham Pharmacia), DMSO, and 0.1U of Accu DNA polymerase (Sigma Aldrich). PCR consisted of initial denaturation step at 94^{o}C for 5min followed by 35 cycles of denaturation at 94^{o}C for 30sec, annealing at 58^{o}C for 30sec and polymerization at 72^{o}C for 2min. Final extension was carried out at 72^{o}C for 7min. The PCR product was cloned into PCR2.1 (Invitrogen). It was later subcloned into the gene fusion system pET23a (Novagen) at the *Eco*RI and *Xho*I sites. The plasmid was transformed into *E.coli* BL21 (DE3) p*Lys* S (Novagen) cells and over-expressed in the presence of inducer-IPTG (1mM) with induction time 5hrs. Crude cellular extracts were prepared by suspending cells in loading buffer [0.1M Tris-HCl (pH6.8), 2% sodium dodecyl sulphate (SDS), 5% glycerol and 0.1M 2-mercaptoethanol] and incubating them for 5min at 100^{o}C. The extracts were immediately loaded onto 10% SDS-polyacrylamide gel. Electrophoresis was performed at a constant 100volts for 1-1.5hrs.

For western blot analysis, the resolved proteins were electrophoretically transferred from polyacrylamide gel to nitrocellulose membrane at 30mA overnight at 4^{o}C. Nonspecific binding was blocked by incubation with 1% bovine serum albumin (BSA) at room temperature for 2hrs, and the membranes were incubated for 1hr with monoclonal anti-His antibodies at 4^{o}C. The membranes were washed three times with 1X phosphate buffered saline (PBS), incubated with anti-mouse antibody conjugated with horse radish peroxidase at 4^{o}C for 40min, and again washed five times in 1X PBS and 0.05% Tween-20. Immunoreactive

bands were visualized using Western blotting luminol reagent (Santa Cruz Biotechnology).

3.2.3 Purification of PeIF5B: PeIF5B was expressed under T7 promoter in pET23a using Bl21 (DE3) p*Lys* S cells and 1mM concentration of IPTG at 37^{o}C for 5hrs. Induced culture was then harvested and the cell pellet resuspended in lysis buffer [8M urea, 20mM Tris-HCl (pH7.8) and 300mM NaCl] and sonicated. The lysed cells were centrifuged at 13,000rpm for 20min. Supernatant carrying the recombinant protein was collected and loaded onto the TALON affinity column (Clontech) pre-equilibrated with the lysis buffer. Washes were given with lysis buffer containing 10mM imidazole. The His-tagged protein was eluted in 8M urea, 20mM Tris-HCl (pH8.0), 300mM NaCl and 200mM imidazole. Finally, the purified protein was dialyzed against 20mM Tris-HCl (pH 8.0) and 100mM NaCl and stored in small aliquots at -20^{o}C.

3.2.4 Initiator tRNA charging: Charging reactions contained 50□g of formylmethioinine specific *E.coli* initiator tRNA, 2mM ATP, 100mM Tris-HCl (pH7.5), 5mM $MgCl_2$, 400□M methionine and 100units of *E.coli* aminoacyl tRNA synthetase in a total volume of 50□l. The reaction mixture was incubated at 37^{o}C for 10min and then terminated by adding 30□l of 1M sodium acetate (pH5.0). The *in vitro* synthesized Met-$tRNA_f^{Met}$ was deproteinized by mixing thoroughly with phenol saturated with 0.2M Tris-HCl (pH8.0) followed by centrifugation at 12,000rpm for 10min at 4^{o}C. The aqueous layer was then dialyzed against a high

salt dialysis buffer containing 50mM sodium acetate (pH5.0) and 0.5M NaCl for 2hrs at 4^{o}C followed by dialysis with low salt dialysis buffer containing 20mM sodium acetate (pH5.0) for 2hrs at 4^{o}C. Charged $tRNA_f^{Met}$ was added in 1:1 molar ratio to the protein. The reaction was incubated at 37^{o}C for 10min.

3.2.5 Fluorescence spectroscopy: All the fluorescence measurements were carried out on a Cary Eclipse Fluorescence Spectrophotometer. The temperature-based denaturation studies were performed on Perkin Elmer LS55 luminescence Spectrometer equipped with a thermostated cell holder. Protein concentration used was in the range 0.52□M-1.04□M. For tryptophan fluorescence of the protein, excitation wavelength selected was 280nm and emission spectrum range was from 300-400 nm with 10 and 5nm slit widths for excitation and emission, respectively. For ANS fluorescence, excitation was at 380nm and emission spectrum was collected between 400-600nm with a slit width of 10nm for both excitation and emission. The molar ratio of protein and ANS was 1:100.

3.2.6 Circular dichroism: Circular dichroism (CD) measurements were taken on Jasco J-810 spectropolarimeter. The instrument was calibrated with a 0.1% *d*-10-camphor sulphonic acid solution (Cassim and Yang, 1969). The results are shown as mean residual ellipticity (MRE) in deg.cm^2.dmol^{-1} according to the following equation (Hackeng *et al*., 2000):

$$MRE = \theta_{obs} / n \times l \times c \times 10$$

where θ_{obs} is the observed ellipticity in millidegrees, n is the number of aminoacid residues, l is the pathlength in centimeters and c is the concentration in moles of residue per litre. Base line recorded for the buffer under similar conditions was substracted from the values obtained. Alterations in the secondary structure of the protein were monitored in the far-UV region in the range 200-250nm with a protein concentration of 1.04 μM in 1mm pathlength cuvette. Changes in the tertiary structure of protein were measured with a 10mm pathlength cuvette in the region of 250-300nm at a protein concentration of 10.42 μM. All the samples were spun before spectroscopic measurements. The percent α-helix was calculated by the method of Chen *et al* (1972) according to the following equation:

$$\% \ \alpha\text{-helix} = \frac{MRE_{222} - 2340}{30300} \times 100$$

3.2.7 Stability studies: The structural stability of the protein towards guanidine hydrochloride- and temperature- mediated denaturation was studied by monitoring the changes in tryptophan fluorescence intensity and far-ultraviolet CD spectra. 1.04 μM protein was incubated with increasing concentration of denaturant (0-6M GnHCl) for 6hrs at room temperature before taking the measurements. To study the temperature-induced unfolding of PeIF5B, protein sample was incubated in a thermostatically controlled cell holder fitted with the Julabo-F25 waterbath and spectroscopic scans were taken at regular intervals of temperature.

3.3 Results

3.3.1 Cloning, expression, western blotting and purification of PeIF5B: PeIF5B open reading frame of size 2.6kb was PCR-amplified from the cDNA clone, pPeIF5B, that carries a 3.5kb insert with 5'-3' UTRs and 2.6kb ORF. This PCR-amplified product was ligated into linearized pCR2.1 vector (Invitrogen) (Figure 3.1). The clones were confirmed by digestion with *Eco*RI and *Xho*I restriction enzymes present in the forward and reverse primers, respectively. The digested product released a fragment of expected 2.6kb with a 3.9kb backbone (Figure 3.2A). Using the same restriction enzymes the *PeIF5B* ORF was subcloned in pET23a expression vector (Novagen) in *EcoR*I and *Xho*I sites under T7 promoter (Figure 3.1). The positive clones carrying the PeIF5B insert gave a 2.6kb fallout from a 3.7kb pET23a backbone (Figure 3.2B). The clone was expressed in *E. coli* Bl21 (DE3) p*Lys* S strain. The induction conditions of 1mM IPTG/5hrs/37^{o}C were found to be optimal to obtain the highest yield of the recombinant protein. PeIF5B was expressed as C-terminal His-tagged protein (Figure 3.3A). The induced band could be located at a position higher than that corresponding to the predicted molecular weight of 96kDa. This is in agreement with our earlier observation where the *in vitro* transcribed and translated PeIF5B protein migrated at 116kDa position on 10% SDS-polyacrylamide gel (Chapter 2, Figure 2.6). In western blot analysis, the band of interest was quite visible using monoclonal anti-His antibodies (Figure 3.3B). The recombinant protein was purified to homogeneity

Figure 3.1

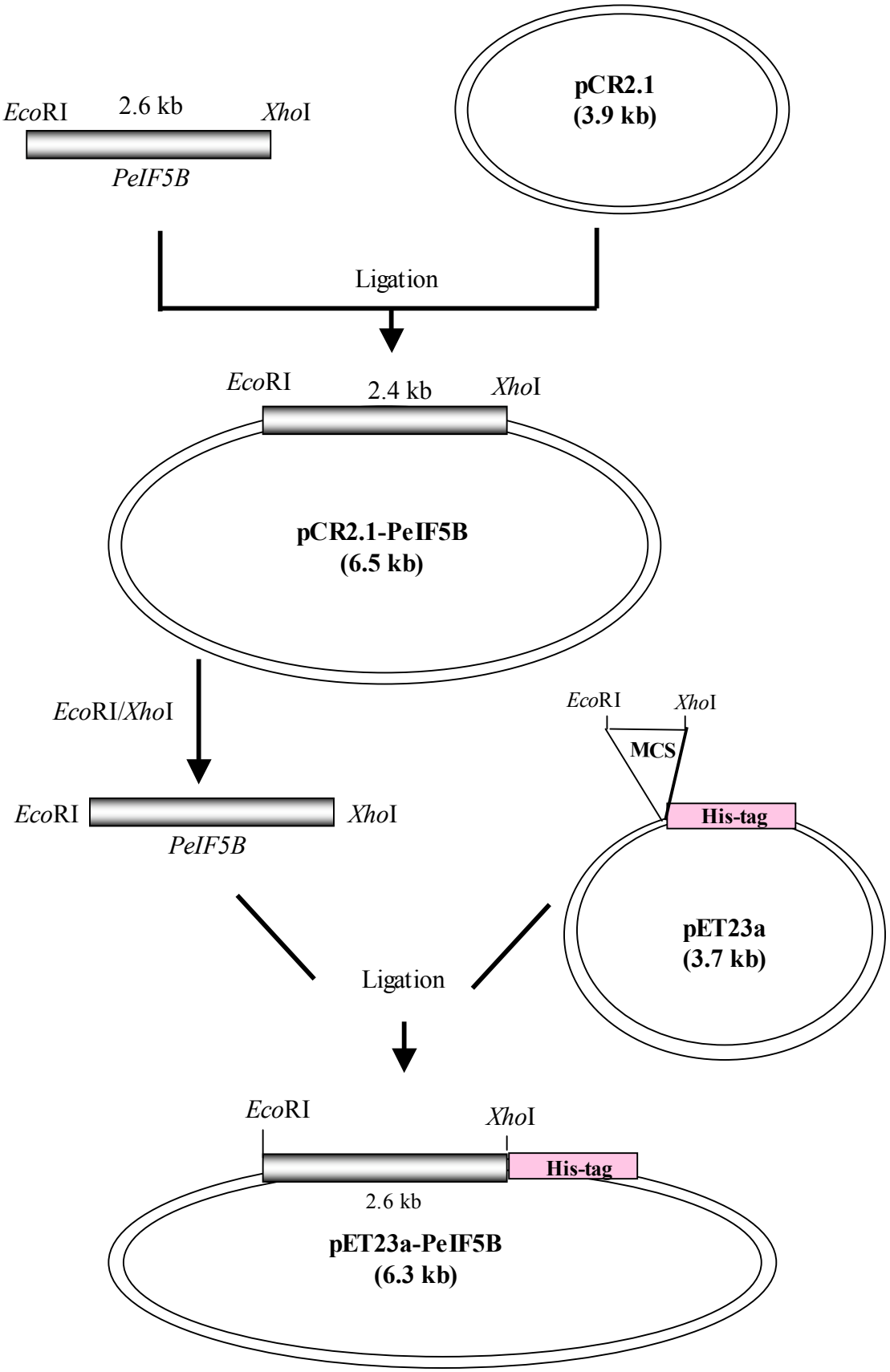

EcoRI
2.6 kb
XhoI
PeIF5B
pCR2.1
(3.9 kb)
Ligation
EcoRI
2.4 kb
XhoI
pCR2.1-PeIF5B
(6.5 kb)
EcoRI/XhoI
EcoRI
XhoI
PeIF5B
EcoRI
XhoI
MCS
His-tag
pET23a
(3.7 kb)
Ligation
EcoRI
XhoI
His-tag
2.6 kb
pET23a-PeIF5B
(6.3 kb)

FIGURE 3.1: *Schematic representation of cloning of PeIF5B ORF in bacterial expression vector under the control of T7 promoter as a His-tagged fusion protein. The 2.6kb PeIF5B ORF was PCR amplified from cDNA clone, pPeIF5B and was ligated in pCR2.1 (3.9kb). Using EcoRI and XhoI, 2.6kb fragment was released from pCR2.1-PeIF5B plasmid and cloned in same sites in pET23a (3.7kb).*

Figure 3.2

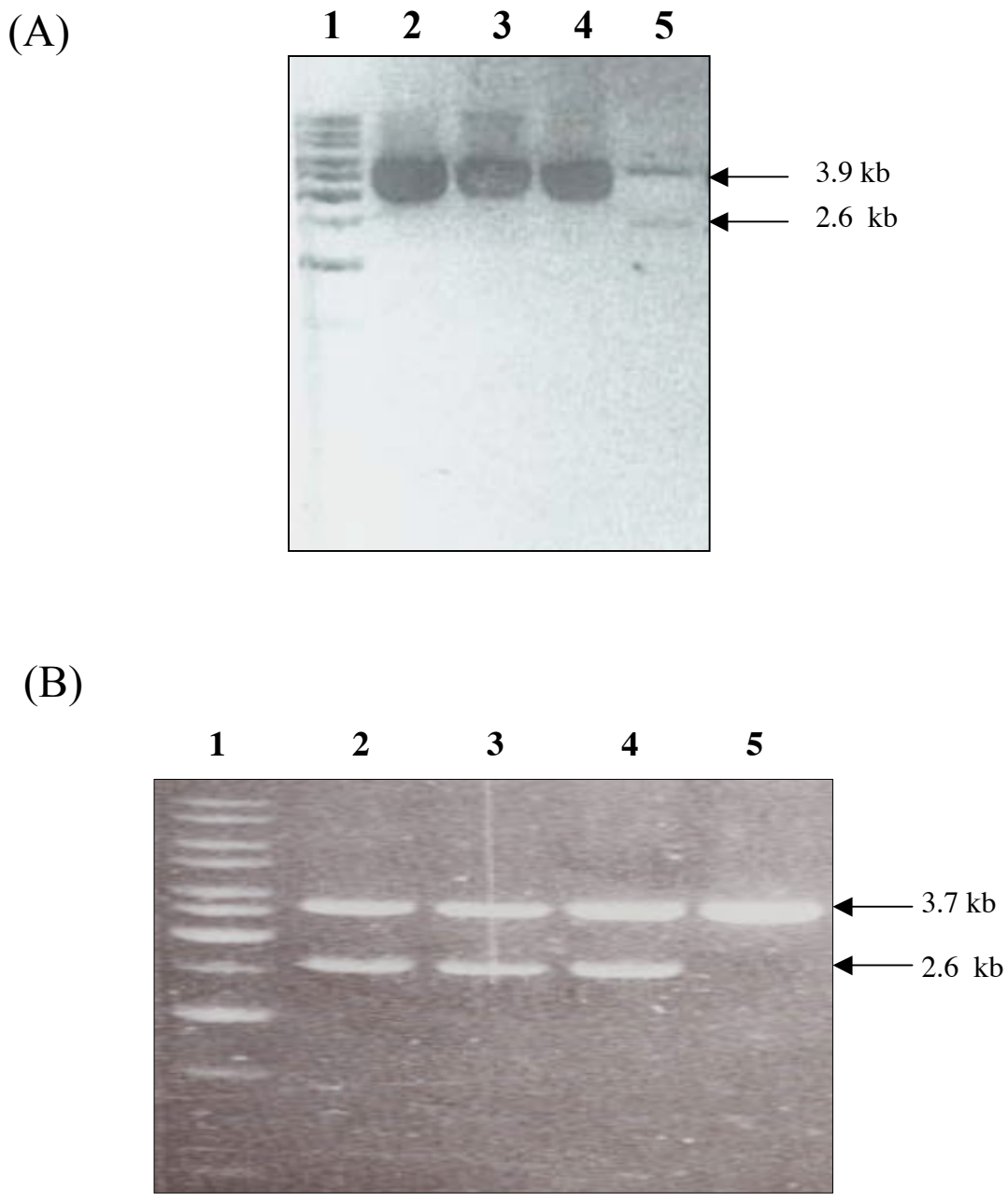


FIGURE 3.2: *Cloning of PeIF5B open reading frame into E. coli plasmids for expression of recombinant protein.* ***(A)*** *Screening of clones of PeIF5B (2.6kb) in pCR2.1 vector (3.9kb) by digestion with restriction enzymes EcoRI and XhoI. Positive clones were expected to give a band pattern of 2.6kb fragment and 3.9kb backbone (lane 5). Lane 2-4 are the negative clones. Lane 1 is the 1kb DNA ladder from MBI Fermentas.* ***(B)*** *PeIF5B ORF was cloned under T7 promoter in pET23a expression vector (3.7kb) in EcoRI and XhoI sites. Digestion of pET23a-PeIF5B clones with the corresponding enzymes released the 2.6kb insert DNA from the 3.7kb vector backbone (lane 2-4). Lane 5 is a self-ligated vector. Lane 1 in the DNA ladder.*

Figure 3.3

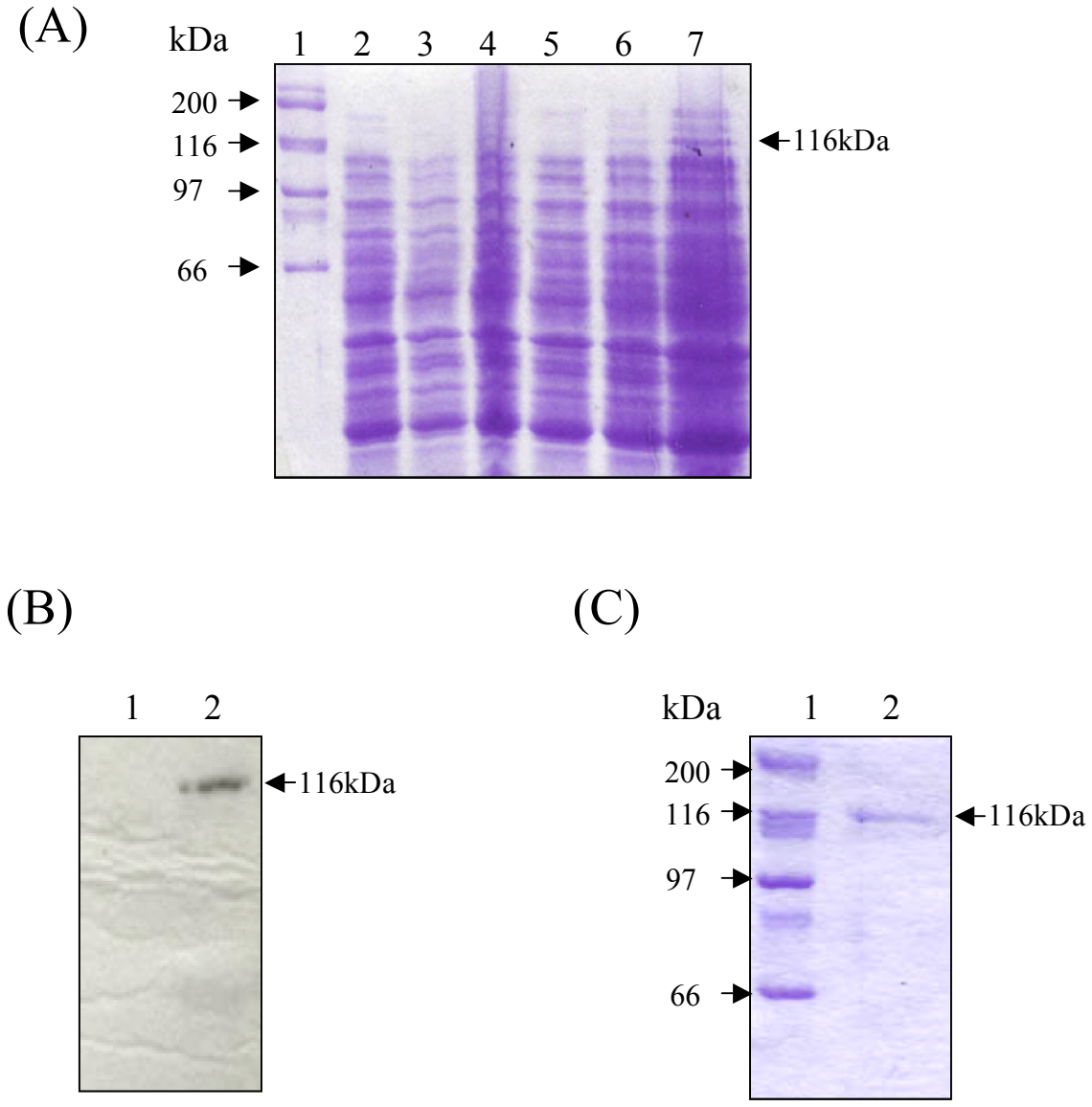


FIGURE 3.3: *PeIF5B expression in E. coli Bl21(DE3) pLys S cells. **(A)** SDS-PAGE profile of expression of PeIF5B in bacterial cells. Lanes 5,6,7 represent uninduced, 1hr and 4hrs induced total cell lysate, respectively while lanes 2-4 are vector control of uninduced (lane 2), 1hr (lane 3) and 4hrs (lane 4) induced total cell lysate. Lane 1 is protein molecular size marker. **(B)** Western blot with anti-His antibodies showing recombinant PeIF5B in total cell lysate (lane 2) and corresponding vector control (lane 1). **(C)** SDS-PAGE profile of affinity purified His-tagged PeIF5B protein from E.coli. Lane 1 is protein molecular size marker. The PeIF5B protein band of size 116kDa is marked by an arrow.*

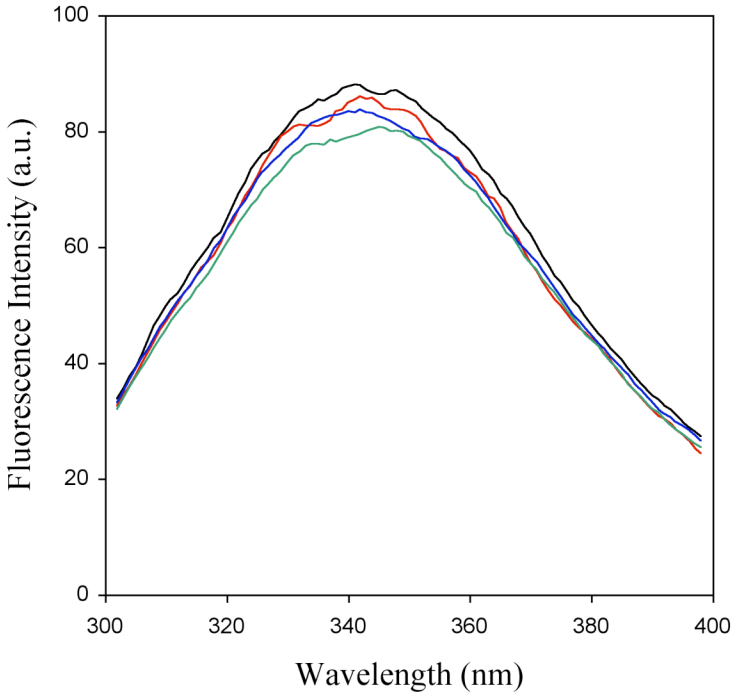
from insoluble fraction in denaturing conditions and refolded by dialysis against 20mM Tris-HCl (pH 8.0) and 100mM NaCl (Figure 3.3C).

3.3.2 Ligand induced conformational changes in PeIF5B: The delicate process of the final assembly of the protein synthesis machinery occurring during translation initiation involves the binding of various factors as well as multiple sequential protein-RNA and protein-protein interactions. Hence, biophysical spectroscopic techniques like fluorescence and circular dichroism were exploited to study the interaction of GTP, metal-ion and initiator tRNA with PeIF5B, a eukaryotic homologue of bacterial IF2 from *Pisum sativum*.

3.3.2.1 Metal ion-binding to PeIF5B: Metal ion-binding to PeIF5B was checked by measuring the intrinsic fluorescence of the protein in the presence of magnesium ion (Figure 3.4A). Native protein showed strong fluorescence at 341nm when excited at 280nm. The emission spectrum in the range 300-400nm after exciting the protein at 280nm is contributed by Trp and Tyr residues. PeIF5B has five Trp and fourteen Tyr residues in its polypeptide chain. With increasing concentration of magnesium ion added (0-50mM) to PeIF5B protein, there was only a slight decrease in its fluorescence. No apparent change in the wavelength of maximum emission (λ_{max}=341nm) could be observed. Thus, effect of Mg^{2+} binding to its pocket on the protein did not lead to any major change in its tertiary conformation. Moreover, upto 10mM Mg^{2+} concentration no change in ANS fluorescence could be found (Figure 3.4B). However, further increase in the ion

Figure 3.4

(A)



(B)

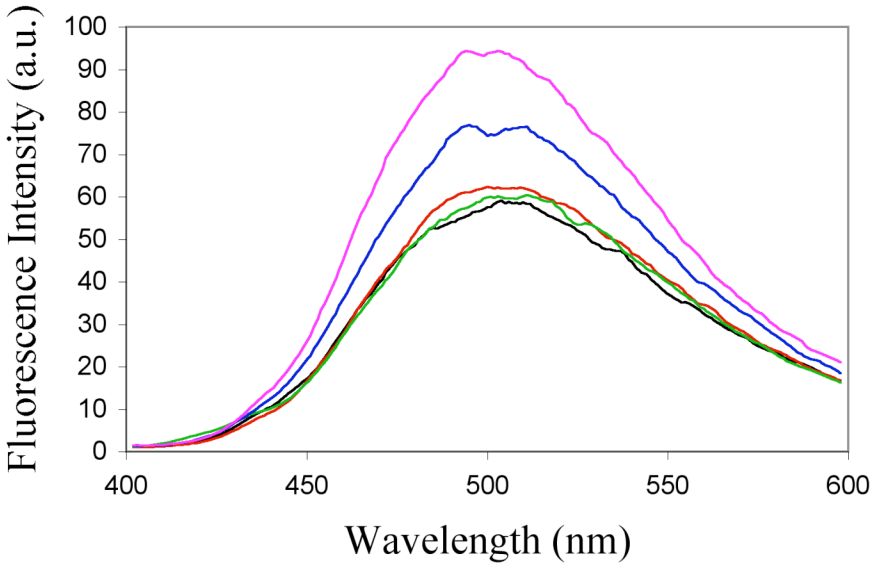


FIGURE 3.4: *Magnesium ion-binding to PeIF5B.* ***(A)*** *Intrinsic fluorescence spectra of PeIF5B when excited at 280nm in presence of increasing concentration of Mg^{2+}ion: 0mM (black), 5mM (red), 10mM (blue) and 50mM (green).* ***(B)*** *ANS fluorescence spectrum of PeIF5B when excited at 380nm in presence of increasing concentration of Mg^{2+}: 0mM (black), 1mM (red), 10mM (green), 20mM (blue) and 50mM (pink).*

concentration showed relatively enhanced intensity (and hence increased binding) of the extrinsic fluorophore to the protein. It suggests that the Mg^{2+} at higher concentrations causes conformational reorganization leading to some exposure of certain hydrophobic groups, previously buried at lower metal ion concentrations.

Far-UV CD spectroscopy analysis was further employed to investigate the metal ion-binding property of PeIF5B. CD spectra of PeIF5B showed the characteristic profile of a protein with α- and β- secondary structures. A double minima at 208nm and 217nm was evident (Figure 3.5). The helical content of the protein is 7% of the total secondary conformations, calculated as described in materials and methods. Figure 3.5 shows far-UV CD spectra of PeIF5B in presence of increasing concentrations of Mg^{2+} ion (0-6mM). As shown in the figure, an overall decrease in the far-UV signal of the protein in the presence of metal ion could be observed. At 6mM Mg^{2+}, there was around 43% loss in the α-helical content. Hence, binding of the metal ion to its pocket led to changes in the secondary structure of the protein. On further increasing the concentration of the ligand, no apparent change could be observed. Hence it can be concluded that Mg^{2+} -binding to the factor does not cause any major change in the tertiary structure of the protein, however, there are some changes in the secondary structure.

3.3.2.2 GTP-binding to PeIF5B: Figure 3.6 shows the effect of increasing concentration of GTP on protein conformation. A steady, sharp decrease in fluorescence intensity upto 100μM GTP followed by a gradual decrease upto

Figure 3.5

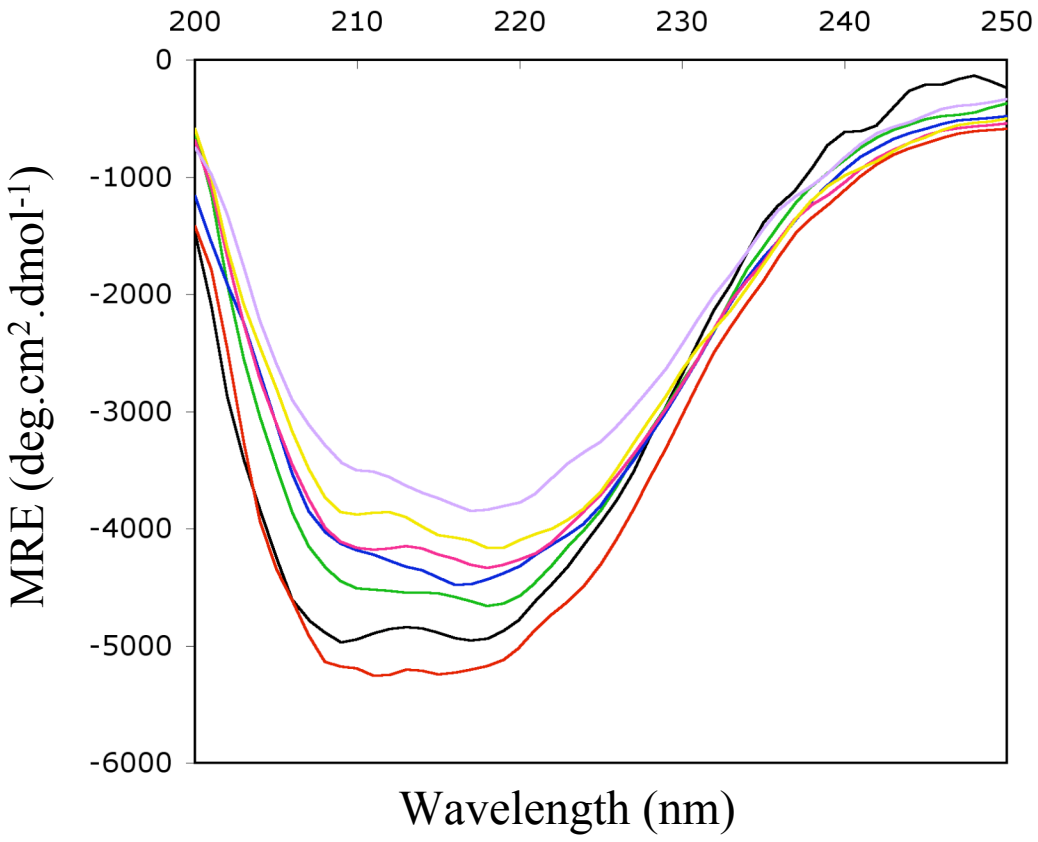


FIGURE 3.5: *Far-UV CD spectra of PeIF5B in presence of Mg^{2+}: 0mM (black), 1mM (red), 2mM (green), 3mM (blue), 4mM (pink), 5mM (yellow) and 6mM (purple).*

Figure 3.6

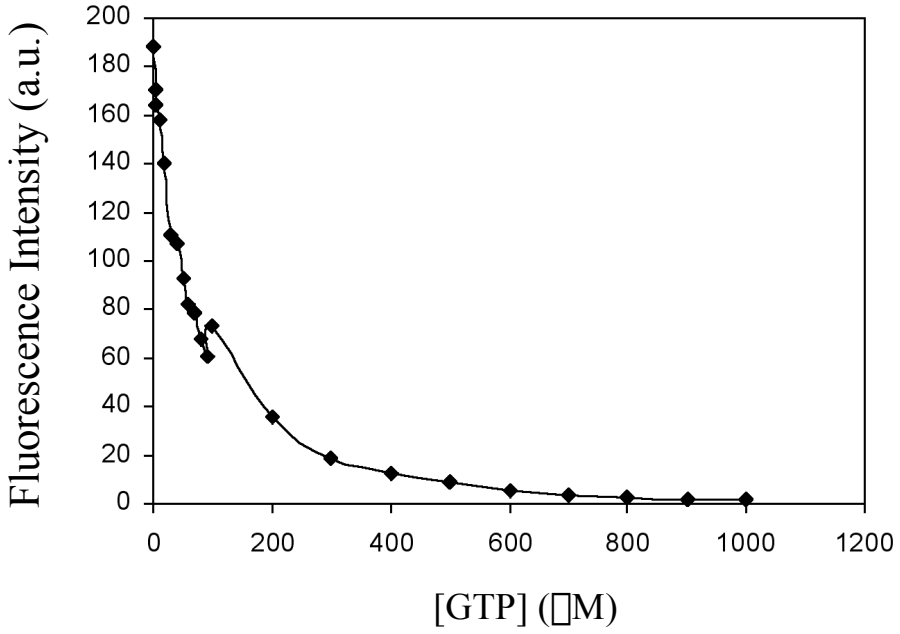


FIGURE 3.6: *Plot of change in intrinsic fluorescence intensity of PeIF5B at 340nm when excited at 280nm with increasing GTP concentration (0-1000□M).*

500μM concentration could be seen (Figure 3.6). At higher GTP concentrations, protein fluorescence was almost totally quenched. However there was no significant shift in λ_{max} of emission. Nucleotide-binding to its pocket in the protein does not alter the hydrophobic patches on the protein molecule as seen by ANS binding. GTP-binding not only seems to alter the overall microenvironment of the aromatic residues but also affected the secondary structure of the protein as seen by monitoring the changes in the far-UV CD spectra in the presence of increasing concentrations of GTP (Figure 3.7). Very interestingly, GTP-binding appears to produce measurable changes in the far-UV region around 213nm with no effect at 208nm and 232nm leading to two isosbestic points. At 500μM GTP the protein almost lost all its α-helix.

3.3.2.3 GTP-binding to PeIF5B in the presence of Mg^{2+}: As mentioned earlier, translation factor PeIF5B exhibits strong negative circular dichroism bands between 208 and 217nm indicating considerable secondary structure. As could be seen in figure 3.8A, addition of 2mM Mg^{2+} to the protein caused slight alteration in the secondary structure as observed from the decrease in 208nm band corresponding to slight structure reorganization or decrease in α-helical structure. However, relatively less change was observed at around 217nm rather than 208nm. At 500μM GTP concentration, unlike Mg^{2+}, ellipticity decreased in the wavelength range 208-222nm. Probably, GTP binds to a pocket or crevice neighbouring the helical and β secondary structures. However, when GTP was added to the protein

Figure 3.7

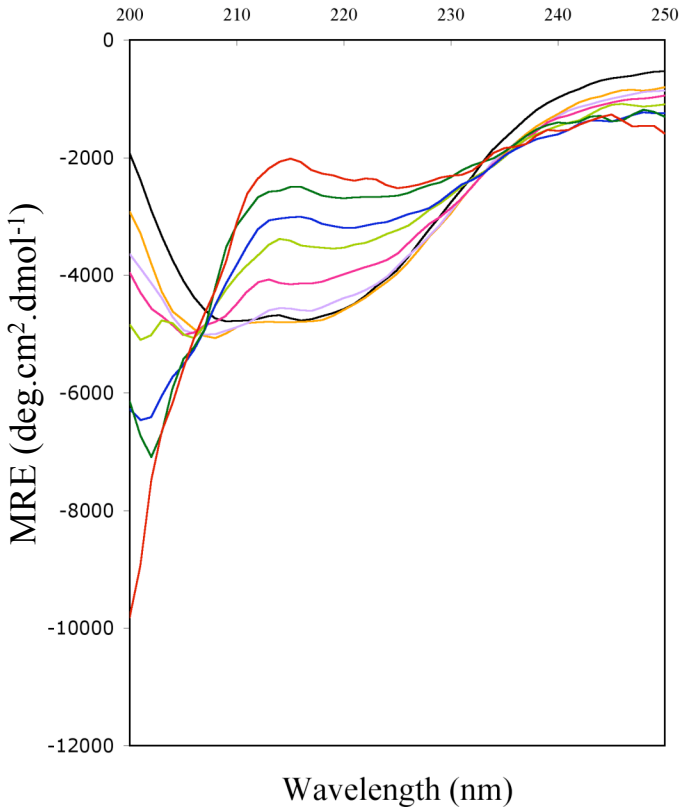
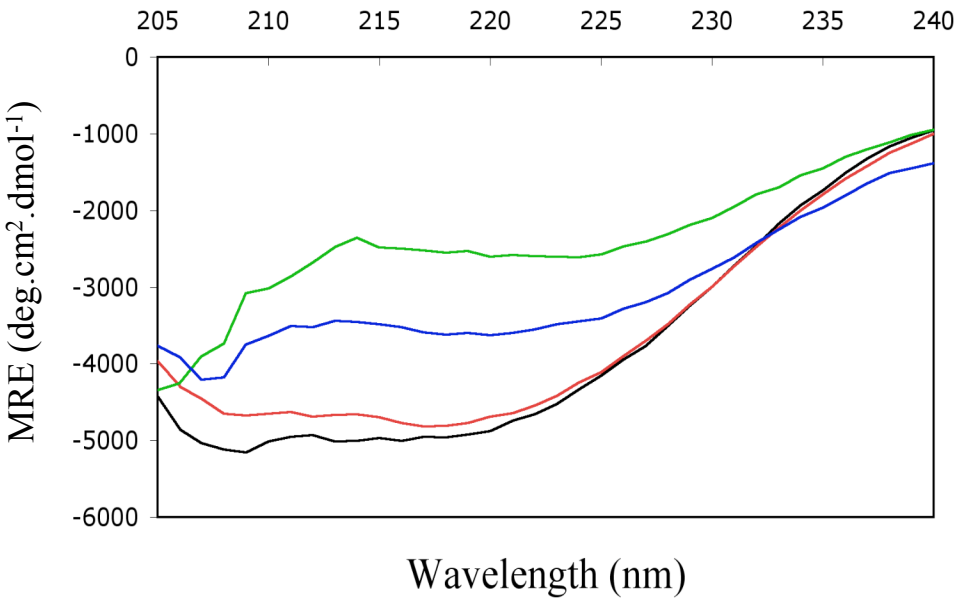


Figure 3.7: *Far-UV CD spectra of PeIF5B in the presence of GTP: 0□M (black), 25□M (orange), 50□M (purple), 100□M (pink), 200□M (light green), 300□M (blue), 400□M (dark green) and 500□M (red).*

Figure 3.8

(A)



(B)

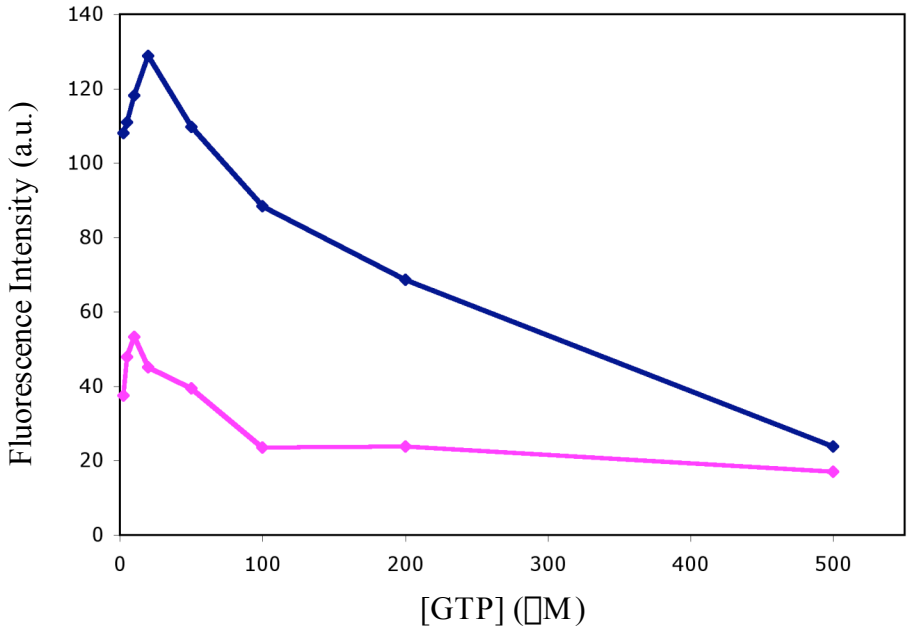


FIGURE 3.8 (A) *Far-UV CD spectra of PeIF5B : native (black), in presence of 2mM Mg^{2+} (red), in presence of 500□M GTP (green) and in presence of 2mM Mg^{2+} and 500□M GTP (blue).* ***(B)*** *Change in intrinsic (blue) at 342nm and extrinsic (pink) at 510nm fluorescence on GTP-binding (0-500□M) in presence of 10mM Mg^{2+}.*

preparation in the presence of metal ion, the changes were less intense. These results suggest that Mg^{2+} ion stabilizes the protein.

Later the effect of Mg^{2+} ions was checked on GTP-binding to PeIF5B by measuring the intrinsic and extrinsic fluorescence (Figure 3.8B). Maximal quenching (~80%) at ~500 M concentration of GTP in the presence of Mg^{2+} was slightly less as compared to 95% decrease in intensity in the absence of Mg^{2+} (compare figures 3.6 and 3.8B). ANS fluorescence was also monitored in the presence of Mg^{2+}. GTP-binding caused significant reduction in the ANS fluorescence emission intensity in presence of metal ion whereas in the absence of metal ion, change was insignificant as mentioned earlier. At 100 M GTP, maximum decrease in extrinsic fluorescence can be seen (50%) indicating that GTP-binding in presence of Mg^{2+} causes burial of some hydrophobic groups.

3.3.2.4 tRNA-binding to PeIF5B: Flourescence studies to examine *in vitro* binding of charged initiator tRNA, Met-$tRNA_f^{Met}$, to PeIF5B were performed. No difference in the fluorescence emission spectrum could be observed upon Met-$tRNA_f^{Met}$-binding to PeIF5B (Figure 3.9) in the absence of cofactors. However, in the presence of 2mM Mg^{2+} and 200mM GTP, significant decrease in fluorescence emission intensity was observed. This clearly indicates that charged initiator tRNA binding to PeIF5B is a cofactor-dependent process. When charged initiator tRNA was added to the reaction mixture containing 2mM Mg^{2+} and 200 M GTP, the fluorescence was enhanced relative to that obtained with metal ion and the

Figure 3.9

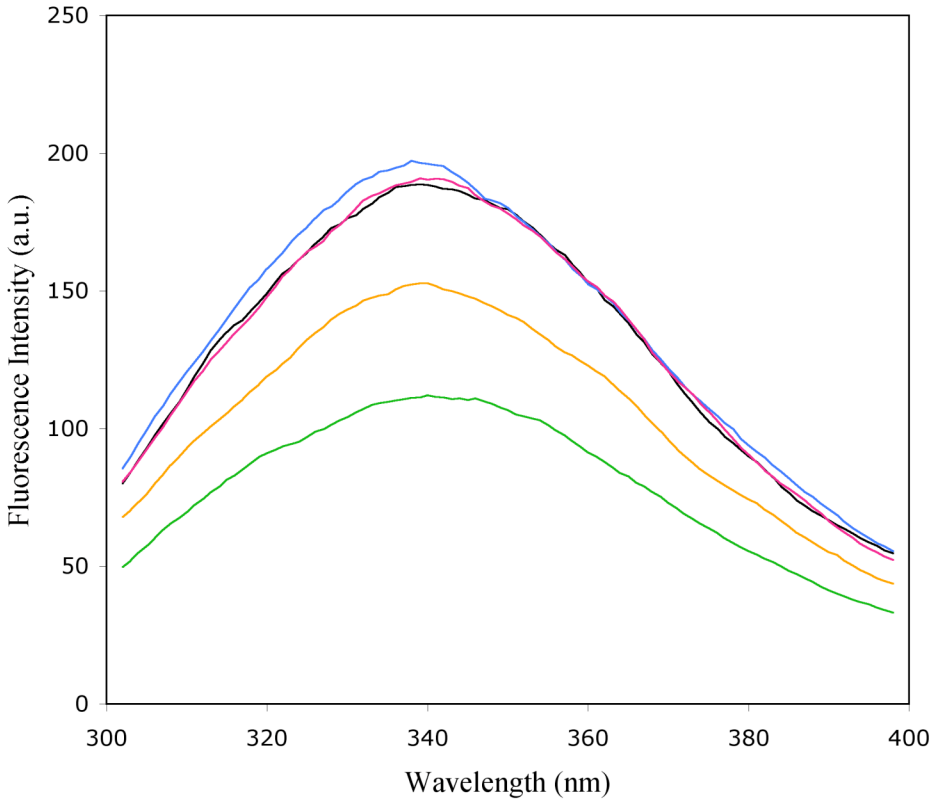


FIGURE 3.9: *Intrinsic fluorescence emission spectra of PeIF5B (black), in presence of 2mM Mg^{2+} (blue), charged initiator tRNA (pink), 2mM Mg^{2+} and 200□M GTP (green) and 2mM Mg^{2+}, 200□MGTP and charged initiator tRNA (orange) to study in vitro binding of charged initiator tRNA to the pea translation initiation factor.*

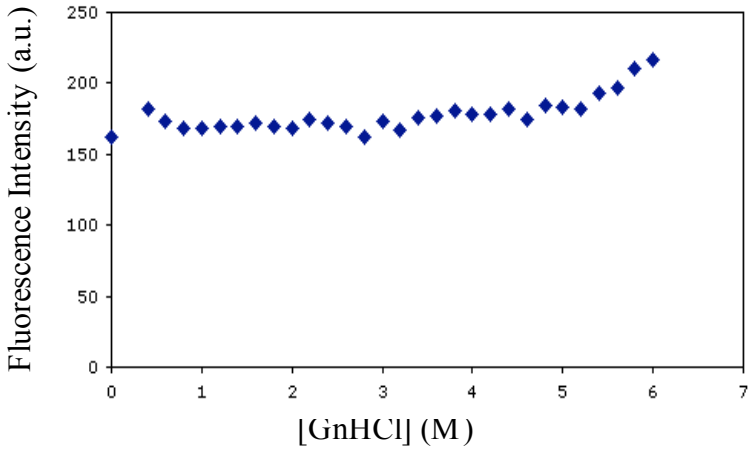
nucleotide. This may be due to conformational optimization of the protein for forming a ternary complex with GTP and tRNA in the presence of magnesium ion.

3.3.3 Stability studies of peIF5B:

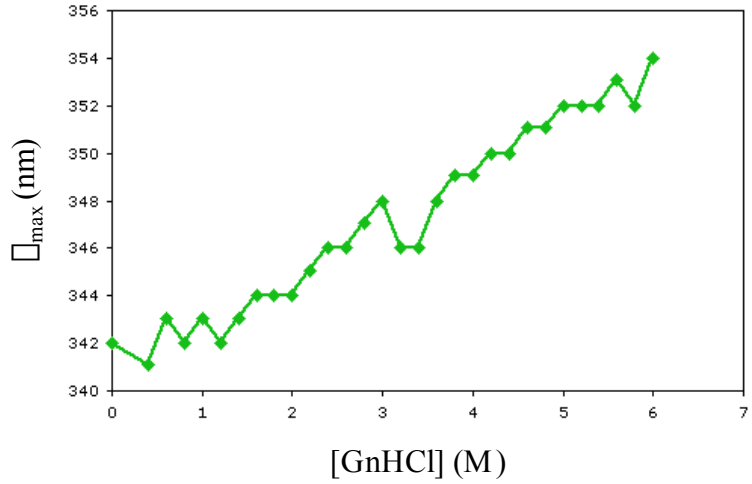
3.3.3.1 Guanine hydrochloride mediated denaturation: Intrinsic tryptophan fluorescence is a sensitive probe of local and global conformation in proteins and has commonly been used to investigate protein conformational stability consequent to the addition of chemical denaturants such as urea and GnHCl. Changes in the protein conformation due to the action of guanidine hydrochloride were monitored by measuring the intrinsic fluorescence of the protein. Figure 3.10A shows the denaturation pattern of PeIF5B induced by GnHCl in the concentration range of 0-6M. As can be seen from the figure, no appreciable change in the intrinsic fluorescence of the protein could be seen in presence of the denaturant. However, a change in λ_{max} was detected with an eminent red shift. A net shift of 12nm from 0-6M GnHCl (Figure 3.10B) accounts for substantial change in the microenvironment of aromatic residues of the protein. Figure 3.10C shows the decrease in ANS-binding with increasing denaturant concentration due to rearrangement (burial) of hydrophobic patches on the protein.

3.3.3.2 Thermal denaturation of PeIF5B: Stability of PeIF5B against temperature was monitored by studying the changes in the tertiary and secondary structures of the protein using intrinsic fluorescence measurement at 340nm and far-UV circular

(A)



(B)



(C)

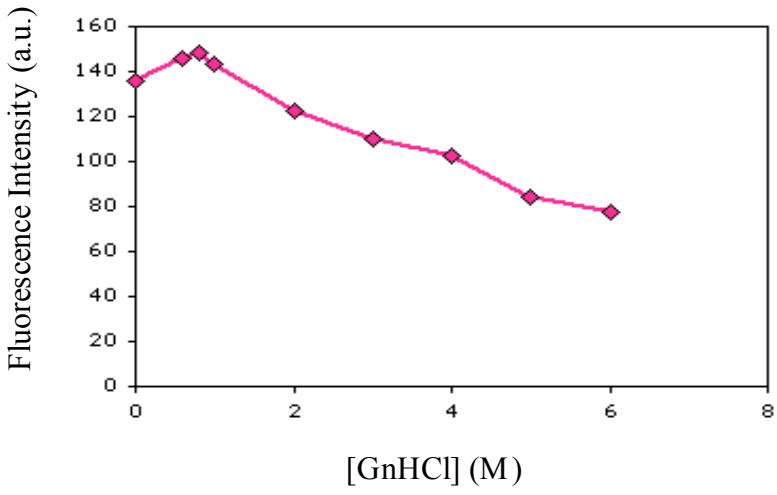


FIGURE 3.10: *GnHCl-induced denaturation of PeIF5B* ***(A)*** *Plot of intrinsic fluorescence intensity of PeIF5B at 340nm against increasing concentrations of GnHCl.* ***(B)*** *Plot of □$_{max}$ in nm against increasing concentrations of GnHCl.* ***(C)*** *Changes in ANS fluorescence at 480nm with increasing concentrations of GnHCl.*

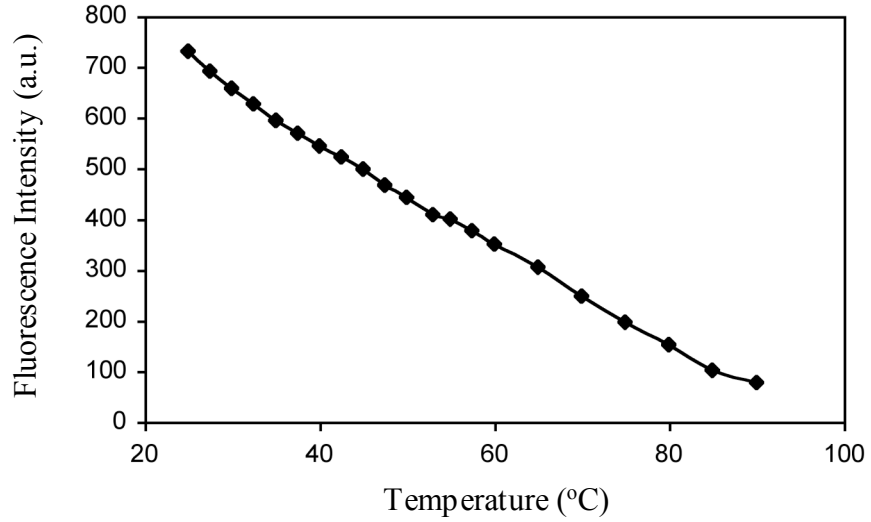
dichroism. With increasing temperature, there is a steady decrease in the fluorescence intensity (Figure 3.11A) where as not much alteration in the secondary structure of the protein could be seen as apparent from far-UV CD spectra (Figure 3.11B).

3.4 Discussion

The translation of a eukaryotic mRNA into a polypeptide is a complicated process requiring twelve or more initiation factors. Out of an array of translation initiation factors, only two are common between eukaryotes and eubacteria/archaea: IF2/eIF5B and IF1/eIF1A (Roll-Mecak *et al*., 2000). First identified in *Saccharomyces cerevisiae* (Choi *et al*., 1998), eIF5B has subsequently been detected in other eukaryotes (Lee *et al.*, 1999; Wilson *et al.*, 1999, Carrera *et al.*, 2000) and archaebacteria (Lee *et al.*, 1999). PeIF5B, resembling IF2/eIF5B, adds one more player in the translation initiation machinery. On the basis of functional domain prediction, it is suggested to function as translation initiation factor and therefore should bind to GTP and Mg^{2+} ion and recruit charged initiator tRNA. Hence, the current study was undertaken to follow the changes in the protein conformation and secondary structure upon binding to its cofactors: Mg^{2+} and GTP as well as initiator tRNA. The conformational differences between free and bound forms of the protein are important for the molecular mechanisms involved in the functioning. Owing to its similarity to e/aIF5B and the universal bacterial translation initiation factor IF2 and the structural information available from

Figure 3.11

(A)



(B)

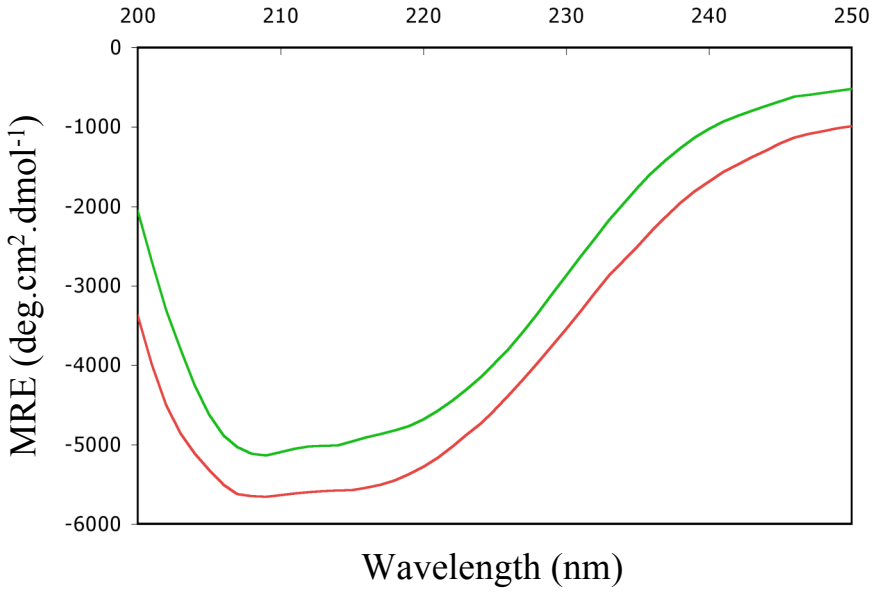


FIGURE 3.11: *Temperature-induced denaturation of PeIF5B* ***(A)*** *Changes in intrinsic fluorescence at 345nm on thermal denaturation of PeIF5B (25°C-95°C) when excited at 280nm.* ***(B)*** *Far-UV CD spectra of PeIF5B at 25°C (-) and at 95°C (---).*

different translation initiation and elongation factors, the results obtained here were correlated with the structural details obtained by them, wherein they provide conclusive evidence for conformational changes occurring on Mg^{2+}/GTP-binding to the protein.

The three-dimensional structure of the two structured modules of C-terminal domain of *Bacillus stearothermophilus* IF2 has been recently solved. The solution structure of IF2-C1 shows that it forms a flattened fold with a core of four parallel β-strands flanked by three α-helices (Wienk *et al.*, 2005). The NMR-structure of the other module (IF2-C2) of the factor provides evidence for direct interaction with fMet-$tRNA_f^{Met}$ (Meunier *et al*., 2000). The X-ray structure of *M. thermoautotrophicum* aIF5B by Roll-Mecak *et al.* (2000) was the first attempt to study the structural details of e/aIF5B translation initiation factor (Figure 3.12). The elucidated crystal structure of the archaeal aIF5B protein is divided into four domains arranged in the form of a molecular "chalice", with domains I-III forming the cup and connecting downwards through a long α-helix to domain-IV which forms the base. Domain I (residues 1-225), called the G-domain, is an eight stranded β-sheet of mixed polarity (seven parallel and one anti-parallel β-strand) flanked by six α-helices and a single 3_{10}helix. It carries four conserved motifs (G1, G2, G3 and G4) that are characteristic features of G-proteins (Brock *et al.,* 1998; Lee *et al.*, 1999). Domain II (residues 231-327) is structurally similar to corresponding domain of EF-Tu and EF-G. The three β-strands (of the total 11 antiparallel strands forming a β-barrel) of domain-II are responsible for interacting

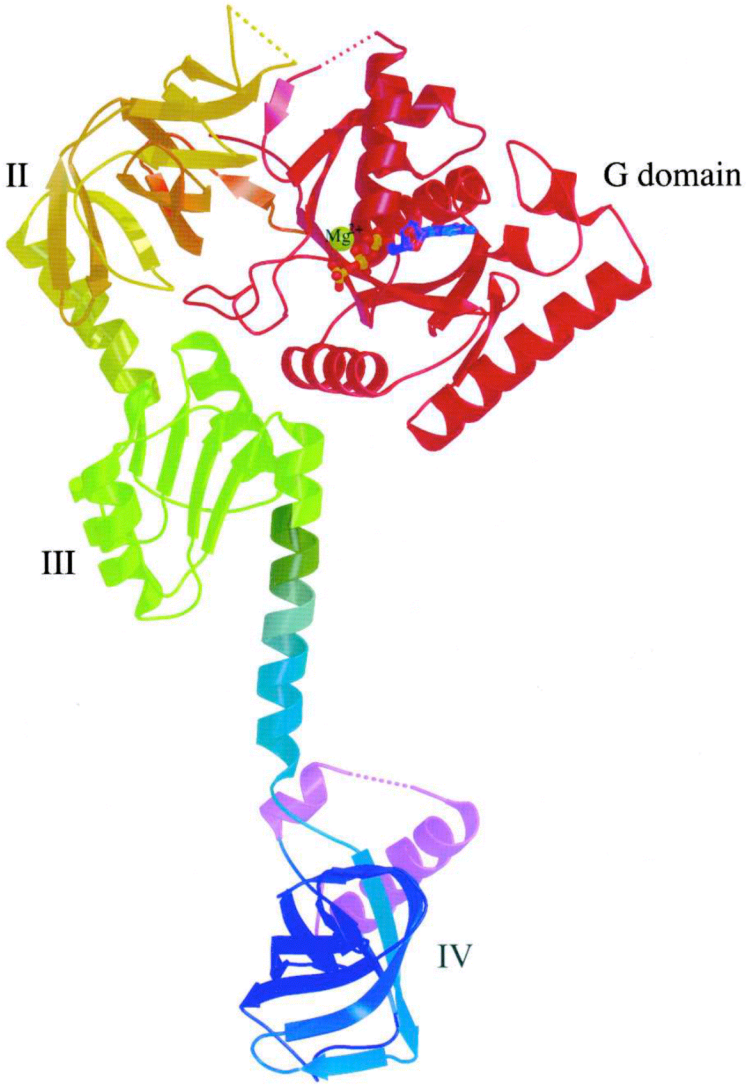


FIGURE 3.12: *Crystal structure of aIF5B from Methanococcus thermoautotrophicum*

with the G-domain in the vicinity of Switch 2 region, where they stabilize the relative orientations of domains I and II. Domain II connects through a 17 residues long α-helix to domain III (residues 344-445) that consists of a four-stranded parallel β-sheet flanked on both sides by two α-helices. A 40Å long α-helix forms the stem of the chalice, connecting domain III to the C-terminal domain IV (residues 462-550) that forms the base of the chalice. Domain IV consists of an eight-stranded anti-parallel β-barrel followed by two α-helices. Thus, PeIF5B is clearly an α-helix and β-sheet rich protein, as can also be seen from the far-UV CD spectrum (Figure 3.5).

In *M. thermoautotrophicum* translation initiation factor aIF5B, guanine moiety binds to a shallow hydrophobic pocket in the G domain whereas the catalytic Mg^{2+} is situated in a cleft separating the GTP-binding site from Switch 2 of the G domain. Similar observation was reported in *E. coli* translation elongation factor EF-Tu•GDP where guanine moiety is bound to a shallow hydrophobic pocket formed by two of the four loops connecting β-strands with the helices (Kjeldgaard and Nyborg, 1992). The other two loops in the G-domain are involved in the binding of the phosphate groups and the magnesium ion. The hydrophobic pocket is in between Leu175 and Lys136. The guanine base interacts with Asp138, Asn135, Ala174 and Ser173. It is suggested that Asn51 would interact with the ribose moiety. The phosphate-binding loop is a rigid structure with a consensus sequence G^{18}XXXXGK(S/T). The four main-chain nitrogen atoms from Asp21 to Lys24 are in close proximity with the β-phosphate group. The β-phosphate group

is bonded to Thr26. The magnesium ion-binding site is situated in a cleft between nucleotide-binding site and switch-loop Asp80-His84. It is shown in *Thermus thermophilus* EF-Tu•GTP crystal structure that the metal ion coordinates with the β- and γ- phosphates as well as Thr25 and Thr62 (Berchtold *et al*., 1993). This correlates well with results obtained with fluorescence spectroscopy and circular dichroism analysis of PeIF5B in presence of ligands. As seen in Figure 3.4A and B, Mg^{2+} binds to PeIF5B leading to negligible decrease in tryptophan fluorescence intensity and minimal enhancement in ANS fluorescence. This implies that only minor conformational alterations occur upon metal ion-binding to the factor. GTP, on the other hand, was observed to bind PeIF5B in a concentration-dependent manner causing a significant decrease in intrinsic fluorescence intensity (Figure 3.6). This reflects that upon GTP-binding local conformational changes occur near aromatic amino acids that result in decrease in fluorescence. However, no gross change in the conformation of the protein occurred on nucleotide-binding as evident from absence of any change in the λ_{max} of the protein (λ_{max} =341nm) as well as no effect on ANS-binding with increasing concentration of GTP. It is known that binding of GTP/Mg^{2+} causes local conformational changes in Switch 1 and Switch 2 regions in the G-domain (Roll-Mecak *et al.,* 2000). However, conformational changes can be visualized from the far-UV CD spectra of PeIF5B in the presence of Mg^{2+}/GTP that show a significant change in the molar ellipticity values in the range 208 to 217nm (Figure 3.5 and 3.7). It is shown that during GTP-GDP exchange, repositioning of domains occur (Roll-Mecak *et al.*, 2000;

Berchtold *et al*., 1993). However, it is reported that the structures of free EF-G and GDP-bound forms are almost identical (Ævarsson *et al*., 1994).

As PeIF5B is considered as a representative of eIF5B-like factor from plant system, its tRNA-binding characteristic was studied by fluorescence. The C2 domain of IF2 from *B.stearothermophilus* is involved in tRNA-binding (Spurio *et al*., 2000). Solution structure of the fMet-$tRNA_f^{Met}$ –binding domain of IF2 from *B.stearothermophilus* reveals that six antiparallel β-strands, connected by loops, form a β-barrel (Meunier *et al*., 2000). In case of aIF5B from *M. thermoautotrophicum*, two copies of such β barrels are present (domains II and IV). Domain II has been suggested to constitute part of the ribosome-binding platform, leaving domain IV free to interact with tRNA (Roll-Mecak *et al*., 2000). It has been suggested that the binding of the aminoacyl-tRNA occurs in a positively charged cleft between domain I and II of EF-Tu from *Thermus thermophilus* (Berchtold *et al*., 1993). This cleft is created only when GTP-binding induces domain rearrangement. Intrinsic fluorescence results of the binding of Met-charged initiator *E.coli* tRNA molecule to PeIF5B in the absence and presence of cofactors suggest that Mg^{2+} ion and nucleotide facilitate tRNA-binding (Figure 3.9). The ternary complex of protein-GTP/Mg^{2+}-tRNA shows an intermediate conformation. Roll-Mecak *et al.* (2000) suggested that the conserved acidic surface on G-domain and part of domain II on the ventral surface of *M. thermoautotrophicum* aIF5B serves as a ribosome-binding site.

Although GnHCl induced a shift in the λ_{max} and ANS quenching of PeIF5B, there was negligible effect on the fluorescence intensity of aromatic groups as a function of denaturant concentration (Figure 3.10A, B, C). This suggests local conformational rearrangements in the protein upon GnHCl-binding without any gross effect on global protein tertiary structure. Thermal denaturation studies showed that PeIF5B is quite a stable protein that resists conformational changes with increase in temperature as seen by far-UV CD spectra (Figure 3.11B). The progressive decrease in the intrinsic fluorescence intensity of the protein with increasing temperature may be indicative of deactivation of fluorophore or changes in the tertiary structure that might bring the fluorophore in the vicinity of a quencher (Bhattacharjee and Das, 2000) (Figure 3.11A). The far-UV study of thermal denaturation of PeIF5B showed that even at 95^{o}C protein retained its native-like secondary structure. The chemical and thermal denaturation data indicate that PeIF5B is a remarkably stable protein. Similar thermal stability is shown by β-crystallin, protein found in the vertebrate eye lens. It acts as a molecular chaperon and protects other proteins from denaturing under stress or high temperature conditions (del Valle *et al.*, 2002). The translation elongation factor EF-Tu from *Zea mays* L has been related to imparting heat tolerance by acting as a molecular chaperon and hence protecting cellular proteins from thermal aggregation and inactivation (Rao *et al.,* 2004). Caldas *et al.* (2000) have stated a similar role for elongation factor EF-G and initiation factor IF2. These factors interact with unfolded and denatured proteins and promote protein folding and renaturation after stress. Based on these results one can conclude that PeIF5B

factor from *Pisum sativum* may function in the cell as a translation initiation factor and at the same time display chaperon-like activity. It may act as a molecular chaperone and protect other proteins from denaturing under stress or high temperature conditions. The functional characterization of this factor therefore becomes very important, and this is presented in the next chapter.

Chapter 4

FUNCTIONAL CHARACTERIZATION OF PeIF5B

Chapter 4: FUNCTIONAL CHARACTERIZATION OF PeIF5B

Contents

FUNCTIONAL CHARACTERIZATION OF PeIF5B

4.1 Rationale

One of the important steps in the regulation of gene expression is at the level of translation where mRNAs are 'read' by factories called ribosomes and are translated into proteins (Pain, 1996). In prokaryotes, it is apparent that transcription and translation of mRNAs are coupled (Mangiarotti and Schlessinger, 1967; Miller *et al*., 1970). But, it has been assumed for a long time that in eukaryotes, this coupling is truncated due to compartmentalization; transcription occurring in the nucleus while translation in the cytoplasm. In organelles like mitochondria and chloroplast, this scenario is quite different. It has been known that mitochondria and chloroplast possess their own DNA that is expressed by specific transcription and translation systems. Overall, the process of translation in these membranous organelles resembles prokaryotic protein synthesis more closely than eukaryotic translation.

Unlike these semi-autonomous organelles, for all these years, nucleus was considered to harbor the machinery involved in transcription only. This spatial separation between the nucleus and cytoplasm was supposed to be of significant importance. But, against this line of thought, some earlier studies propounded the concept of nuclear translation. As per this theory, the functional significance of nucleus harboring translation process is to carry out nonsense-mediated mRNA

decay. It is required to recognize any internal nonsense codon in the nascent mRNA by a translating ribosome. Hence, the nonsense codon should be recognized by a nuclear ribosome before its translocation into the nucleus. Frenster *et al.* (1960, 1961) isolated ribonucleoprotein particles from isolated nuclei of calf thymus lymphocytes. These isolated particles were shown to possess the ability to incorporate radioactive precursors into polypeptide in a nucleus-free system. Protein synthesis was observed within the isolated calf thymus cell nucleus. These thymus nuclear ribosomes were found to be ribonuclease sensitive while in the nucleus and required sodium rather than potassium cation environment for their metabolic activity in the nucleus. The addition of deoxyribonucleic acid of diverse sources stimulates amino acid incorporation by isolated nuclear ribosomes, but other polyanions were inhibitory. Similar active ribonucleoprotein particles were reported in rat liver nuclei (Rendi, 1960). The nuclear fractions isolated from rat liver were capable of incorporating labeled amino acids *in vitro.* Mangiarotti and Chiaberge (1997) found biologically active newly-assembled 40S and 60S ribosomal subunits in the nuclei of *Dictyostelium* cells. Furthermore, cytoplasmic ribosomal subunits containing immature RNA enter polyribosomes with the same efficiency as mature ribosomal subunits (Mangiarotti *et al*., 1997). This suggests the probability of coupled transcription and translation even in the nuclei. Later evidence of coupled transcription-translation in eukaryotes was given by Mangiarotti (1999) in *Dictyostelium discoideum*. It was shown that biochemically active polyribosomes occur in the nuclei of *Dictyostelium* cells. The presence of active nuclear polyribosomes is a prerequisite for the coupling of transcription and

translation. mRNA molecules enter nuclear polyribosomes while they are being synthesized. They show that nonsense-mediated decay occurs while the mRNAs are still bound to newly formed nuclear ribosomes. It is seen that RNA-RNA duplexes occur within the cell nucleus that are involved in antisense inhibition of ribosome translation. Lund and Dahlberg (1998) found that all tRNAs can be aminoacylated in nuclei of *Xenopus* oocytes. Finally, it has been accepted that most of the components of translation machinery are present inside the nucleus. The final breakthrough into this concept came in when Iborra *et al*. (2001) conclusively proved coupled transcription and translation within the nucleus of mammalian cells. They localized distinct translation sites within mammalian nuclei. This nuclear translation and transcription by RNA polymerase II was found to be concurrent. Hence, despite several controversies against the emerging concept of "nuclear translation", stronger evidences are continuously building up in support of it. This idea of nuclear translation based on all the above results, refined through past few decades, would have tremendous implications for research on nonsense-mediated decay, the process by which flawed messenger RNAs are identified and degraded. As far as the nature of nuclear translation is concerned, it is claimed that transcription and nuclear translation are coupled as in prokaryotes.

Till date, there is no evidence to show translation in the nucleus of plant cells. The functional characterization of the eukaryotic translation initiation factor, PeIF5B, from the plant system and its cellular distribution suggests that PeIF5B may

probably form part of the nuclear translation machinery of *Pisum sativum.* Earlier it was shown that this factor displays extensive homology to eIF5B factor from *Homo sapiens, Saccharomyces cerevisiae* and *Drosophila melanogaster* as well as archaeal aIF5B. Domain search showed the presence of various functional motifs in the central and C-terminal domains. Fluorescence and circular dichroism studies with PeIF5B showed prominent changes in the factor following ligand-binding. Hence, functional characterization of PeIF5B was carried out to establish whether this factor has a major role in the translation process. Here we examine a number of features of this factor important for concluding that nucleus harbors both transcription and translation.

4.2 Materials and Methods

4.2.1 Charging $tRNA_f^{Met}$ with [^{35}S]methionine in presence of aminoacyl tRNA synthetase: Charging reactions contained 50 g of formyl methioinine specific *E.coli* tRNA (Sigma Chemicals), 2mM ATP, 100mM Tris-HCl (pH7.5), 5mM $MgCl_2$, 2 M [^{35}S]methionine (1000Ci/mmol) and 100units of *E.coli* aminoacyl tRNA synthetase (Sigma Chemicals) in a total volume of 50 l. The reaction mixture was incubated at 37^{o}C for 10min and then terminated by adding 30 l of 1M sodium acetate (pH5.0). The *in vitro* synthesized [^{35}S]Met-$tRNA_f^{Met}$ was deproteinized by mixing thoroughly with phenol saturated with 0.2M Tris-HCl (pH8.0) followed by centrifugation at 12,000rpm for 10min at 4^{o}C. The aqueous layer was then dialyzed against a high salt dialysis buffer containing 50mM sodium acetate (pH5.0) and 0.5M NaCl for 2hours at 4^{o}C followed by dialysis with

low salt dialysis buffer containing 20mM sodium acetate (pH5.0) for 2hours at 4°C.

4.2.2 Met-tRNA$_f^{Met}$ binding assay: 2μg of purified recombinant PeIF5B protein was incubated in a 50μl reaction mixture containing 20mM Tris-HCl (pH7.8), 2mM DTT, 1.5mM $MgCl_2$, 250μM GTP, 75mM KCl, 130μg BSA and dialyzed [^{35}S]Met-tRNA$_f^{Met}$ (~100,000cpm). The reaction mixture was incubated at 37°C for 10min and the reaction terminated by addition of ice-cold wash buffer containing [20mM Tris-HCl (pH7.8), 100mM KCl and 5mM $MgCl_2$] and then vacuum filtered through pre-wetted 0.45μ nitrocellulose filters (Millipore type HA) using a Millipore filter unit. The filters were washed with another 10ml of ice-cold wash buffer and dried in hot air oven at 60°C for 15-20min and then counted by liquid scintillation counter in 8ml of scintillation fluid [PPO (5gm/l) and POPOP (0.5gm/l)].

4.2.3 GTP-binding by UV-crosslinking: Purified PeIF5B (1.5μM) was incubated with 20μCi of [α-^{32}P]GTP (2,000Ci/mmol; BARK India Ltd.) in 50μl of 1X binding buffer [50mM Tris-HCl (pH8.0), 50mM KCl, 2mM dithiothreitol, 5μM ATP, 1mM EDTA, and 10%(wt/vol) glycerol] supplemented with 10mM Mg^{2+}. In competition samples, 40μM nonradioactive nucleotide was added separately. Samples were incubated on ice for 5min then crosslinked by UV (1200×100μJ/cm^2 for 30min).

4.2.4 GTPase activity: 25nmolar final concentration of PeIF5B was incubated with 70mM KCl, 25mM Tris-HCl (pH7.5), 7mM $MgCl_2$, 1mM DTT and 35μM [γ-^{32}P]GTP in a reaction volume of 20μl for 5min at 65^{o}C. 1μl of sample was spotted onto poly(ethyleneimine) cellulose thin layer chromatography (TLC) plate. The solvent used was 0.75M potassium phosphate buffer (pH3.5). The conversion of NTP to NDP and P_i was observed in a PhosphoImager.

4.2.5 Raising polyclonal antibodies against PeIF5B: Polyclonal antisera against recombinant PeIF5B were raised in rabbit in moderately high titers. 80μg of purified recombinant protein antigen was mixed with an equal volume of complete Freund's adjuvant and then injected at 4 sites intramuscularly on day 0. Animal was given two boosters: first on day 8 and second on day 22. Rabbit was bled at different time intervals and 8-10ml blood collected and serum stored at 4^{o}C. The titer of the antibodies in the antiserum was checked by dot blot analysis.

For western blot analysis, the resolved protein bands were electrophoretically transferred from polyacrylamide gel to nitrocellulose membranes at 30mA overnight at 4^{o}C. Nonspecific binding was blocked by incubation with 5% bovine serum albumin (BSA) at room temperature for 1-2hrs. The membrane was incubated for 1hr with polyclonal anti-PeIF5B antiserum at room temperature. In western analyses, antiserum was used at a dilution of 1:8000 as the source of primary antibodies. Later the membrane was washed three times with 1X PBS, incubated with 1:10,000 diluted horse radish peroxidase-conjugated anti-rabbit

IgGs (Santa Cruz) at room temperature for 40min, and again washed five times in 1X PBS and 0.05% Tween-20. Immunoreactive bands were visualized using 3,3'-Diaminobenzidine (DAB) (Sigma-Aldrich) substrate for the detection of peroxidase activity or by using Western blotting luminol reagent (Santa Cruz Biotechnology).

4.2.6 Ribosome isolation: Ribosomes were isolated from *E.coli* as described previously (Primm *et al.,* 2000). 250ml of *E. coli* culture expressing recombinant PeIF5B was grown to OD_{600nm} of 0.8. The cells were centrifuged at 6000rpm for 5min. The cell pellet was resuspended in 4ml of Buffer-1 [10mM Tris-HCl (pH7.6), 30mM KCl, 15mM $MgCl_2$ and 6mM β-mercaptoethanol]. The resuspended bacterial cells were lysed by sonication in Branson Sonifier 450 for 5min [Duty cycle: 50%, Output: 4 microtip limit]. The lysed cells were centrifuged at 15,000 × g for 20min followed by another spin at 30,000 × g for another 20min. The supernatant was collected and centrifuged at 150,000 × g for 3hrs. The resultant pellet containing ribosomes was again resuspended in 1ml Buffer-1 and layered over Buffer-2 [Buffer-1 containing 30%(wt/vol) sucrose]. It was followed by a spin at 150,000 × g for 3hrs. The pellet was washed with 1ml of ribosome buffer [50mM Tris-HCl (pH7.6), 50mM KCl and 10mM $MgCl_2$] and centrifuged at 30,000 × g for 5min. The resultant ribosomal pellet was finally resuspended in 500μl of ribosome buffer. Ribosome concentration was calculated spectrophotometrically using OD_{260nm}= 1unit for 18pmol of ribosomes (Spedding, 1990).

4.2.7 Immunoprecipitation: Immunoprecipitation was performed as shown by Ehtesham *et al.,* 1999. The isolated ribosomes were incubated with anti-PeIF5B antibodies in the presence or absence of PeIF5B overnight at 4^{o}C in 1X RIPA buffer [5mM Tris-HCl (pH8.0), 70mM NaCl, 2.5mM iodoacetamide, 0.25% Triton-X100, 0.05% SDS and 0.05% sodium deoxycholate) in the presence of 1mM PMSF, 1□M pepstatin and 1□g/□l benzamidine. The reaction was later incubated with protein G-Sepharose beads (Pharmacia) equilibrated with 1X RIPA buffer at 25^{o}C for 1hr with constant shaking. The beads were washed 20X bead volume with 1X RIPA buffer at 4^{o}C. The bound proteins were eluted in 1X SDS sample buffer and resolved on 10% SDS-polyacrylamide gel.

4.2.8 Generating PeIF5B clone in mammalian vector to express with green fluorescent protein (GFP): Green fluorescent protein (GFP) fusion of PeIF5B was generated by cloning full-length ORF into pEGFP-N1 (Clontech), a eukaryotic expression vector that carries sequence for GFP-tag 3' to the multiple cloning site. The ORF was obtained from pET23a clone of PeIF5B (Chapter 3) using *Nhe*I and *Xho*I enzymes and ligated into the same sites in pEGFP-N1. The nuclear localization signal (NLS)-deleted clone of PeIF5B was created by deleting first 180 nucleotides from the complete open reading frame. Such a truncated ORF of PeIF5B was amplified from the cDNA clone, pPeIF5B (Chapter 2) using primers: FP- GGAATTCGAAGGTAAATTGTTAACCGGTAAG carrying *EcoR*I restriction site and RP- TCTCGAGTTGTATCTTGAAAAGACTCTTCAATTTC

carrying *Xho*I restriction site. The PCR conditions used were same as that for the amplification of complete ORF (Chapter 3) using the same template. The amplified fragment was cloned in TA-cloning vector pCR2.1 (Invitrogen). Using the restriction enzymes *Kpn*I and *Apa*I, the fragment was isolated and cloned into pEGFP-C3, linearized with same enzymes. pEGFP-C3 (Clontech) carries GFP-sequence towards the 5' end of multiple cloning site.

4.2.9 Transient transfection assay and confocal imaging: For lipofectin mediated transient transfection, HeLa cells were seeded in 35mm dishes (0.2 □ 10^6 cells/well), washed with serum-free DMEM and left for 2hrs. Again two washes were given before adding DNA. Filter-sterilized DNA (10□g) was added to 8□l of Lipofectin reagent (Invitrogen) in 400□l of serum-free medium. This mixture was incubated at room temperature for 30min and later added to the culture well. The Lipofectin-containing medium was removed after 6hrs incubation at 37^{o}C and 1ml of complete medium (CM) was added. After 10hrs the cells were observed under fluorescence microscope. For confocal imaging the cells were fixed on 18mm diameter No.1 glass coverslips with 4% paraformaldehyde in 1X PBS for 15min at room temperature. Coverslips were mounted on slides with Fluormount (Virotech). Laser scanning confocal microscopy was performed on a Zeiss Axiophot microscope. Excitation of GFP was performed with standard fluorescein isothiocyanate (FITC) filters. To localize nuclei, cells were fixed and 4', 6'-Diamidino-2-phenylindole (DAPI) was added at a concentration of 100ng/ml to observe the DNA at an excitation wavelength of 395nm.

4.2.10 Phosphorylation: Phosphorylation of 1.25 M of purified PeIF5B by 20 units each of protein kinase A (PKA), protein kinase C (PKC), DNA-dependent protein kinase (DNA-PK) and casein kinase II (CKII) was carried out in 25 l reaction volume as per manufacturer's instructions. The reaction mixture contained kinase-specific buffer and 30 M unlabelled ATP. The composition of specific buffers of different kinases is given in Table 4.1. Treatments were incubated at 30^{o}C for 10min. Reactions were stopped by addition of 2X SDS-sample buffer and boiled briefly. Samples were resolved on 10% SDS-PAGE and transferred to the nitrocellulose membrane by applying 30mA of current for 12hrs at 4^{o}C. Later the membrane was subjected to autoradiography.

4.2.11 Cytoplasmic-nuclear extract preparation: Cytoplasmic and nuclear extracts were prepared as previously described (Phan *et al.,* 2003). The *O. sativa* nuclear extract was prepared from 40g of young 10 days old leaves. The leaves were washed and submerged in ice-cold STM buffer [0.55M sucrose, 50mM Tris-HCl (pH8.0), 10mM $MgCl_2$, 25mM KCl, 10mM $Na_2S_2O_3$, 7mM 2-mercaptoethanol and 0.5mM PMSF] and homogenized in a kitchen mixture. To remove the suspended particles, the homogenate was then passed through two layers of cotton cloth. The filtrate was then centrifuged at 1000 g for 10min at 4^{o}C. The cell pellet was then slowly resuspended in STM buffer containing 2.5% Triton-X100 and incubated at 4^{o}C with slow shaking to lyse the chloroplasts. This was followed by centrifugation at 2000 g for 30min at 4^{o}C. The supernatant was later used as

Table 4.1

5X buffer for Protein kinase A (PKA):

Reagents	Stock conc	Final conc.	Amt. in 1ml
Tris (pH7.4)	1M	200mM	200µl
Mg acetate	0.5M	100mM	200µl
ATP	1mM	500µM	500µl

5X buffer for Protein kinase C (PKC):

Reagents	Stock conc	Final conc.	Amt. in 1ml
HEPES (pH7.5)	1M	100mM	100µl
$CaCl_2$	83.5mM	8.35mM	100µl
DTT	1M	5mM	5µl
$MgCl_2$	500mM	50mM	100µl
ATP	1mM	500µM	500µl
Phosphotidyl Serine (Activator)	1µg/µl (use 1µl/reaction)		

Table 4.1 conti.

5X buffer for DNA-dependent protein kinase (DNA-PK) :

Reagents	Stock conc	Final conc.	Amt. in 1ml
HEPES (pH7.5)	1M	250mM	250□l
KCl	2M	500mM	250□l
$MgCl_2$	500mM	50mM	100□l
EDTA	50mM	500□M	10□l
DTT	1M	5mM	5□l
BSA	10mg/ml	0.4mg/ml	40□l
ATP	10mM	500□M	50□l
Calf thymus DNA (Activator)	50□g/□l (use 1□l/reaction)		

5X buffer for casein kinase II (CKII):

Reagents	Stock conc	Final conc.	Amt. in 1ml
Tris (pH7.4)	1M	50mM	50□l
KCl	2M	400mM	200□l
$MgCl_2$	500mM	10mM	20□l
ATP	1mM	500□M	500□l
DTT	1M	5mM	5□l

Table 4.1 *The composition of buffers for specific protein kinases*

cytoplasmic extract. If the pellet obtained was still green in colour, the above step was repeated till clean white chloroplast-free pellet was obtained. The resultant nuclear pellet was resuspended in NE buffer [600mM KCl, 50mM Tris-HCl (pH7.9), 1.5mM $MgCl_2$, 0.2mM EDTA, 0.5mM DTT, 25% glycerol, 0.5 M leupeptin, 0.5mM PMSF and 1mM pepstatin] and incubated for 30min at 4°C with gentle shaking. It was finally centrifuged at 12000 g for 30min at 4°C and the supernatant was used as the nuclear extract for western blotting.

4.2.12 Exon-intron analysis: Different sets/regions covering the entire *PeIF5B* gene were amplified using different primers (Table 4.2). PCR amplifications were carried out using both the cDNA clone, pPeIF5B as well as *Pisum sativum* genomic DNA as template. PCR products were visualized on 1% agarose gel.

4.3 Results

4.3.1 PeIF5B binds to charged initiator tRNA: The formation of ternary complex (TC) by PeIF5B was evaluated by incubating the charged initiator tRNA (Met-$tRNA_f^{Met}$) from *E.coli* with increasing concentrations of protein (0-4 g) in the presence of 1.5mM $MgCl_2$ and 250 M GTP (Figure 4.1A). As shown in the graph a significant increase is evident in the radioactive counts with the addition of increasing amounts of the protein till it reached a plateau. The optimum binding was observed at 2 g of PeIF5B protein and any further increase could not be observed. All the reactions were performed in the presence of BSA control protein

Table 4.2

Name	Sequence	Position in pPeIF5B
Set-I/V FP (ERev1)	AAAAAGAAGAAAAAGAAGA	204-222
Set-I RP (Race1)	ACATCATCCCAGCTTCTAG	923-941
Set-II FP (Rev1)	GAAGATGATGTTGAGGATG	895-913
Set-II RP (AT3-2)	CAGGGGTGACTTTGACAT	2414-2431
Set-III FP (Rev5)	GGTTATTGAAGGCCATGG	1887-1904
Set-III RP (For2)	GTCTACCTCTATAAACCGG	3097-3115
Set-IV FP (JT3)	GCAAAGAAAGGGCAGAAAGTA	2752-2772
Set-IV/V RP (For1)	CTTGTACACCATGGAAACC	3329-3347

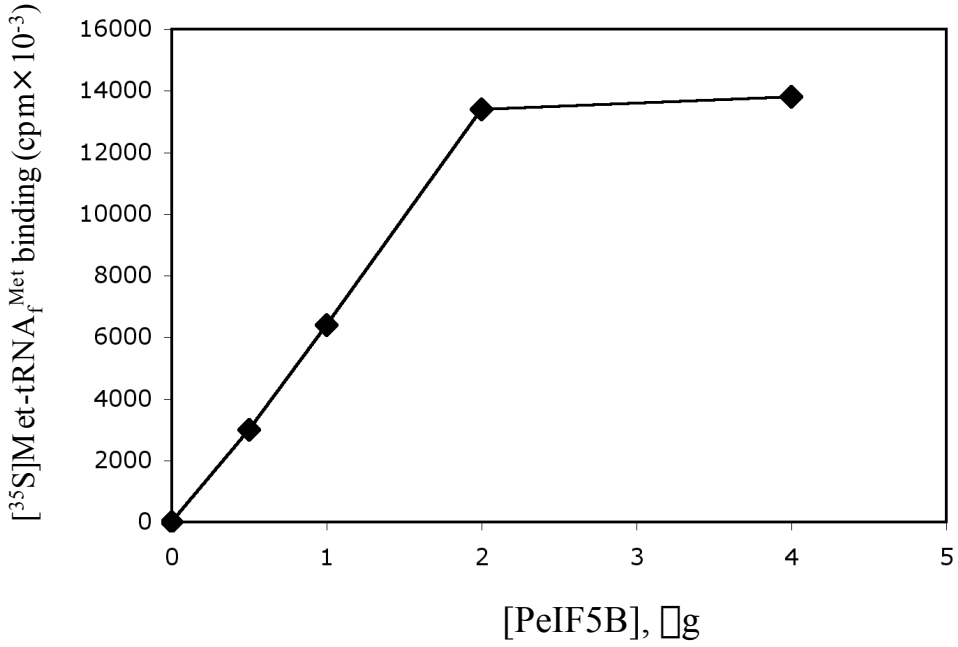
TABLE 4.2: *Primers used to amplify different regions of the open reading frame in pPeIF5B clone*

to prevent non-specific binding. To evaluate the importance of nucleotide in Met-$tRNA_f^{Met}$-binding to the recombinant PeIF5B, the ternary complex formation was monitored in the presence of GTP and GDP (Figure 4.1B). It could be seen that GTP enhances tRNA-binding to the protein factor. As shown in figure 1B, the formation of TC increased in a dose-dependent manner with increasing concentration of GTP till it reached saturation at 250 M GTP. However, minimal counts could be detected in the presence of GDP. The formation of ternary complex by PeIF5B was found to be optimum at 1mM Mg^{2+} ion concentration and further increase in concentrations of the metal ion appears to be inhibitory (Figure 4.1C). To identify the specificity of formation of ternary complex in a nucleotide-dependent manner, 2 g of PeIF5B was incubated in the presence of 250 M of one of the nucleotides (UTP, ATP, CTP, dGTP or GTP) and 1.5mM $MgCl_2$. The formation of ternary complex appears to be favored by GTP and dGTP but not by CTP, ATP or UTP (Figure 4.1D).

4.3.2 PeIF5B is a GTP-binding protein as evident from UV-crosslinking: As discussed in chapter 2, *in silico* study had predicted a GTP-binding domain in PeIF5B polypeptide. In order to determine the *in vitro* guanine-binding properties of PeIF5B, purified recombinant protein was subjected to UV-crosslinking with [-^{32}P]GTP. The protein-nucleotide complex was resolved on 10% SDS-PAGE and exposed to X-ray film. The autoradiograph shows a clear band at the corresponding position of 116kDa (Figure 4.2, lane 2). Lane 3 shows the competition with 25X molar excess of cold GTP. The ability of the cold nucleotide

Figure 4.1

(A)


16000
14000
12000
10000
8000
6000
4000
2000
0
0
1
2
3
4
5
[35S]Met-tRNAfMet binding (cpm×10-3)
[PeIF5B], □g

(B)

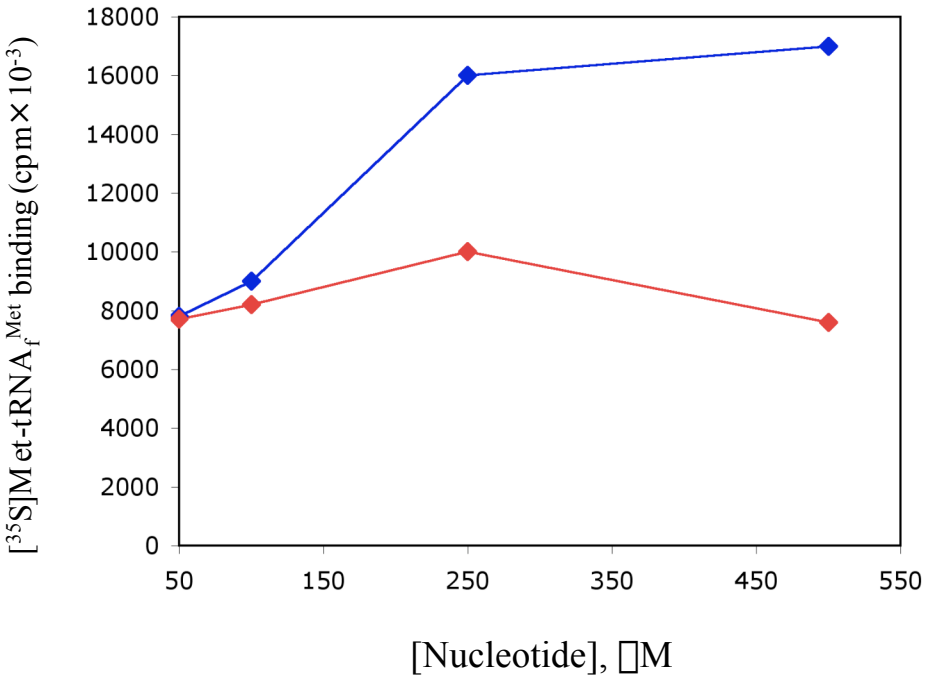
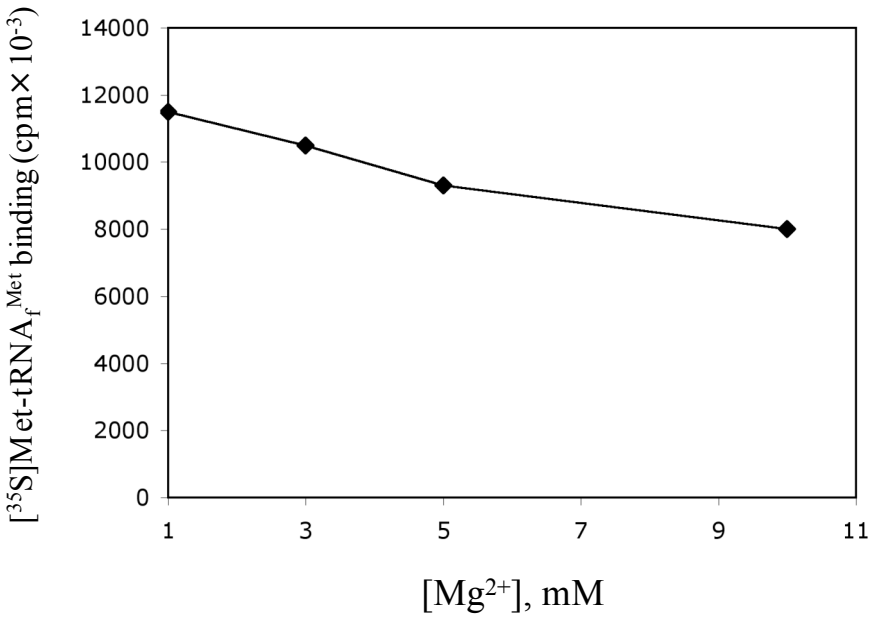

18000
16000
14000
12000
10000
8000
6000
4000
2000
0
50
150
250
350
450
550
[35S]Met-tRNAfMet binding (cpm×10-3)
[Nucleotide], □M

Figure 4.1 conti.

(C)



(D)

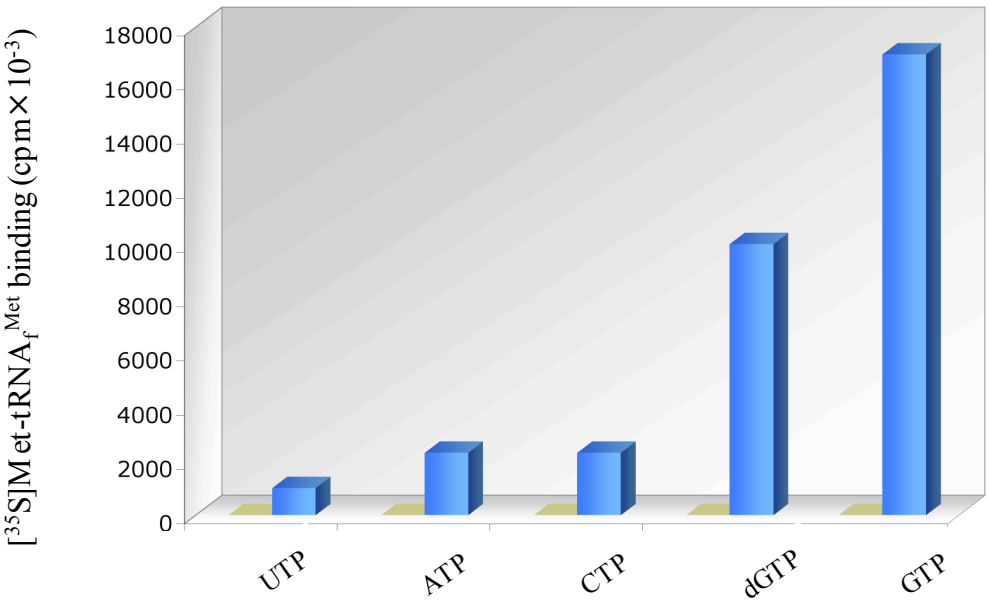


FIGURE 4.1: *Interaction of PeIF5B with charged initiator tRNA is dependent on the nucleotide and metal ion.* ***(A)*** *Binding of methionine charged E.coli fMet specific tRNA to PeIF5B occurs as a direct function of protein concentration. Increasing amounts of the protein (0-4 g) was incubated with a fixed amount of [^{35}S]Met-charged initiator tRNA in presence of 250 M GTP and 1.5mM $MgCl_2$.* ***(B)*** *GTP and not GDP activates [^{35}S]Met-$tRNA_f^{Met}$-binding to PeIF5B. Charged initiator tRNA and protein interaction was studied in presence of 1.5mM $MgCl_2$ as a function of 50-500 M GTP (blue line) and GDP (red line).* ***(C)*** *Higher concentrations of Mg^{2+} inhibit the formation of ternary complex between PeIF5B, Met-$tRNA_f^{Met}$ and GTP. 2 g of protein was incubated with charged initiator tRNA and 250 M GTP in presence of 1-10mM $MgCl_2$.* ***(D)*** *The interaction between PeIF5B translation initiation factor and charged initiator tRNA is promoted by GTP and dGTP whereas UTP, ATP and CTP show no effect. 2 g of protein was incubated with [^{35}S]Met-$tRNA_f^{Met}$ in presence of 1.5mM $MgCl_2$ and 250 M of different nucleotides (UTP, ATP, CTP, dGTP or GTP).*

Figure 4.2

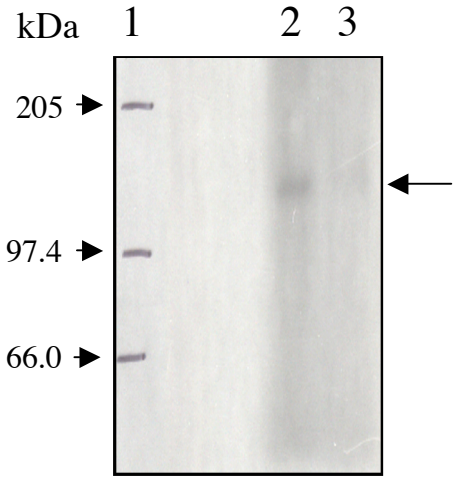


FIGURE 4.2 *GTP-binding to PeIF5B as seen after UV-crosslinking on 10% SDS-PAGE. Lane 1 is the protein molecular weight marker. Lane 2 is the □-^{32}PGTP binding to the protein at a molecular size of 116kDa. Lane 3 is the competition with 25X molar excess of cold GTP.*

Figure 4.3

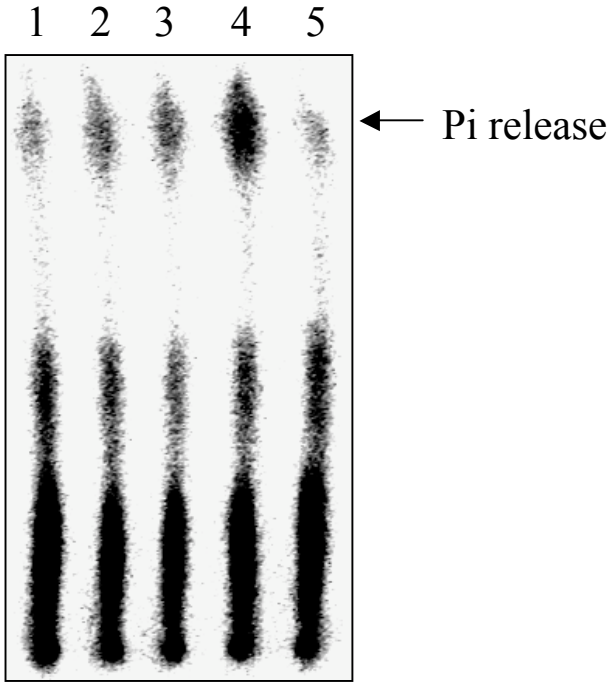


FIGURE 4.3 *Metal ion-dependent GTPase activity shown by PeIF5B. 0, 5, 10 and 20pmoles protein was incubated with □-^{32}PGTP (lanes 1, 2, 3 and 4, respectively) in the presence of 5mM Mg^{+2} ions. Lane 5 is the reaction containing 20pmoles of PeIF5B and □-^{32}PGTP in the absence of Mg^{2+} and presence of 10mM EDTA.*

to directly outcompete the binding of [□-^{32}P]GTP confirms that this binding to GTP is indeed very specific for the nucleotide.

4.3.3 PeIF5B shows metal ion-dependent GTPase activity: In order to further assess whether PeIF5B also exhibits GTPase activity, the purified recombinant protein was incubated with [□-^{32}P]GTP as a function of protein concentration (0-20pmoles) in the presence of 5mM $MgCl_2$ (Figure 4.3, lanes 1-4). The reaction product was fractionated by chromatography on TLC plate that was later autoradiographed. It is apparent from the figure that PeIF5B shows significant dose-dependent GTPase activity that can be visualized as the cleavage of □-phosphate as seen on the X-ray film in the form of a black spot. On the TLC plate, the cleaved terminal phosphate group generated after GTP hydrolysis by PeIF5B, runs faster than the rest of the complex. Interestingly, it could also be seen that in presence of 10mM EDTA, the GTPase activity of the translation factor was inhibited (Figure 4.3, lane 5). Hence, it can be concluded that PeIF5B has inherent metal ion-dependent GTPase activity.

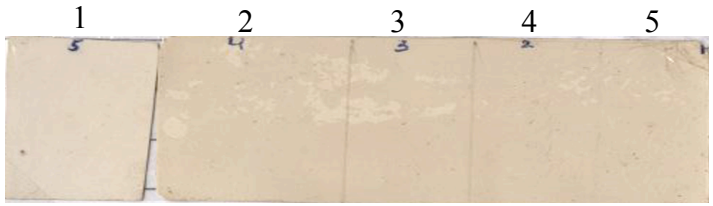
4.3.4 High titer polyclonal antibodies raised against PeIF5B: To show interaction between this noval translation initiation factor and ribosome particles by immunoprecipitation, antibodies were raised in rabbit against recombinant, bacterially expressed PeIF5B. As discussed earlier in chapter 2, the N-terminal region of PeIF5B polypeptide was found by *in silico* analysis, to be highly hydrophilic and antigenic. Hence the anti-PeIF5B antibodies could be raised at a

high titer. As shown in figure 4.4A, the purified protein was spotted on nitrocellulose paper (25-100ng) (lanes 3-5) and coated with 1:1000 diluted anti-PeIF5B antiserum collected after 8 days of primary exposure. The secondary antibodies used were anti-rabbit IgGs. No signal was detected indicating a very low titer of the antibodies. As a control 50 and 100ng of the protein was spotted and dot blot analysis carried out in the absence of primary antibodies (lanes 1 and 2). On 8^{th} day of primary exposure, first booster was given. Later after 14 days of first booster i.e. 22^{nd} day of primary exposure, again the titer was checked by dot blot analysis (Figure 4.4B). 1:4000 dilution of anti-serum gave very high signal with 25ng and 50ng of purified protein (lanes 1 and 2). Even 1:8000 diluted anti-serum recognized 25ng and 50ng of protein to some extent (lanes 3 and 4). Lane 5 is the control with no primary antibodies. On the same day second booster was given. After 14 days of second booster, once again dot blot analysis was carried out with 25ng and 50ng of protein using anti-PeIF5B anti-serum at dilutions 1:1000 (Figure 4.4C, lanes 1,2), 1:4000 (Figure 4.4C, lanes 3,4) and 1:8000 (Figure 4.4C, lanes 5,6). It was found that 1:8000 dilution of the anti-serum worked very well.

Western blot analysis was carried out using anti-PeIF5B anti-serum at 1:8000 dilution (Figure 4.5). Total cell lysate of *E.coli* cells expressing recombinant PeIF5B was prepared as described in chapter 3 and used for western blotting. Lanes 2 and 3 show the 116kDa PeIF5B band recognized by polyclonal anti-PeIF5B antibodies in uninduced and induced bacterial cell lysate, respectively.

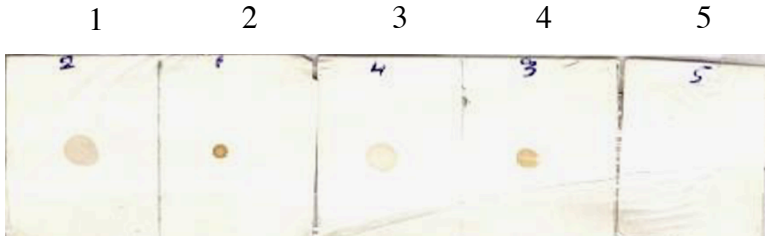
Figure 4.4

(A) Titer check after 8 days of primary exposure



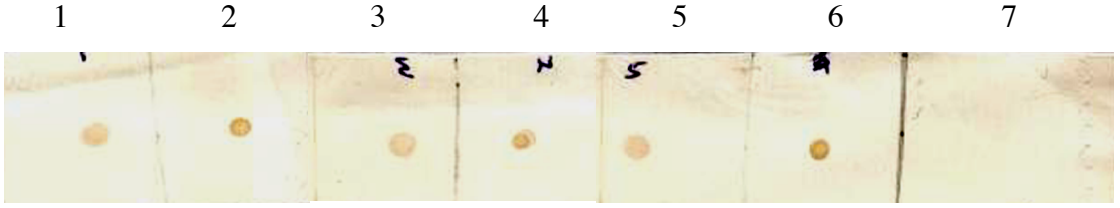
1.NIF(50ng)(-Primary antibodies)
2.NIF(100ng)(-Primary antibodies)
3.NIF(25ng)(Anti-NIF-1:1000)
4.NIF(50ng)(Anti-NIF-1:1000)
5.NIF(100ng)(Anti-NIF-1:1000)

(B) Titer check after 14 days of I booster



1.NIF(25ng)(Anti-NIF-1:4000)
2.NIF(50ng)(Anti-NIF-1:4000)
3.NIF(25ng)(Anti-NIF-1:8000)
4.NIF(50ng)(Anti-NIF-1:8000)
5.NIF(50ng)(-Primary antibodies)

(C) Titer check after 14 days of II booster



1.NIF(25ng)(Anti-NIF-1:1000)
2.NIF(50ng)(Anti-NIF-1:1000)
3.NIF(25ng)(Anti-NIF-1:4000)
4.NIF(50ng)(Anti-NIF-1:4000)
5.NIF(25ng)(Anti-NIF-1:8000)
6.NIF(50ng)(Anti-NIF-1:8000)
7.NIF(50ng)(-Primary antibodies)

FIGURE 4.4: *Dot blot analysis to check the titer of the anti-PeIF5B antibodies raised in rabbit against purified, recombinant PeIF5B.* ***(A)*** *Titer check after 8 days of primary exposure. 25ng, 50ng and 100ng of protein was spotted (lanes 3-5) and treated with 1:1000 diluted anti-serum. Lanes 1 and 2 are the control dot blots with 25ng and 50ng of protein, respectively, without primary antibodies.* ***(B)*** *Titer check after 14 days of I booster. Lanes 1,2 and 3,4 are dot blots with 25ng and 50ng of protein using 1:4000 and 1:8000 diluted antisera, respectively. Lane 5 is the control dot blot with 50ng of protein.* ***(C)*** *Titer check after 14 days of II booster. 1:1000, 1:4000 and 1:8000 diluted anti-sera was used (lanes 1-2, 3-4 and 5-6, respectively) each with 25ng and 50ng of protein. Lane 7 is the control dot blot with 50ng of protein.*

Figure 4.5

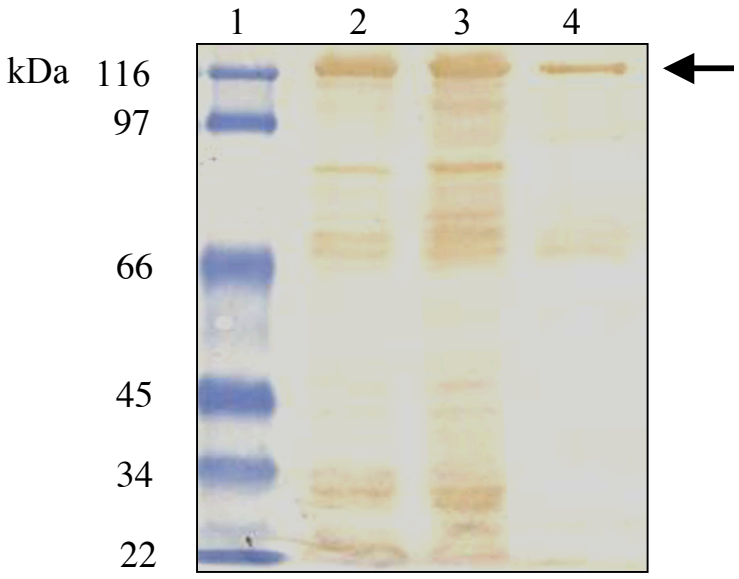


FIGURE 4.5: *Western blot of PeIF5B with anti-PeIF5B antibodies raised in rabbit against recombinant full-length protein. Lane 1 is the protein marker. Lanes 2 and 3 show the uninduced and induced total bacterial cell lysate expressing recombinant PeIF5B. Lane 4 is the affinity purified PeIF5B protein. The 116kDa size of PeIF5B is indicated by an arrow.*

Figure 4.6

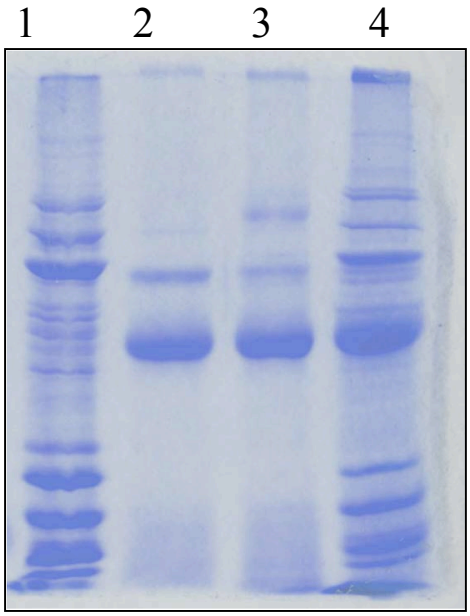


FIGURE 4.6: *PeIF5B interaction with E. coli ribosomes as observed by immunoprecipitation using anti-PeIF5B antibodies. Lane 1 is the crude preparation of E. coli ribosomes. Lane 2 is anti-PeIF5B antibodies. Lane 3 and 4 is the immunoprecipitation of ribosomes by anti-PeIF5B antibodies in the absence and presence of purified PeIF5B.*

Lane 4 shows same band was picked up when affinity-purified protein (Chapter 3) was loaded. It is to be noted that anti-PeIF5B antisera also has anti-*E. coli* IgGs that generate non-specific bands as seen in the western blot with total bacterial cell lysate (lane 2 and 3).

4.3.5 PeIF5B-ribosome interaction: After high titer antibodies were raised against PeIF5B translation initiation factor, the cross-talk between this factor and ribosomes was studied by immunoprecipitation (Figure 4.6). The crude preparation of *E.coli* ribosomes (lane 1) was incubated with anti-PeIF5B antibodies (lane 2) in absence and presence of purified, recombinant PeIF5B protein. This was followed by the addition of protein G-Sepharose beads that have affinity for IgGs. Later when the anti-PeIF5B antibodies were separated out with the help of these beads and resolved on SDS-PAGE to check for any interacting protein, it was found that several ribosomal proteins were pulled down in the presence of purified PeIF5B (lane 4). However, in the absence of PeIF5B there was no cross-talk between anti-PeIF5B antibodies and ribosomal proteins (lane 3). This conclusively proves that the translation initiation factor from *Pisum sativum* interacts with ribosomes.

4.3.6 A functional NLS is present at the N-terminus of PeIF5B protein: During *in silico* analysis of PeIF5B polypeptide, a nuclear localization signal was predicted towards the N-terminal portion of PeIF5B (http://cubic.bioc.columbia.edu/cgi/var/nair/resonline.pl) (Figure 4.7A and B). It was predicted as a short sequence stretch of 16 positively charged amino acids,

Figure 4.7 (A)

(B)

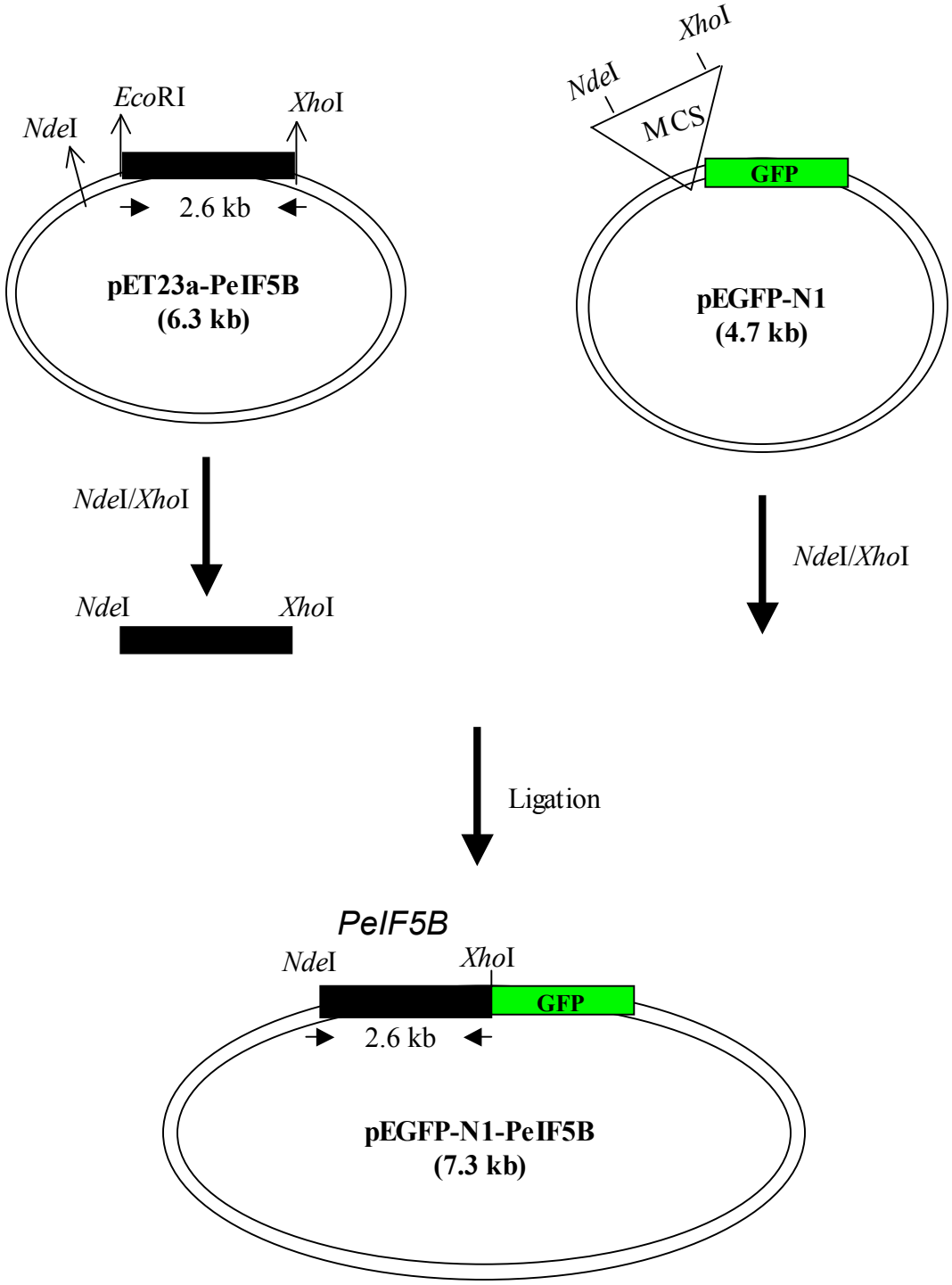
MQELLARRKEAEEKQKREEEEKLRKEEEERQRLEELER
QAEEAK**RRKKEKEKEKLQKKKL**EGKLLTGKQK
EEARRLEAMRRQILNSTGGVTLPAGDTGAPAKKPIYQT
KKGKSTSRNYNGAASVKADESIEAKETTADLD
SEEPKKVEEVVSVQKEDIIELPEAVVEDRVEEDDVEDE
WDARSWDDVNLNDKGAFADEEVDSEPELIVKK
EIKTGIPAKNAGATSKTVSKHVAEEIEDRKQAKIGVEA
KKKKQDQQQSAAFSKPSDANLRSPICCIMGHV
DTGKTKLLDCIRGTNVQEGEAGGITQQIGATYFPAENIR
DRTKELKADATLKVPGLLVIDTPGHESFNNL
RSRGSGLCDIAILVVDIMHGLEPQTKESLDLLKMRNTEF
IVALNKVDRLYGWKTCRNAPIRKAMLQQSKD
VQNEFNMRLDQIVTEFKEQGLNTALYYKNKEMGETFSI
VPTSAISGEGIPDMLLLLVQWTQKTMIEKLTY
SDEVQCTVLEVKVIEGHGTTIDVVLVNGVLHEGDQIVV
AGMQGPIVTSIRALLTPHPMKELRVKGSYIHH
KEIKAAMGIKITAQGLEHAIAGASLYVVKPDDDLEHIK
TAALEDVESVLSRIDRSGEGVCVQASTLGSLE
ALLEFLKTPAVNIPVSAISIGPVHKKDVMKASVMLEKK
REYSTILAFDVKVTPEARELADELGVKIFIAD
IIYHLFDQFKAYMENIKDEKKKESADEAVFPCVLKILPN
CVFNKKDPIVLGVDILEGILKIGTPICIPSQ
DFIDIGRIASIENNHKPVDYAKKGQKVAIKIVGSNSEEQ
QKMFGRHFEIDDELVSHISRRSIDVLKSDYR
DELSNEEWKLVVKLKSLFKIQ

FIGURE 4.7: *Nuclear localization signal (NLS) prediction in PeIF5B* ***(A)*** *Diagrammatic representation of the position of NLS in PeIF5B polypeptide.* ***(B)*** *Whole sequence of PeIF5B protein with amino acids in bold letters forming NLS*

predominantly rich in lysine residues, lying between 45th to 60th position in the polypeptide (Figure 4.7B).

4.3.7 PeIF5B cloned with GFP in mammalian vector: To check for the functionality of predicted NLS, *in vivo* localization of the full-length and NLS-deleted protein was checked in mammalian cells by confocal microscopy. For this purpose, the full-length and NLS-deleted *PeIF5B* ORF were cloned in mammalian expression vector under the control of CMV promoter to express as GFP-fusion protein. The 2.6kb full-length gene was obtained from pET23a PeIF5B clone (Chapter 3) using *Nde*I and *Xho*I restriction enzymes and inserted into pEGFP-N1 vector in the same sites (Figure 4.8A). pEGFP-N1 carries sequence for GFP towards the 3' end of the multiple cloning site. As shown in figure 4.8B, four colonies were screened for the full-length *PeIF5B* clone by restriction digestion with *Nde*I and *Xho*I. The positive clones were expected to release a fragment of 2.6kb and a backbone of 4.7kb. All the four colonies screened were positive *PeIF5B* clones (lanes 2-5). To check for the importance of NLS, the first 180 nucleotides were deleted (that carried the nuclear localization signal) and truncated *PeIF5B* ORF lacking first 60 amino acids was expressed in mammalian cells. The truncated ORF was amplified from pPeIF5B cDNA clone (Chapter 2) using FP carrying *EcoR*I site and RP carrying *Xho*I sites and cloned in pCR2.1 (Figure 4.9A). The colonies were screened by digestion with *Eco*RI and *Xho*I (Figure 4.9B). The positive clones are expected to release a fragment of 2.4kb. From pCR2.1 clone of NLS-deleted PeIF5B, the ORF was isolated using *Kpn*I and *Apa*I

Figure 4.8 (A)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NdeI
XhoI
2.6 kb
pET23a-PeIF5B
(6.3 kb)
XhoI
NdeI
MCS
GFP
pEGFP-N1
(4.7 kb)
NdeI/XhoI
NdeI/XhoI
NdeI
XhoI
Ligation
PeIF5B
NdeI
XhoI
GFP
2.6 kb
pEGFP-N1-PeIF5B
(7.3 kb)

Figure 4.8

(B)

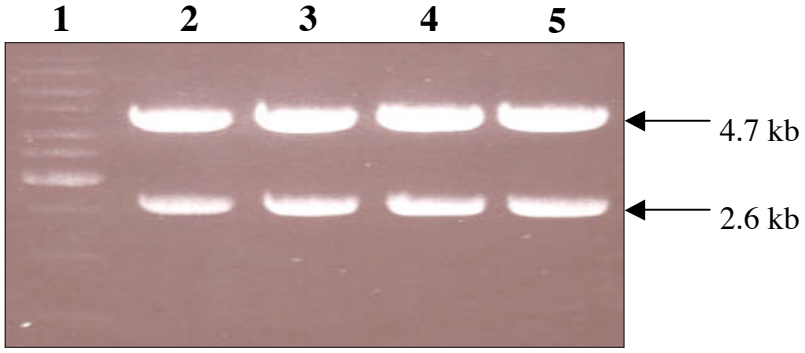


FIGURE 4.8: *Cloning complete PeIF5B ORF in mammalian expression vector under the control of CMV promoter as a GFP-tagged fusion protein.* ***(A)*** *Schematic representation of the cloning strategy of full-length 2.6kb PeIF5B ORF in pEGFP-N1 in NdeI and XhoI sites.* ***(B)*** *Screening of colonies for positive clones of pEGFP-N1 carrying 2.6kb PeIF5B insert. Lanes 2-5 are the different colonies screened to check for the presence of positive PeIF5B clone by check digestion with NdeI and XhoI. Positive clone gives a 2.6kb and 4.7kb band pattern with NdeI and XhoI. Lane 1 in the DNA ladder.*

Figure 4.9 (A)

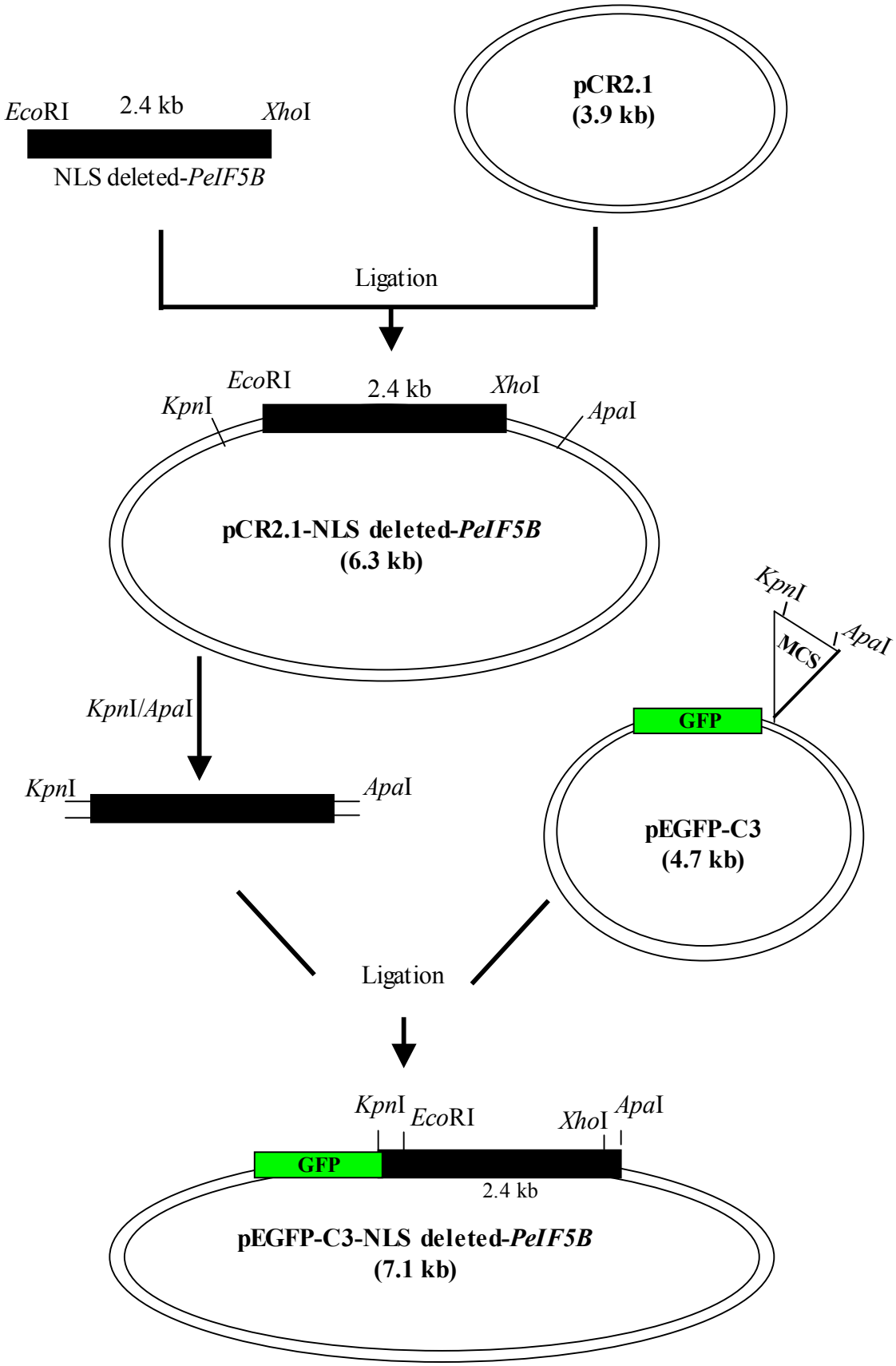
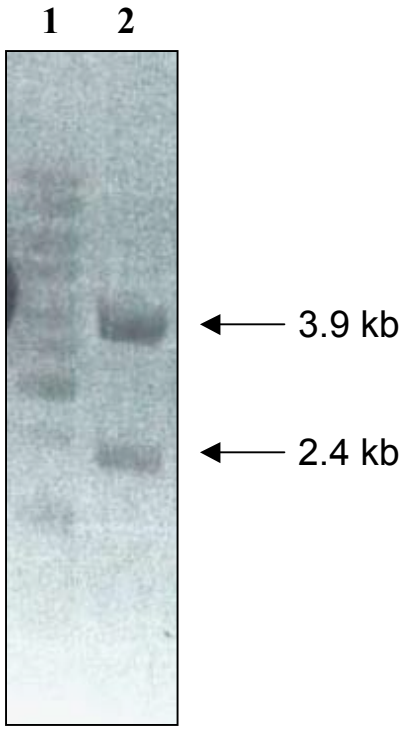

EcoRI
2.4 kb
XhoI
NLS deleted-PeIF5B
pCR2.1
(3.9 kb)
Ligation
EcoRI
2.4 kb
XhoI
KpnI
ApaI
pCR2.1-NLS deleted-PeIF5B
(6.3 kb)
KpnI
ApaI
MCS
GFP
KpnI/ApaI
KpnI
ApaI
pEGFP-C3
(4.7 kb)
Ligation
KpnI
EcoRI
XhoI
ApaI
GFP
2.4 kb
pEGFP-C3-NLS deleted-PeIF5B
(7.1 kb)

Figure 4.9

(B)



(C)

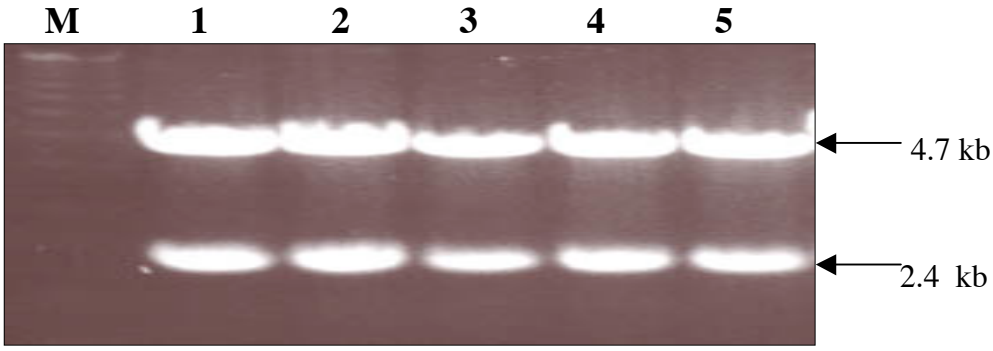


FIGURE 4.9: *Cloning NLS-deleted PeIF5B ORF in mammalian expression vector under the control of CMV promoter as a GFP-tagged fusion protein.* ***(A)*** *Schematic representation of the cloning strategy of NLS-deleted PeIF5B in pEGFP-C3. The 2.4kb NLS-deleted PeIF5B ORF was PCR amplified from cDNA clone and was ligated in pCR2.1. Using KpnI and ApaI, 2.4kb fragment was released from pCR2.1-NLS-deleted PeIF5B and cloned in same sites in pEGFP-C3.* ***(B)*** *Digestion check of pCR2.1-NLS-delated PeIF5B clone with EcoRI and XhoI to give a fall-out of 2.4kb (lane 2). Lane 1 in the DNA ladder.* ***(C)*** *Screening of colonies for NLS-deleted clone of PeIF5B in pEGFP-C3 by digestion with EcoRI and XhoI. Lanes 2-5 are the different colonies screened for the presence of positive PeIF5B clone. Positive clone gives a 2.4kb and 4.7kb band pattern with EcoRI and XhoI.*

enzymes and cloned in same sites in pEGFP-C3 (Figure 4.9A). pEGFP-C3 carries sequence for GFP towards the 5' end of the multiple cloning site. The colonies were screened by digestion with *Eco*RI and *Xho*I to give two bands of sizes 4.7kb and 2.4kb (Figure 4.9C).

4.3.8 The NLS drives PeIF5B into the nucleus: To address the question of localization of PeIF5B factor in the cell, mammalian cells were transfected with full-length and NLS-deleted PeIF5B-expressing plasmids and confocal imaging was carried out. Transient transfection assay in HeLa cells was performed with both full-length and NLS-deleted clones of *PeIF5B* using lipofectamine-mediated plasmid delivery. As a control, GFP-expressing vector was also transfected. Efficiency of transformation was as low as 5-10%. As shown in Figure 4.10A and B, the cells transfected with complete *PeIF5B* fused with GFP show fluorescence mainly in the nucleus. However, the NLS-deleted (the first 60 amino acids removed) form of PeIF5B that lacks the nuclear localization signal was mainly restricted to the cytoplasm (Figure 4.10C and D). On the other hand, only GFP transfected, as a control, expressed all over the cytoplasm and in the nucleus uniformly (Figure 4.10E and F). This conclusively shows that PeIF5B has a functional nuclear localization signal that drives the protein to the nucleus. This signal is present within first 60 amino acid stretch, within the 45^{th} to 60^{th} residue as predicted by *in silico* analysis. When this NLS was deleted, the GFP fluorescence was confined to the cytoplasm indicating that the truncated protein was restricted to the cytoplasm only.

Figure 4.10

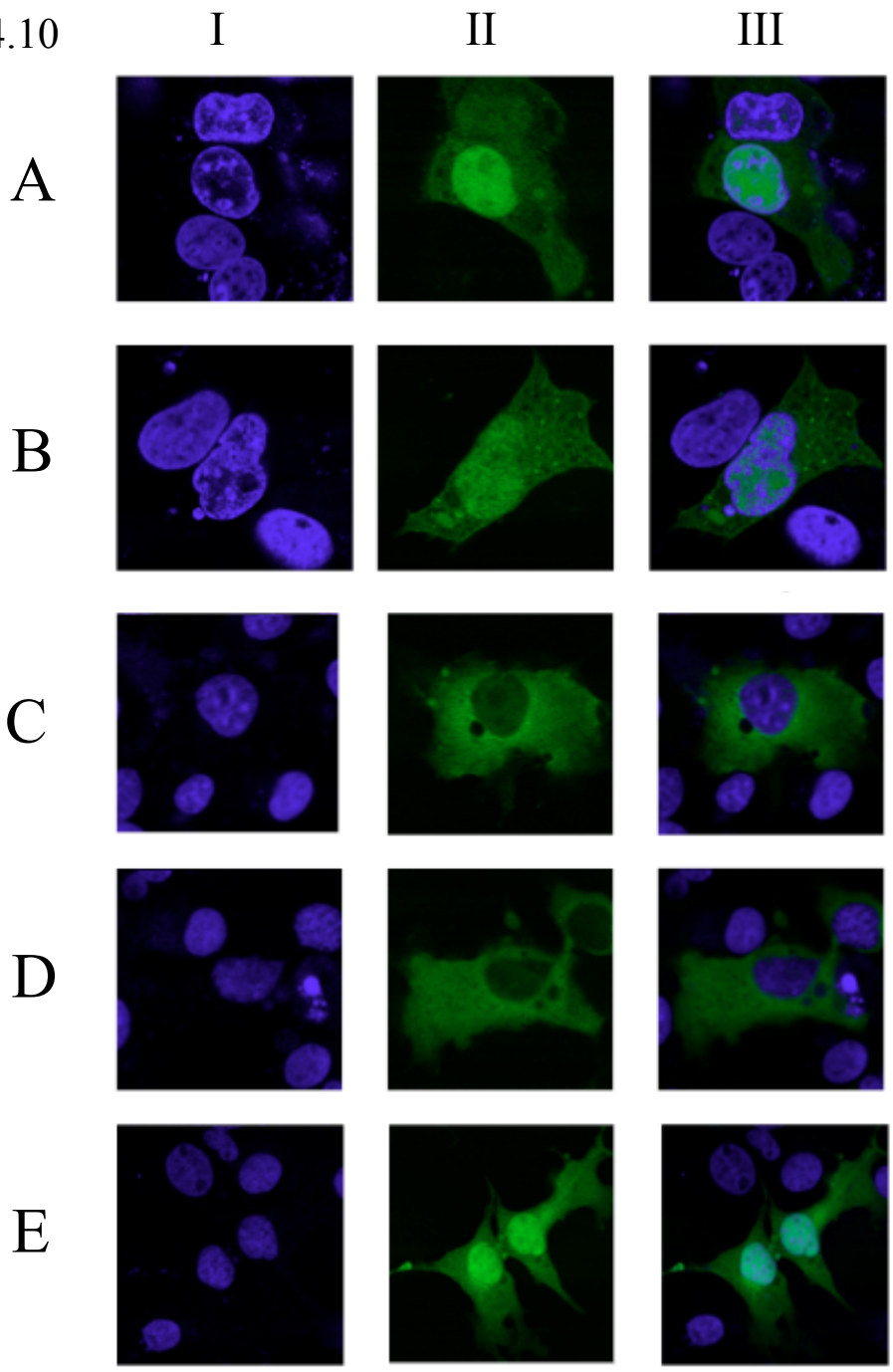


FIGURE 4.10: *Subcellular localization of full-length and NLS-deleted forms of PeIF5B in Hela cells. Full-length PeIF5B mainly found in the nucleus of Hela cells whereas NLS-deleted PeIF5B was confined only to the cytoplasm as localized by confocal microscopy. Hela cells were transfected with full-length PeIF5B, NLS-deleted PeIF5B and GFP expressing plasmids and confocal imaging done.* ***(A)*** *and* ***(B)*** *show two different fields captured of Hela cells transfected with full-length PeIF5B clone.* ***(C)*** *and* ***(D)*** *show Hela cells expressing NLS-deleted protein.* ***(E)*** *shows the expression of GFP in Hela cells. Blue fluorescent signal refers to DAPI fluorescence (Column I); Green fluorescent signal refers to GFP fluorescence (Column II); Column III refers to merged images of I and II when blue and green fluorescent signals were overlaid by computer assistance.*

4.3.9 Western blot analysis of different cellular fractions: Having shown that PeIF5B mainly localizes in the nucleus of mammalian cells, study was designed to check for the cellular distribution of this factor in plant system. As mentioned earlier in chapter 2, PeIF5B has a homologue in *Oryza sativa* with which it shares 66% sequence identity and 80% sequence similarity. Hence cytoplasmic and nuclear extracts were prepared from 10-days old leaves of *Oryza sativa* and western blot analysis of various cellular fractions was performed using antisera raised against PeIF5B. Increasing amounts 20□g (Figure 4.11 lanes 1 and 2) and 40□g (lane 3 and 4) of the nuclear and cytoplasmic extracts were loaded on 10% SDS-polyacrylamide gel and western blot performed. It could be seen from figure 4.11 that a clean signal at a position corresponding to 132kDa is visible only in the nuclear extract lanes (lanes 1 and 3). It is known from the protein sequence that the homologue in *Oryza sativa* has a theoretical molecular weight of 132kDa. However, no such band was detected in cytoplasmic extract (lane 2 and 4). It is to be noted that in 20□g of cytoplasmic extract loaded lane (lane 2) a band can be seen which appears to be an artifact because no such signal was detected when 40□g of the cytoplasmic extract was loaded (lane 4). Hence, this result further confirms the earlier confocal finding that PeIF5B is mainly present within the nucleus.

4.3.10 PeIF5B is phosphorylated by PKA and PKC: After it was shown that PeIF5B is distributed mainly in the nucleus, further study was focused to understand the mechanism of transport from cytoplasm to the nucleus. It is well

Figure 4.11

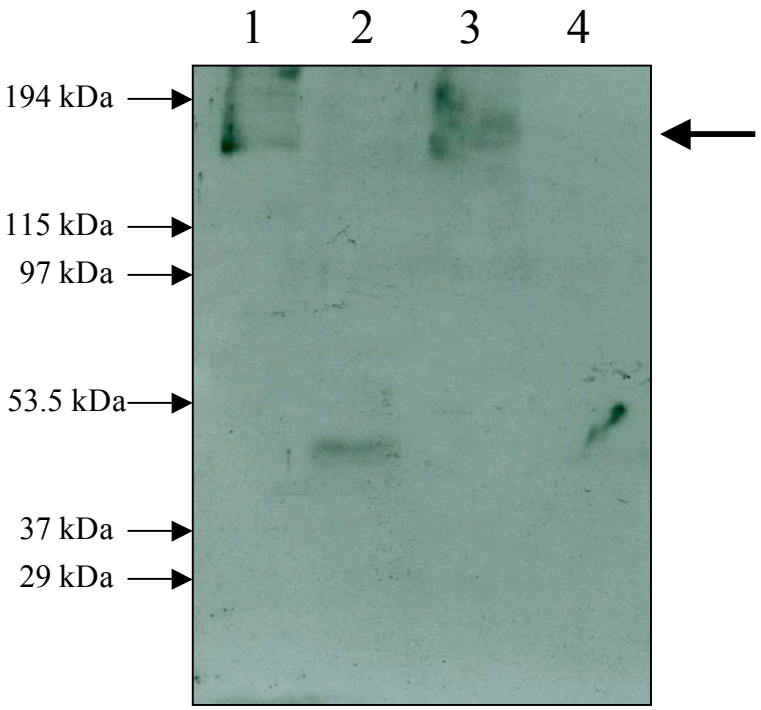


FIGURE 4.11*: Western blot of cellular fractions of Oryza sativa using anti-PeIF5B antibodies. Lane 1 and 3 show 20□g and 40□g, respectively, of nuclear extract loaded while lane 2 and 4 refer to 20□g and 40□g, respectively, of cytoplasmic fraction. The 132kDa band of PeIF5B homologue is shown by arrow.*

Figure 4.12

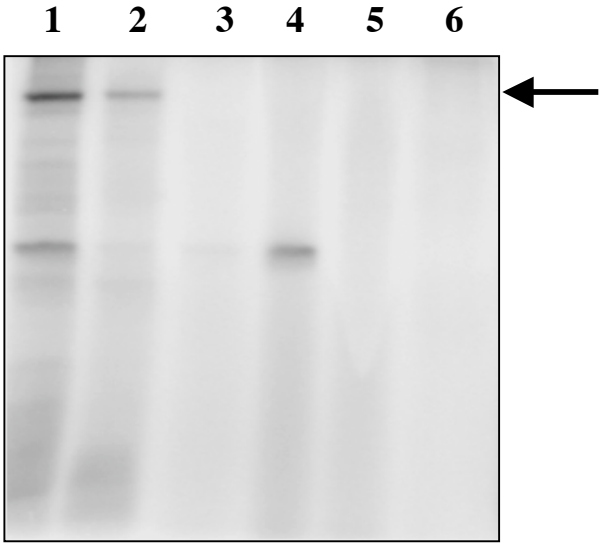


FIGURE 4.12: *PeIF5B is phosphorylated specifically by PKA and PKC. Phosphorylation of PeIF5B with kinases-PKA (lane 1), PKC (lane 2), CK2 (lane 5) and DNAPK (lane 6) is shown. Lanes 3 and 4 are the control lanes of PKC and PKA reactions, respectively, in the absence of PeIF5B. The position of PeIF5B band is marked by an arrow.*

documented in several cases that the phosphorylation status of a protein influences the transport across the nuclear membrane. Hence, as a first step to address this issue, PeIF5B was checked *in vitro* for phosphorylation by different kinases (Figure 4.12). PeIF5B was treated with protein kinase A (lane 1), protein kinase C (lane 2), casein kinase II (lane 5) and DNA-dependent protein kinase (lane 6) in the presence of [□-32P]ATP. In lane 1 and 2, a distinct band at 116kDa size could be seen that corresponds to radiolabeled PeIF5B protein phosphorylated by PKA and PKC, respectively. However no such band could be seen in lanes 5 and 6 suggesting that CK2 and DNAPK were unable to phosphorylate the factor. This proves the specificity of the reaction. Lanes 3 and 4 show the control reactions where the auto-phosphorylation status was checked for PKC and PKA, respectively. It was observed that PKA undergoes auto-phosphorylation (lane 4). Hence the additional band in lane 1 apart from 116kDa band is due to the auto-phosphorylation of the kinase itself. Hence it can be concluded that PKA and PKC phosphorylate PeIF5B specifically.

4.3.11 Pea eIF5B is an intron-less gene: The conclusion drawn so far from the above experiments suggests that PeIF5B acts as a translation initiation factor as predicted from *in silico* analysis and biochemical approach. From blast results it was apparent that peIF5B is a plant homologue of eIF5B translation initiation factor characterized from humans, yeast, *Drosophila* and archaea. The finding from confocal imaging in HeLa cells and western blot of nuclear-cytoplasmic extract from *Oryza sativa,* that PeIF5B is present in the nucleus raises the

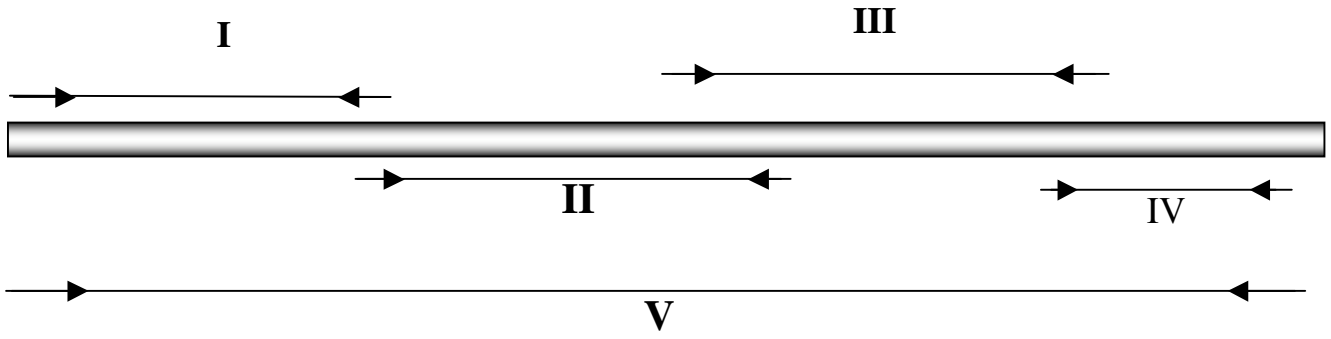
possibility of its participation in nuclear translation. It is suggested that nuclear translation is more like that in prokaryotes than like eukaryotic cellular translation. Hence there were strong reasons to believe that PeIF5B may have characters shared by prokaryotic translation initiation factors. Keeping this rationale in mind, experiment was designed to check for the exon-intron status in *PeIF5B* gene using PCR-based approach. Two templates were taken: first cDNA clone of *PeIF5B* (pPeIF5B) that would lack introns and second template taken was the genomic DNA isolated from *Pisum sativum* that is expected to carry all the introns present in *PeIF5B* gene. The *PeIF5B* ORF was divided into several overlapping sets/regions to cover the complete ORF (Sets I-V) as shown in figure 4.13A. Primers were designed to amplify these sets (Table 4.2). It was found by PCR amplification that the size of PCR amplified band for a particular region of the gene was the same in the case of both cDNA as well as genomic DNA when taken as template (Figure 4.13B). This could only be possible if there is no intron present in the genomic DNA within *PeIF5B* open reading frame. Hence, it shows that *PeIF5B* is an intron-less gene.

4.4 Discussion

The concept of nuclear translation is not a very new one and has been quite enigmatic ever since. This piece of work is a step ahead supporting this school of thought. For the first time in plant system, a probable key player of the nuclear translation process is presented. eIF5B is clearly a novel gene from *Pisum sativum*

Figure 4.13

(A)



(B)

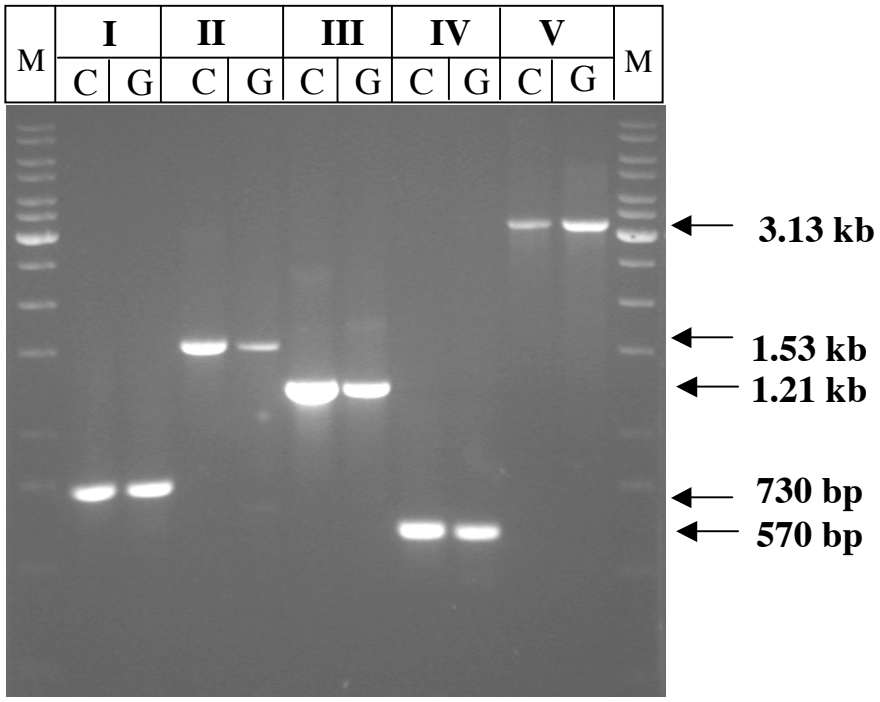


FIGURE 4.13: *Exon-intron analysis of pPeIF5B cDNA library clone carrying PeIF5B open reading frame with 5' and 3' UTRs. (A) Diagrammatic representation of positions of different primer sets spanning whole pPeIF5B clone. (B) PCR amplification from primer sets-I, II, III, IV, V using cDNA (C) and genomic DNA (G) as templates.*

that acts as a probable translation initiation factor in nuclear translation process. It was picked up from a cDNA library of *Pisum sativum* using antisera against synthetic peptide for DNA polymerase consensus sequence. Quite surprisingly it showed extensive homology to eukaryotic translation initiation factor eIF5B. The gene was cloned, expressed and recombinant PeIF5B protein was purified from *E. coli.* Based on fluorescence and circular dichroism studies its probable function as eIF5B in the cellular context was shown. Having shown by *in silico* and biophysical analyses that PeIF5B may form part of the translation machinery, biochemical characterization of this noval factor was carried out. Several lines of evidence indicate that eIF5B facilitates initiator tRNA-binding to the small ribosomal subunit (Choi *et al.*, 1998) and also help in the ribosomal subunit joining (Pestova *et al*., 2000; Unbehaun *et al*., 2004). GTP-hydrolysis is required for the release of the factor from the 80S ribosome (Lee *et al*., 2002; Shin *et al.,* 2002). PeIF5B was checked for binding to charged initiator tRNA from *E. coli* by filter-binding assay. It showed dose-dependent tRNA-binding that was facilitated by GTP and not GDP. Guillon *et al*. (2005) showed the formation of a complex between charged initiator tRNA and aIF5B from *Sulfolobus solfataricus* or *Pyrococcus abyssi* using an assay based on protection against deacylation. The discovery of bacterial IF2 ortholog in archaea and eukaryotes has suggested that a second GTP hydrolysis step exists in eukaryotic translation (Choi *et al*., 2000; Lee *et al*., 1999). eIF5B was demonstrated to bind GTP and hydrolyze GTP in the presence of ribosomes. Through UV-crosslinking experiment GTP-binding to PeIF5B was confirmed. Interestingly, PeIF5B showed metal ion- and dose-

dependent GTPase activity in the absence of any added ribosome component. It may be suggested at this point that the possibility of minimal contamination of ribosomal proteins in PeIF5B preparation during purification of recombinant PeIF5B expressed in *E. coli* cannot be ruled out. This small amount of ribosomal components may be enough to facilitate the inherent property of PeIF5B to hydrolyze GTP. Further as suggested for eIF5Bs, PeIF5B shows interaction with ribosomal proteins as evident from immunoprecipitation. eIF5B is suggested to mediate joining of ribosomal subunits (Pestova *et al.,* 2000). The biochemical evidences very well establish the role of PeIF5B as the translation initiation factor eIF5B from *Pisum sativum*.

PeIF5B was predicted to carry a nuclear localization signal towards the N-terminal region of the polypeptide. To check for its functionality, *in vivo* localization of PeIF5B was studied by confocal microscopy in HeLa cells (Figure 4.10). When PeIF5B was transiently expressed in mammalian cells as a GFP-tagged fusion protein, the green fluorescence was mainly confined to the nucleus. When similar transfections were carried out using NLS-deleted form of *PeIF5B* gene that lacks first 60 amino acids, the expressed protein was limited to the cytoplasm only. This clearly proves that the N-terminal NLS drives the factor into the nucleus of the cell. Further PeIF5B distribution was checked in plant system. As discussed earlier in chapter 2, BLAST results had shown that PeIF5B has homologue in *Oryza sativa* that shows 66% sequence identity and 80% similarity. Hence, nuclear and cytoplasmic extracts were prepared from *Oryza sativa* and western done with anti-

PeIF5B antibodies. It was observed that a signal was immuno-detected in the nuclear extract at a size of 132kDa using anti-PeIF5B antibodies whereas no signal was apparent in the cytoplasmic fraction (Figure 4.11). This clearly proves that PeIF5B is present in the nucleus also. When checked for nuclear localization signal in eIF5B from *Homo sapiens* using the same tool as used for NLS prediction in PeIF5B, a distinct signal was predicted. Several translation factors have been detected in the nucleus as shown by a growing number of reports. Cellular fractionation and immunofluorescence analysis have demonstrated the occurrence of significant fraction of eukaryotic initiation factor eIF4E in the nucleus of mammalian cells (Lejbkowicz *et al*., 1992). Immuno-electron microscope studies later showed that *Saccharomyces cerevisiae* eIF4E is also present both in the nucleus and cytoplasm similar to that in higher cells (Lang *et al*., 1994). Human eIF3 large subunit p170 is also shown to carry a functional NLS that helps in its targeting into the nucleus (Chudinova *et al*., 2004).

One of the two major hypotheses for the origin of the nucleus states that nucleus originated through some gradual unspecified segregating process within the cell itself (Lake, 1983). The other theory supports its symbiotic origin that like other double-membrane bound organelles, nucleus is derived through capture by an engulfing species (Wilson, 1928). Later the engulfed species had taken over the control of the host. Hence, the origin of eukaryotic nucleus is linked to an endosymbiotic event between two very different prokaryotes. The existence of nuclear translation also supports the latter hypothesis. And this is the reason we

believe that the nature of nuclear translation is quite primitive and prokaryote-like rather than that of eukaryotes. Mangiarotti (1999) showed that nascent mRNA molecules are included in polyribosomes that are active in protein synthesis in the nuclei of *Dictyostelium* cells. There seem fair chances to believe that mRNA molecules are incorporated into polyribosomes while they are still being synthesized. It suggests that like prokaryotes, there is possibility of transcription and translation being coupled in a eukaryotic nucleus. Interestingly, PeIF5B also displays several features that make it a probable candidate to form part of the primitive translation process in the eukaryotic nucleus. It shows significant sequence similarity to bacterial IF2 (42% similarity). PeIF5B is a single polypeptide chain molecule similar to prokaryotic IF2. As apparent from exon-intron analysis, PeIF5B is an intron-less gene, a prerequisite for coupled transcription and translation process (Figure 4.13). This is a unique feature of PeIF5B not shared by other eukaryotic eIF5Bs. Hence all these evidences together make it a probable factor participating in the nuclear translation process.

SUMMARY

SUMMARY

Translation initiation process in bacteria and eukarya shows several common features but involves distinct set of factors. The translation initiation step in prokaryotes and eukaryotes is evolutionarily linked by two common initiation factors. Amino acid sequence comparisons of the translation initiation factors in prokaryotes, archaea and eukaryotes reveal that IF1/eIF1A and IF2/eIF5B form a pair of universally conserved translation initiation factors (Kyrpides and Woese, 1998a, b). Bacterial IF2 has its sequence homologue in eukaryotes (eIF5B) and archaea (aIF5B) (Choi *et al*., 2000; Lee *et al*., 1999). Deletion of the *FUN12* gene, encoding eIF5B factor in yeast, is reported to hamper the growth that can be complemented by recombinant yeast eIF5B. Pestova *et al*. (2000) reported that eIF5B is necessary for ribosome subunit joining. It also harbors ribosome-dependent GTPase activity. GTP hydrolysis is not required for the association of the two subunits but to facilitate dissociation of eIF5B for its recycling.

Till date, among eukaryotes, full-length eIF5B protein sequences have been characterized from *Saccharomyces cerevisiae* (Choi *et al*., 1998)*, Homo sapiens* (Lee *et al*., 1999) and *Drosophila melanogaster* (Carrera *et al*., 2000) however, eIF5B-like factor has not been reported from plant system. In the present study we characterized an eIF5B-like factor from *Pisum sativum*. The discovery of this novel gene, *PeIF5B,* is the first report of a full-length eIF5B-like factor from plant

system. The present study was originally initiated with the intention to isolate the gene coding for DNA polymerase from *Pisum sativum* genome. Around one hundred million plaques were immunoscreened to finally obtain one single clone carrying 3kb insert (pP1). It was a partial clone lacking 5'end of the ORF. With the help of this truncated clone, the cDNA library was rescreened to obtain the full-length clone. A 500bp fragment released after digestion with *Eco*RI-*Hin*dIII present near the 5'end and the 3' end of the pP1 partial clone was used as a radiolabeled probe to screen the library. Finally a clone carrying 3.5kb insert was obtained carrying complete ORF of 2.6kb (pPeIF5B). The deduced protein sequence of this gene indicated that it shows sequence similarity to eukaryotic translation initiation factor eIF5B. Northern blot analysis from total RNA from *Pisum sativum* showed the presence of the transcript of *PeIF5B* gene. In the *in vitro* transcribed-translated *PeIF5B* gene, the single polypeptide could be seen at a size of 116kDa rather than at an expected size of 96kDa. This size discrepancy between the predicted size of the protein (96kDa) and that observed after *in vitro* synthesis of the polypeptide (116kDa) is perhaps a reflection of the presence of highly charged N-terminus of PeIF5B thereby contributing to its aberrant movement in SDS-PAGE.

Going well with earlier examples, PeIF5B shows high amount of similarity with the corresponding genes from yeast, human and archaea, that is, 63%, 64% and 57%, respectively. Being an IF2 homologue, PeIF5B displays 42% similarity to *E. coli* IF2. Apart from sequence similarity to eIF5Bs from other systems, several

major domains were predicted in PeIF5B that strengthened the idea of it being a translation initiation factor. It carries a GTP-binding domain, as predicted by Pfam, consists of G-I, G-II and G-III motifs that are known to be important for GDP/GTP exchange, GTP-induced conformational changes and GTP hydrolysis. Other Pfam predicted domain was EF-TU-D2 domain. This EF-Tu-domain 2 possesses β-barrel structure and is known to interact with charged transfer ribonucleic acids (tRNAs) (Nissen *et al*., 1995). Similar G-domain and EF-TU-D2 domain are also predicted for *E.coli* IF2. Interestingly, like other IF2 and e/aIF5B factors, the central and C-terminal regions of PeIF5B were found to be highly conserved. Thus *in-silico* analysis suggests that PeIF5B probably plays a role in the cellular translation process. This novel factor is phylogenetically related to other known e/aIF5Bs from eukaryotes and archaea and prokaryotic IF2.

Further this gene was cloned, expressed and recombinant protein was purified to homogeneity from *E.coli.* Fluorescence spectroscopy and circular dichroism were employed to study the structural details of the novel translation initiation factor eIF5B from *Pisum sativum.* Study was undertaken to follow the changes in the protein conformation and secondary structure upon binding to its cofactors: Mg^{2+} and GTP as well as initiator tRNA. The conformational differences between free and bound forms of the protein are important for the molecular mechanisms involved in the functioning. Stability studies were also performed on PeIF5B by measuring the structural changes in the protein after guanidine hydrochloride (GnHCl)- and temperature- induced denaturation. PeIF5B was found to be an β-

helix and □-sheet rich protein, as seen from the far-UV CD spectrum. It was observed that Mg^{2+} binds to PeIF5B leading to negligible decrease in tryptophan fluorescence intensity and minimal enhancement in ANS fluorescence. This implies that only minor conformational alterations occur on metal ion-binding to the factor. GTP, on the other hand, was observed to bind PeIF5B in a concentration dependent manner causing a significant decrease in intrinsic fluorescence intensity. This reflects that on GTP-binding local conformational changes occur near aromatic amino acids that result in decrease in fluorescence. However, no gross change in the conformation of the protein occurred on nucleotide-binding as seen by no change in the λ_{max} of the protein (λ_{max} =341nm) as well as no effect on ANS-binding with increasing concentration of GTP. Conformational changes could be visualized from the far-UV CD spectra of PeIF5B in the presence of Mg^{2+} /GTP that shows a significant change in the molar ellipticity values in the range 208 to 217nm. As PeIF5B is considered as a representative of eIF5B-like factor from plant system, its tRNA-binding characteristic was studied by fluorescence. Intrinsic fluorescence results of the binding of Met-charged initiator *E.coli* tRNA molecule to PeIF5B in the absence and presence of cofactors suggested that Mg^{2+} ion and nucleotide facilitates tRNA-binding. The ternary complex of protein-GTP/Mg^{2+}-tRNA showed an intermediate conformation. The chemical and thermal denaturation data indicate that PeIF5B is a remarkably stable protein. This suggests that PeIF5B factor from *Pisum sativum* may function in the cell as a translation initiation factor and at the same time

exhibits chaperon-like activity. It may thus act as a molecular chaperone and protect other proteins from denaturing under stress or high temperature conditions.

In prokaryotes, it is apparent that transcription and translation of mRNAs are coupled. But, it has been assumed for a long time that in eukaryotes, this coupling is truncated due to compartmentalization; transcription occurring in the nucleus while translation in the cytoplasm. For all these years, nucleus was considered to harbor the machinery involved in transcription only. But, against this line of thought, some earlier studies pointed to the concept of nuclear translation. As per this theory, the functional significance of nucleus harboring translation machinery is to carry out nonsense-mediated mRNA decay. After several reports it has been accepted that most of the components of translation machinery are present inside the nucleus. The final breakthrough into this concept was the report by Iborra *et al*. (2001) conclusively proving coupled transcription and translation within the nucleus of mammalian cells. Hence, despite several controversies against the emerging concept of "nuclear translation", stronger evidences are continuously building up in support of it. As far as the nature of nuclear translation is concerned, it is claimed that transcription and nuclear translation are coupled as in prokaryotes.

As of today, there is no evidence to show translation in the nucleus of plant cells. The functional characterization of the eukaryotic translation initiation factor, PeIF5B, from the plant system and its cellular distribution suggests that PeIF5B

may probably form part of the nuclear translation machinery of *Pisum sativum*. For the first time in plant system, a probable key player of the nuclear translation process is presented. *PeIF5B* is suggested to be a novel gene from *Pisum sativum* that acts as a probable translation initiation factor in nuclear translation process. PeIF5B was checked for binding to charged initiator tRNA from *E. coli* by filter-binding assay. It showed dose-dependent tRNA-binding that was facilitated by GTP and not GDP. eIF5B was demonstrated to bind GTP and hydrolyze GTP in the presence of ribosomes. Through UV-crosslinking experiment we confirm GTP-binding to PeIF5B. Interestingly, PeIF5B showed metal ion- and dose-dependent GTPase activity in the absence of any added ribosome component. Further as suggested for eIF5Bs, PeIF5B showed interaction with ribosomal proteins as evident by immunoprecipitation. eIF5B is suggested to mediate joining of ribosomal subunits (Pestova *et al.,* 2000). The biochemical evidences very well establish the role of PeIF5B as the translation initiation factor eIF5B from *Pisum sativum*.

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proves that the N-terminal NLS drives the export of this factor into the nucleus of the cell. Further PeIF5B distribution was checked in plant system. Nuclear and cytoplasmic extracts were prepared and western analysis was carried out with anti-PeIF5B antibodies. The presence of a signal in the nuclear extract using anti-PeIF5B antibodies and the absence of the same in the cytoplasmic fraction clearly proved that PeIF5B is present in the nucleus. There are several reasons to believe that the nature of nuclear translation is quite primitive and prokaryote-like rather than that of eukaryotes (Mangiarotti, 1999). Interestingly, PeIF5B also displayed several features that make it a probable candidate forming part of the primitive translation process in the eukaryotic nucleus. It showed significant sequence similarity to bacterial IF2 (42% similarity). PeIF5B is a single polypeptide chain molecule similar to prokaryotic IF2. As found from exon-intron analysis, PeIF5B is an intron-less gene, a prerequisite for coupled transcription and translation process. This is a unique feature of PeIF5B not shared by other eukaryotic eIF5Bs. Hence all the evidences together make it a probable factor participating in the nuclear translation process.

REFERENCES

REFERENCES

Ævarsson. A, Brazhnikov, E., Garber, M., Zheltonosova, J., Chirgadze, Y., Al-Karadaghi, S., Svensson, L.A., and Liljas, A. (1994) Three-dimensional structure of the ribosomal translocase: elongation factor G from *Thermus thermophilus*. *The EMBO J*. **13**, 3669-3677.

Altchul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990) Basic local alignment search tool. *J. Mol. Biol*. **215**, 403-410.

Asano, K., Krishnamoorthy, T., Phan, L., Pavitt, G.D., and Hinnebusch, A.G. (1999) Conserved bipartite motifs in yeast eIF5 and eIF2B, GTPase-activating and GDP-GTP exchange factors in translation initiation, mediate binding to their common substrate eIF2. *The EMBO J.* **18**, 1673-1688.

Asano, K., Phan, L., Anderson, J., and Hinnebusch, A.G. (1998) Complex Formation by All Five Homologues of Mammalian Translation Initiation Factor 3 Subunits from Yeast *Saccharomyces cerevisiae. J. Biol. Chem*. **273**, 18573-18585.

Ban, N., Nissen, P., Hansen, J., Capel, M., Moore, P.B., and Steitz, T.A. (1999) Placement of protein and RNA structures into a 5 Å resolution map of the 50S ribosomal subunit. *Nature* **400,** 841-847.

Bandyopadhyay, A., and Maitra, U. (1999) Cloning and characterization of the p42 subunit of mammalian translation initiation factor 3 (eIF3): Demonstration that eIF3 interacts with eIF5 in mammalian cells. *Nucleic Acids Research* **27**, 1331-1337.

Battiste, J.B., Pestova, T.V., Hellen, C.U.T., and Wagner, G. (2000) The eIF1A solution structure reveals a large RNA-binding surface important for scanning function. *Mol Cell* **5**, 109–119.

Benne, R., Brown-Luedi, M.L., and Hershey, J.W.B. (1978) Purification and characterization of protein synthesis initiation factors eIF-1, eIF-4C, eIF-4D, and eIF-5 from rabbit reticulocytes. *J. Biol. Chem*. **253**, 3070-3077.

Berchtold, H., Reshetnikova, L., Reiser, C.O.A., Schirmer, N.K., Sprinzl, M., and Hilgenfeld, R. (1993) Crystal structure of active elongation factor Tu reveals major domain rearrangements. *Nature* **365**, 126-132.

Bhattacharjee, C., and Das, K.P. (2000) Thermal unfolding and refolding of □-lactoglobulin: An intrinsic and extrinsic fluorescence study. *Eur. J. Biochem*. **267**, 3957-3964.

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

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

Boileau, G., Butler, P., Hershey, J.W.B., and Traut, R.R. (1983) Direct crosslinks between initiation factors 1, 2, and 3 and ribosomal proteins promoted by 2-iminothiolane. *Biochemistry* **22**, 3162-3170.

Brock, S., Szkaradkiewicz, K., Sprinzl, M. (1998) Initiation factors of protein biosynthesis in bacteria and their structural relationship to elongation and termination factors. *Mol. Microbiol*. **29**, 409-417.

Bult, C.J., White, O., Olsen, G.J., Zhou, L., Fleischmann, R.D., Sutton, G.G., Blake, J.A., FitzGerald, L.M., Clayton, R.A., Gocayne, J.D., Kerlavage, A.R., Dougherty, B.A., Tomb, J.F., Adams, M.D., Reich, C.I., Overbeek, R., Kirkness, E.F., Weinstock, K.G., Merrick, J.M., Glodek, A., Scott, J.L., Geoghagen, N.S., and Venter, J.C. (1996) Complete genome sequence of the methanogenic archaeon, *Methanococcus jannaschii. Science* **273**, 1058-1073.

Caldas, T., Laalami, S., and Richarme, G. (2000) Chaperone properties of bacterial elongation factor EF-G and initiation factor IF2. *J. Biol. Chem*. **275**, 855-860.

Canonaco, M.A., Calogero, R.A., and Gualerzi, C.O. (1986) Mechanism of translational initiation in prokaryotes. Evidence for a direct effect of IF2 on the activity of the 30S ribosomal subunit. *FEBS Lett*. **207**, 198-204.

Carrera, P., Johnstone, O., Nakamura, A., Casanova, J., Jackle, H., Lasko, P. (2000) VASA mediates translation through interaction with a *Drosophila* yIF2 homolog. *Mol Cell*. **5**, 181-187.

Carter, A.P., Clemons, Jr., W.M., Brodersen, D.E., Morgan-Warren, R.J., Hartsch, T., Wimberly, B.T., and Ramakrishnan, V. (2001) Crystal Structure of an Initiation Factor Bound to the 30S Ribosomal Subunit. *Science* **291**, 498-501.

Cassim, J.Y., and Yang, J.T. (1969) A computerized calibration of the circular dichrometer. *Biochemistry* **8**, 1947-1951.

Cate, J.H., Yusupov, M.M., Yusupova, G.Z., Earnest, T.N., and Noller, H.F. (1999) X-ray crystal structures of 70S ribosome functional complexes. *Science* **285**, 2095-2104.

Celano, B., Pawlik, R.T., and Gualerzi, C.O. (1988) Interaction of *Escherichia coli* translation-initiation factor IF-1 with ribosomes. *Eur. J. Biochem*. **178**, 351-355.

Chen, Y.H., Yang, J.T., and Martinez, H.M. (1972) Determination of the secondary structures of proteins by circular dichroism and optical rotatory dispersion. *Biochemistry* **11**, 4120-4131.

Choi, S.K., Lee, J.H., Zoll, W.L., Merrick, W.C., and Dever, T.E. (1998) Promotion of Met-tRNA$_i^{Met}$ binding to ribosomes by yIF2, a bacterial IF2 homolog in yeast. *Science* **280,** 1757-1760.

Choi, S.K., Olsen, D.S., Roll-Mecak, A., Martung, A., Remo, K.L., Burley, S.K., Hinnebusch, A.G., and Dever, T.E. (2000) Physical and functional interaction between the eukaryotic orthologs of prokaryotic translation initiation factors IF1 and IF2. *Mol. Cell Biol*. **20,** 7183-7191.

Chudinova, E.M., Ivanov, P.A., and Nadezhdina, E.S. (2004) Nuclear localization signals in p170, the large subunit of translation initiation factor 3. *Molecular Biology* **38**, 575-581.

Cigan, A.M., Pabich, E.K., and Donahue, T.F. (1988) Mutational analysis of the HIS4 translational initiator region in *Saccharomyces cerevisiae. Mol. Cell Biol.* **8**, 2964-2975.

Clemons, W.M., Jr., May, J.L.C., Wimberly, B.T., McCutcheon, J.P., Capel, M.S., and Ramakrishnan, V. (1999) Structure of a bacterial 30S ribosomal subunit at 5.5Å resolution. *Nature* **400**, 833-840.

Craigen, W.J., Lee, C.C., and Caskey, C.T. (1990) Recent advances in peptide chain termination. *Mol Microbiol.* **4**, 861–865.

Dahlquist, K.D., and Puglisi, J.D. (2000) Interaction of Translation Initiation Factor IF1 with the *E. coli* ribosomal A site. *J. Mol. Biol*. **299**, 1-15.

Dahlseid, J.N., Puziss, J., Shirley, R.L., Atkin, A.L., Hieter, P., and Culbertson, M.R. (1998) Accumulation of mRNA coding for the ctf13p kinetochore subunit of *Saccharomyces cerevisiae* depends on the same factors that promote rapid decay of nonsense mRNAs. *Genetics* **150**, 1019-1035.

Davidson, S.D., Passmore, R., and Brock, J.F. (1973) *Human nutrition and dietetics*, 5th edition, Churchill Livingstone, London, United Kingdom.

del Valle, L.J., Escribano, C., Perez, J.J., and Garriga, P. (2002) Calcium-induced decrease of the thermal stability and chaperone activity of alpha-crystallin. *Biochim. Biophys. Acta.* **1601**, 100-109.

Dennis, P.P. (1997) Ancient ciphers: translation in Archaea. *Cell* **89**, 1007-1010.

Dever, T.E., Wei, C.-L., Benkowski, L.A., Browning, K., Merrick, W.C., and Hershey, J.W.B. (1994) Determination of the amino acid sequence of rabbit, human, and wheat germ protein synthesis factor eIF-4C by cloning and chemical sequencing. *J. Biol. Chem*. **269**, 3212-3218.

Dottavio-Martin, D., Suttle, D.P., and Ravel, J.M. (1979) The effects of initiation factors IF-1 and IF-3 on the dissociation of Escherichia coli 70 S ribosomes. *FEBS Lett.* **97**, 105-110.

Duncan, R., and Hershey, J.W. (1983) Identification and quantitation of levels of protein synthesis initiation factors in crude HeLa cell lysates by two-dimensional polyacrylamide gel electrophoresis. *J. Biol. Chem*. **258**, 7228-7235.

Erickson, F.L., and Hannig, E.M. (1996) Ligand interactions with eukaryotic translation initiation factor 2: role of the γ-subunit. *The EMBO J*. **15**, 6311-6320.

Ehtesham, N.Z., Phan, T.-N., Gaikwad, A., Sopory, S.K., and Tuteja, N. (1999) Calnexin from *Pisum sativum*: Cloning of the cDNA and characterization of the encoded protein. *DNA Cell Biol*. **18**, 853-862.

Finn, R.D., Mistry, J., Schuster-Bockler, B., Griffiths-Jones, S., Hollich, V., Lassmann, T., Moxon, S., Marshall, M., Khanna, A., Durbin, R., Eddy, S.R., Sonnhammer, E.L., and Bateman, A. (2006) Pfam: clans, web tools and services. *Nucleic Acids Res*. **34**(Database issue), D247-251.

Fletcher, C.M., Pestova, T.V., Hellen, C.U.T., and Wagner, G. (1999) Structure and interactions of the translation initiation factor eIF1. *The EMBO J.* **18**, 2631-2637.

Flynn, A., Shatsky. I.N., Proud, C.G., and Kaminski, A. (1994) The RNA-binding properties of protein synthesis initiation factor eIF-2. *Biochim. Biophys. Acta* **1219**, 293-301.

Fraser, C.M., Gocayne, J.D., White, O., Adams, M.D., Clayton, R.A., Fleischmann, R.D., Bult, C.J., Kerlavage, A.R., Sutton, G., Kelley, J.M. *et al.* (1995) The minimal gene complement of *Mycoplasma genitalium*. *Science* **270**, 397-403.

Freistroffer, D.V., Pavlov, M.Y., MacDougall, J., Buckingham, R.H., and Ehrenberg, M. (1997) Release factor RF3 in *E.coli* accelerates the dissociation of release factors RF1 and RF2 from the ribosome in a GTP-dependent manner. *The EMBO J.* **16**, 4126–4133.

Frenster, J.H., Allfrey, V.G., and Mirsky, A.E. (1960) Metabolism and morphology of ribonucleoprotein particles from the cell nucleus of lymphocytes. *Proc Natl Acad Sci USA* **46**, 432-444.

Frenster, J.H., Allfrey, V.G., and Mirsky, A.E. (1961) *In vitro* incorporation of amino acids into the proteins of isolated nuclear ribosomes. *Biochim. Biophys. Acta* **47**, 130-137.

Frolova, L., Le Goff, X., Rasmussen, H.H., Cheperegin, S., Drugeon, G., Kress, M., Arman, I., Haenni, A.L., Celis, L.E., Phillippe, M. Justesen, J., and Kisselev, L. (1994) A highly conserved eukaryotic protein family processing properties of a polypeptide chain release factor. *Nature* **372**, 701-703.

Frolova, L., Le Goff, X., Zhouravleva, G., Davydova, E., Philippe, M., and Kisselev, L. (1996) Eukaryotic polypeptide chain release factor eRF3 is an eRF1- and ribosome-dependent guanosine triphosphatase. *RNA* **4**, 334-341.

Garcia, C., Fortier, P.L., Blanquet, S., Lallemand, J.Y., and Dardel, F. (1995a) ^{1}H and ^{15}N resonance assignments and structure of the N-terminal domain of *Escherichia coli* initiation factor 3. *Eur. J. Biochem*. **228**, 395-402.

Garcia, C., Fortier, P.L., Blanquet, S., Lallemand, J.Y., and Dardel, F. (1995b) Solution structure of the ribosome-binding domain of *E. coli* translation initiation factor IF3. Homology with the U1A protein of the eukaryotic spliceosome. *J. Mol. Biol*. **254**, 247-259.

Gaspar, N.J., Kinzy, T.G., Scherer, B.J., Humbelin, M., Hershey, J.W. and Merrick, W.C. (1994) Translation initiation factor eIF-2. Cloning and expression of the human cDNA encoding the □-subunit. *J. Biol. Chem*. **269**, 3415–3422.

Gillham, N.W., Boynton, J.E., and Hauser, C.R. (1994) Translational regulation of gene expression in chloroplasts and mitochondria. *Annu. Rev. Genet*. **28**, 71-93.

Godefroy-Colburn, T., Wolfe, A.D., Dondon, J., Grunberg-Manago, M., Dessen, P., and Pantaloni, D. (1975) Light-scattering studies showing the effect of

initiation factors on the reversible dissociation of *Escherichia coli* ribosomes. *J Mol Biol*. **94**, 461-478.

Goidl, J.A. (1978) Does protein synthesis occur within the nucleus? Good evidence that it does. *TIBS* **3**, N225–N228.

Goumans, H., Thomas, A., Verhoeven, A., Voorma, H.O., and Benne, R. (1980) The role of eIF-4C in protein synthesis initiation complex formation. *Biochim. Biophys. Acta*. **608**, 39-46.

Grentzmann, G., Kelly, P.J., Laalami, S., Shuda, M., Firpo, M.A., Cenatiempo, Y., and Kaji, A. (1998) Release factor RF-3 GTPase activity acts in disassembly of the ribosome termination complex. *RNA* **4**, 973-983.

Grunberg-Manago, M., Dessen, P., Pantaloni, D., Godefroy-Colburn, T., Wolfe, A.D., and Dondon, J. (1975) Light-scattering studies showing the effect of initiation factors on the reversible dissociation of *Escherichia coli* ribosomes. *J. Mol. Biol.* **94**, 461–478.

Gualerzi, C.O., Brandi, L., Caserta, E., Garofalo, C., Lammi, M., La Teana, A., Petrelli, D., Spurio, R., Tomsic, J., and Pon, C.L. (2001) Initiation factors in the early events of mRNA translation in bacteria. *Cold Spring Harbor Symp. Quant. Biol*. **66**, 363-376.

Gualerzi, C., Risuleo, G., and Pon, C.L. (1977) Initial rate kinetic analysis of the mechanism of initiation complex formation and the role of initiation factor IF-3. *Biochemistry* **16**, 1684-1689.

Gualerzi, C.O., and Pon, C.L. (1990) Initiation of mRNA translation in prokaryotes. *Biochemistry* **29**, 5881-5889.

Gualerzi, C.O., Severini, M., Spurio, R., La Teana, A., and Pon, C.L. (1991) Molecular dissection of translation initiation factor IF2. Evidence for two structural and functional domains. *J. Biol. Chem*. **266**, 16356-16362.

Guillon, L., Schmitt, E., Blanquet, S., and Mechulam, Y. (2005) Initiator tRNA binding by e/aIF5B, the eukaryotic/archaeal homologue of bacterial initiation factor IF2. *Biochemistry* **44**, 15594-15601.

Hackeng, T.M., Fernández, J.A., Dawson, P.E., Kent, S.B.H., and Griffin, J.H. (2000) Chemical synthesis and spontaneous folding of a multidomain protein: Anticoagulant microprotein S. *Proc. Natl. Acad. Sci. U.S.A*. **97**, 14074 - 14078.

Haggerty, T.J., and Lovett, S.T. (1997) IF3-mediated suppression of a GUA initiation codon mutation in the *recJ* gene of *Escherichia coli*. *J. Bacteriol*. **179**, 6705-6713.

Harashima, S., and Hinnebusch, A.G. (1986) Multiple *GCD* genes required for repression of *GCN4*, a transcriptional activator of amino acid biosynthetic genes in *Saccharomyces cerevisiae. Mol. Cell Biol*. **6**, 3990-3998.

Hartz, D., Binkley, J., Hollingsworth, T., and Gold, L. (1990) Domains of initiator tRNA and initiation codon crucial for initiator tRNA selection by *Escherichia coli* IF3. *Genes & Dev*. **4**, 1790-1800.

Hartz, D., McPheeters, D.S., and Gold, L. (1989) Selection of the initiator tRNA by *Escherichia coli* initiation factors. *Genes & Dev*. **3**, 1899-1912.

Hentze, M.W., and Kulozik, A.E. (1999) A perfect message: RNA surveillance and nonsense-mediated decay. *Cell* **96**, 307-310.

Hirokawa, G., Kiel, M.C., Muto, A., Selmer, M., Raj, V.S., Liljas, A., Igarashi, K., Kaji, H., and Kaji, A. (2002) Post-termination complex disassembly by ribosome recycling factor, a functional tRNA mimic. *The EMBO J.* **21**, 2272-2281.

Hui, A., and de Boer, H.A. (1987) Specialized ribosome system: Preferential translation of a single mRNA species by a subpopulation of mutated ribosomes in *Escherichia coli. Proc Natl Acad Sci USA* **84**, 4762-4766.

Hutchison, C.A., Peterson, S.N., Gill, S.R., Cline, R.T., White, O., Fraser, C.M., Smith, H.O., and Venter, J.C. (1999) Global transposon mutagenesis and a minimal Mycoplasma genome. *Science* **286**, 2165-2169.

Iborra, F.J., Jackson, D.A., and Cook, P.R. (2001) Coupled transcription and translation within nuclei of mammalian cells. *Science* **293**, 1139-1142.

Jacob, W.F., Santer, M., and Dahlberg, A.E. (1987) A single base change in the Shine-Dalgarno region of 16S rRNA of *Escherichia coli* affects translation of many proteins. *Proc Natl Acad Sci USA* **84**, 4757-4761.

Jameel, S., Zafrullah, M., Ozdener, M.H., and Panda, S.K. (1996) Expression in animal cells and characterization of the hepatitis E virus structural proteins. *J. Virol.* **70**, 207-216.

Karimi, R., Pavlov, M., Buckingham, R.H., and Ehrenberg, M. (1999) Novel roles for classical factors at the interface between translation termination and initiation. *Mol. Cell* **3**, 601-609.

Kennell, D., and Riezman, H. (1977) Transcription and translation initiation frequencies of the *Escherichia coli lac* operon. *J. Mol. Biol*. **114**, 1–21.

Khurana, R., and Udgaonkar, J.B. (1994) Equilibrium unfolding studies of barstar: Evidence for an alternative conformation which resembles a molten globule. *Biochemistry* **33**, 106-115.

Kjeldgaard, M., and Nyborg, J. (1992) Refined structure of elongation factor EF-Tu from *Escherichia coli*. *J. Mol. Biol*. **223**, 721-742.

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

Kozak, M. (1990) Downstream secondary structure facilitates recognition of initiator codons by eukaryotic ribosomes. *Proceedings of the National Academy of Sciences* **87**, 8301-8305.

Kozak, M., and Shatkin, A.J. (1978) Identification of features in 5' terminal fragments from reovirus mRNA which are important for ribosome binding. *Cell* **13**, 201–212.

Kyrpides, N.C., and Woese, C.R. (1998a) Universally conserved translation initiation factors. *Proc. Nat. Acad. Sci. USA* **95**, 224-228.

Kyrpides, N.C., and Woese, C.R. (1998b) Archaeal translation initiation revisited: The initiation factor 2 and eukaryotic initiation factor 2B □-□-□ subunit families. *Proc. Nat. Acad. Sci. USA* **95**, 3726-3730.

Laalami, S., Putzer, H., Plumbridge, J.A., and Grunberg-Manago, M. (1991) A severely truncated form of translational initiation factor 2 supports growth of *Escherichia coli*. *J. Mol. Biol*. **220**, 335-349.

La Teana, A., Gualerzi, C.O., and Brimacombe, R. (1995) From stand-by to decoding site. Adjustment of the mRNA on the 30S ribosomal subunit under the influence of the initiation factors. *RNA* **1**, 772-782.

Lamphear, B.J., Kirchweger, R., Skern, T., and Rhoads, R.E. (1995) Mapping of functional domains in eukaryotic protein synthesis initiation factor 4G (eIF4G) with picornaviral proteases: Implications for cap-dependent and cap-independent translational initiation. *J. Biol. Chem*. **270**, 21975-21983.

Lang, V., Zanchin, N.I., Lunsdorf, H., Tuite, M., and McCarthy, J.E. (1994) Initiation factor eIF-4E of *Saccharomyces cerevisiae*: Distribution within the cell, binding to mRNA and consequences of its overproduction. *J. Biol. Chem.* **269**, 6117-6123.

Laurino, J.P., Thompson, G.M., Pacheco, E., and Castilho, B.A. (1999) The subunit of eukaryotic translation initiation factor 2 binds mRNA through the lysine repeats and a region comprising the C_2-C_2 motif. *Mol. Cell Biol*. **1**, 173-81.

Laursen, B.S., Kjaergaard, A.C., Mortensen, K.K., Hoffman, D.W., Sperling-Petersen, H.U. (2004) The N-terminal domain (IF2N) of bacterial translation initiation factor IF2 is connected to the conserved C-terminal domains by a flexible linker. *Protein Sci*. **13**, 230-239.

Laursen, B.S., Mortensen, K.K., Sperling-Petersen, H.U., Hoffman, D.W. (2003) A conserved structural motif at the N-terminus of bacterial translation initiation factor IF2. *J. Biol. Chem*. **278**, 16320-16328.

Laursen, B. S., Sorensen, H. P., Mortensen, K. K., and Sperling-Petersen, H. U. (2005) Initiation of Protein Synthesis in Bacteria. *Microbiol. Mol. Biol. Rev.* **69**, 101-123.

Lee, J.H., Choi, S.K., Roll-Mecak, A., Burley, S.K., and Dever, T.E. (1999) Universal conservation in translation initiation revealed by human and archaeal homologs of bacterial translation initiation factor IF2. *Proc. Nat. Acad. Sci. USA* **96**, 4342-4347.

Lee, J.H., Pestova, T.V., Shin, B.-S., Cao, C., Choi, S.K., and Dever, T.E. (2002) Initiation factor eIF5B catalyzes second GTP-dependent step in eukaryotic translation initiation. *Proc. Nat. Acad. Sci. USA* **99**, 16689-16694.

Lejbkowicz, F., Goyer, C., Darvear, A., Neron, S., Lemieux, R., and Sonenberg, N. (1992) A fraction of the mRNA 5'cap-binding protein, eukaryotic initiation factor 4E, localizes to the nucleus. *Proc. Natl. Acad. Sci. USA* **89**, 9612-9616.

Lew, J.E., Enomoto, S., and Berman, J. (1998) Telomere length regulation and telomeric chromatin require the nonsense-mediated mRNA decay pathway. *Mol. Cell Biol.* **18**, 6121-6130.

Li, S., and Wilkinson, M.F. (1998) Nonsense surveillance in lymphocytes? *Immunity* **8**, 135–141.

Lim, S.K., Sigmund, C.D., Gross, K.W., and Maquat, L.E. (1992) Nonsense codons in human β-globin mRNA result in the production of mRNA degradation products. *Mol. Cell. Biol*. **12**, 1149-1161.

Louie, A., and Jurnak, F. (1985) Kinetic studies of *Escherichia coli* elongation factor Tu-GTP-aminoacyl-tRNA complexes. *Biochemistry* **24**, 6533-6539.

Lund, E., and Dahlberg, J.E. (1998) Proofreading and aminoacylation of tRNAs before export from the nucleus. *Science* **282**, 2003-2004.

Ma, L., and Spremulli, L.L. (1990) Identification and characterization of large, complex forms of chloroplast translational initiation factor 2 from *Euglena gracilis*. *J. Biol. Chem.* **265**, 13560-13565.

Ma, L., and Spremulli, L.L. (1995) Cloning and sequence analysis of the human mitochondrial translational initiation factor 2 cDNA. *J. Biol. Chem*. **270**, 1859-1865.

Ma, J., and Spremulli, L.L. (1996) Expression, purification, and mechanistic studies of bovine mitochondrial translational initiation factor 2. *J. Biol. Chem*. **271**, 5805-5811.

Mader, S., Lee, H., Pause, A., and Sonenberg, N. (1995) The translation initiation factor eIF-4E binds to a common motif shared by the translation factor eIF-4γ and the translational repressors 4E-binding proteins. *Mol Cell Biol.* **15**, 4990–4997.

Malhotra, A., Penczek, P., Agrawal, R.K., Gabashvili, I.S., Grassucci, R.A., Jünemann, R., Burkhardt, N., Nierhaus, K.H., and Frank, J. (1998) *Escherichia coli* 70 S ribosome at 15 Å resolution by cryo-electron microscopy: localization of fMet-tRNA$_f^{Met}$ and fitting of L1 protein. *J. Mol Biol.* **280**, 103–116.

Mangiarotti, G. (1999) Coupling of transcription and translation in Dictyostelium discoideum nuclei. *Biochemistry* **38**, 3996-4000.

Mangiarotti, G., and Chiaberge, S. (1997) Reconstitution of functional eukaryotic ribosomes from *Dictyostelium discoideum* ribosomal proteins and RNA. *J. Biol. Chem.* **272**, 19682-19687.

Mangiarotti, G., Chiaberge, S., and Bulfone, S. (1997) rRNA maturation as a "quality" control step in ribosomal subunit assembly in *Dictyostelium discoideum*. *J. Biol. Chem.* **272**, 27818-27822.

Mangiarotti, G., and Schlessinger, D. (1967) Polyribosome metabolism in *Escherichia coli*. II. Formation and lifetime of messenger RNA molecules, ribosomal subunit couples, and polyribosomes. *J. Mol. Biol*. **29**, 395-418.

Mangroo, D., Wu, X.Q., and RajBhandary, U.L. (1995) *Escherichia coli* initiator tRNA: Structure-function relationships and interactions with the translational machinery. *Biochem. Cell Biol*. **73**, 1023-1031.

Maquat, L.E. (1995) When cells stop making sense: effects of nonsense codons on RNA metabolism in vertebrate cells. *RNA* **1**, 453-465.

Maquat, L.E. (1996) Defects in RNA splicing and the consequence of shortened translational reading frames. *Am. J. Hum. Genet.* **59**, 279–286.

Maquat, L.E., Kinniburgh, A.J., Rachmilewitz, E.A., and Ross, J. (1981) Unstable β-globin mRNA in mRNA-deficient β^o-thalassemia. *Cell.* **27,** 543–553.

Marintchev, A., Kolupaeva, V.G., Pestova, T.V., and Wagner, G. (2003) Mapping the binding between the human translation initiation factors eIF1A and eIF5B: new interaction between old partners. *Proc. Nat. Acad. Sci. USA* **100**, 1535-1540.

Meinnel, T., Sacerdot, C., Graffe, M., Blanquet, S., and Springer, M. (1999) Discrimination by *Escherichia coli* initiation factor IF3 against initiation on non-canonical codons relies on complementarity rules. *J. Mol. Biol*. **23**, 825-837.

Meisenberg, G., and Simmons, W.H. (1998) *Principles of medical biochemistry*. 1st edition, Mosby, St. Louis, Missouri.

Merrick, W.C. (1979) Purification of protein synthesis initiation factors from rabbit reticulocytes. *Methods Enzymol.* **60**, 108-123.

M é thot, N., Song, M.S., Sonenberg, N. (1996) A region rich in aspartic acid, arginine, tyrosine, and glycine (DRYG) mediates eukaryotic initiation factor 4B (eIF4B) self-association and interaction with eIF3. *Mol. Cell. Biol*. **16**, 5328-5334.

Meunier, S., Spurio, R., Czisch, M., Wechselberger, R., Guenneugues, M., Gualerzi, C.O., and Boelens, R. (2000) Structure of the fMet-tRNA^{fMet}-binding domain of *B. stearothermophilus* initiation factor IF2. *The EMBO J* **19,** 1918-1926.

Mikuni, O., Ito, K., Moffat, J., Matsumura, K., McCaughan, K., Nobukuni, T., Tate, W., and Nakamura, Y. (1994) Identification of the *prfC* gene, which encodes peptide-chain-release factor 3 of *Escherichia coli*. *Proc Natl Acad Sci USA* **91**, 5798–5802.

Miller Jr., O.L., Hamkalo, B.A., Thomas Jr., C.A. (1970) Visualization of bacterial genes in action. *Science* **169**, 392-395.

Moreno, J.M.P., Dyrskjotersen, L., Kristensen, J.E., Mortensen, K.K., and Sperling-Petersen, H.U. (1999) Characterization of the domains of *E. coli* initiation factor IF2 responsible for recognition of the ribosome. *FEBS Lett*. **455**, 130-134.

Moreno, J.M.P., Kildsgaard, J., Siwanowicz, I., Mortensen, K.K., and Sperling-Petersen, H.U. (1998) Binding of *Escherichia coli* initiation factor IF2 to 30S ribosomal subunits: A functional role for the N-terminus of the factor. *Biochem. Biophys. Res. Commu*. **252,** 465–471.

Moriarty, P.M., Reddy, C.C., and Maquat, L.E. (1998) Selenium deficiency reduces the abundance of mRNA for Se-dependent glutathione peroxidase 1 by a UGA-dependent mechanism likely to be nonsense codon-mediated decay of cytoplasmic mRNA. *Mol. Cell Biol*. **18**, 2932-2939.

Morrison, M., Harris, K.S., and Roth, M.B. (1997) *smg* mutants affect the expression of alternatively spliced SR protein mRNAs in *Caenorhabditis elegans. Proc. Nat. Acad. Sci. USA* **94**, 9782-9785.

Mortensen, K.K., Kildsgaard, J., Moreno, J.M., Steffensen, S.A., Egebjerg, J., and Sperling-Petersen, H.U. (1998) A six-domain structural for *Escherichia coli* translation initiation factor IF2. Characterisation of twelve surface epitopes. *Biochem. Mol. Biol. Int*. **46**, 1027-1041.

Nissen, P., Kjeldgaard, M., Thirup, S., Polekhina, G., Reshetnikova, L., Clark, B.F., Njborg, J. (1995) Crystal structure of the ternary complex of Phe-tRNAPhe, EF-Tu, and a GTP analog. *Science* **270**, 1464-1472.

Naranda, T., Macmillan, S.E., Donahue, T.F. and Hershey, J.W. (1996) SUI1/p16 is required for the activity of eukaryotic translation initiation factor 3 in *Saccharomyces cerevisiae*. *Mol. Cell Biol.* **16**, 2307-2313.

Olsen, D.S., Savner, E.M., Mathew, A., Zhang, F., Krishnamoorthy, T., Phan, L., and Hinnebusch, A.G. (2003) Domains of eIF1A that mediate binding to eIF2, eIF3 and eIF5B and promote ternary complex recruitment in vivo. *The EMBO J.* **22**, 193-204.

Olsen, G.J., and Woese, C.R. (1997) Archaeal genomics: an overview. *Cell* **89**, 991-994.

Olsson, C.L., Graffe, M., Springer, M., and Hershey, J.W. (1996) Physiological effects of translation initiation factor IF3 and ribosomal protein L20 limitation in *Escherichia coli*. *Mol. Gen. Genet.* **250**, 705-714.

Ouzounis, C., and Kyrpides, N. (1996) The emergence of major cellular processes in evolution. *FEBS Lett.* **390,** 119-123.

Pain, V.M. (1996) Initiation of protein synthesis in eukaryotic cells. *Eur. J. Biochem*. **236**, 747-771.

Pestova, T.V., Borukhov, S.I., and Hellen, C.U.T. (1998) Eukaryotic ribosomes require initiation factors 1 and 1A to locate initiation codons. *Nature* **394**, 854-859.

Pestova, T.V., Lomakin, I.B., Lee, J.H., Choi, S.K., Dever, T.E., and Hellen, C.U.T. (2000) The joining of ribosomal subunits in eukaryotes requires eIF5B. *Nature* **403**, 332-335.

Petrelli, D., La Teana, A., Garofalo, C., Spurio, R., Pon, C.L., and Gualerzi, C.O. (2001) Translation initiation factor IF3: two domains, five functions, one mechanism? *The EMBO J.* **20**, 4560–4569.

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

Primm, T.P., Andersen, S.J., Mizrahi, V., Avarbock, D., Rubin, H., and Barry, C.E.III (2000) The stringent response of *Mycobacterium tuberculosis* is required for long-term survival. *J. Bacteriol*. **182**, 4889-4898.

Rao, D., Momcilovic, I., Kobayashi, S., Callegari, E., and Ristic, Z. (2004) Chaperone activity of recombinant maize chloroplast protein synthesis elongation factor, EF-Tu. *Eur. J. Biochem*. **271**, 3684–3692.

Raychaudhuri, P., Stringer, E.A., Valenzuela, D.M., and Maitra, U. (1984) Ribosomal subunit antiassociation activity in rabbit reticulocyte lysates. Evidence

for a low molecular weight ribosomal subunit antiassociation protein factor (Mr = 25,000). *J. Biol. Chem*. **259**, 11930-11935.

Rendi, R. (1960) *In vitro* incorporation of labeled amino acids into nuclei isolated from rat liver. *Experimental Cell Research* **19**, 489-498.

Rogers, Jr., G.W., Richter, N.J. and Merrick, W.C. (1999) Biochemical and kinetic characterization of the RNA helicase activity of eukaryotic initiation factor 4A. *J. Biol. Chem*. **274**, 12236-12244.

Roll-Mecak, A., Cao, C., Dever, T.E., Burley, S.K. (2000) X-ray structures of the universal translation initiation factor IF2/eIF5B: Conformational changes on GDP and GTP binding. *Cell* **103**, 781-792.

Rozen, F., Edery, I., Meerovitch, K., Dever, T.E., Merrick, W.C., and Sonenberg, N. (1990) Bidirectional RNA helicase activity of eucaryotic translation initiation factors 4A and 4F. *Mol. Cell Biol.* **10**, 1134-1144.

Russell, D. W., and Spremulli, L.L. (1979) Purification and characterization of a ribosome dissociation factor (eukaryotic initiation factor 6) from wheat germ. *J. Biol. Chem*. **254**, 8796-8800.

Sacerdot, C., Chiaruttini, C., Engst, K., Graffe, M., Milet, M., Mathy, N., Dondon, J., and Springer, M. (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**, 331-346.

Sacerdot, C., Dessen, P., Hershey, J.W.B., Plumbridge, J.A., and Grunberg-Manago, M. (1984) Sequence of the Initiation factor IF2 Gene: Unusual Protein Features and Homologies with Elongation Factors. *Proc. Nat. Acad. Sci. USA* **81**, 7787-7791.

Sacerdot, C., Fayat, G., Dessen, P., Springer, M., Plumbridge, J.A., Grunberg-Manago, M., and Blanquet, S. (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. *The EMBO J*. **1**, 311-315.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sander, C., and Schneider, R. (1991) Database of homology-derived protein structures and the structural meaning of sequence alignment. *Proteins* **9**, 56-68.

Sanger, F., Nicklen, S., and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci U.S.A*. **74**, 5463-5467.

Schreier, M.H., Erni, B., and Staehelin, T. (1977) Initiation of mammalian protein synthesis. I. Purification and characterization of seven initiation factors. *J. Mol. Biol*. **116**, 727-753.

Sette, M., Tilborg, P.V., Spurio, R., Kaptein, R., Paci, M., Gualerzi, C.O., and Boelens, R. (1997) The structure of the translational initiation factor IF1 from *E. coli* contains an oligomer-binding motif. *The EMBO J*. **16**, 1436-1443.

Shi, J., Blundell, T.L., and Mizuguchi, K. (2001) FUGUE: sequence-structure homology recognition using environment-specific substitution tables and structure-dependent gap penalties. *J. Mol. Biol*. **310**, 243-257.

Shin, B.-S., Maag, D., Roll-Mecak, A., Arefin, M.S., Burley, S.K., Lorsch, J.R., and Dever, T.E. (2002) Uncoupling of initiation factor eIF5B/IF2 GTPase and

translational activities by mutations that lower ribosome affinity. *Cell* **111**, 1015-1025.

Simons, R.W., and Grunberg-Manago, M. Eds. (1998) RNA Structure and Function. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Smith, K.E., and Henshaw, E.C. (1975) Binding of Met-tRNA$_f$ to native 40 S ribosomal subunits in Ehrlich ascites tumor cells. *J. Biol. Chem*. **250**, 6880-6884.

Sørensen, H.P., Hedegaard, J., Sperling-Petersen, H.U., and Mortensen, K.K. (2001) Remarkable Conservation of Translation Initiation Factors: IF1/eIF1A and IF2/eIF5B are Universally Distributed Phylogenetic Markers. *IUBMB Life* **51**, 321-327.

Spedding, G. (1990) in *Ribosomes and Protein Synthesis: A Practical Approach.* (Spedding, G., ed) pp. 1–29, IRL Press, Oxford.

Spurio, R. Brandi, L., Caserta, E., Pon, C.L., Gualerzi, C.O., Misselwitz, R., Krafft, C., Welfle, K., and Welfle, H. (2000) The C-terminal subdomain (IF2 C-2) contains the entire fMet-tRNA binding site of initiation factor IF2. *J. Biol. Chem*. **275**, 2447-2454.

Spurio, R., Severini, M., La Teana, A., Canonaco, M.A., Pawlik, R.T., Gualerzi, C.O., Pon, C.L. (1993) Novel structural and functional aspects of translational initiation factor IF2. In *The Translational Apparatus: Structure, Function, Regulation and Evolution* (ed. Nierhaus, K.H., Franceschi, F., Subramanian, A.R., Erdmann, V.A., and Wittmann-Liebold, B.). Plenum Press, New York, 241–252.

Srivastava, S., Verschoor, A., and Frank, J. (1992) Eukaryotic initiation factor 3 does not prevent association through physical blockage of the ribosomal subunit-subunit interface. *J. Mol. Biol*. **226**, 301-304.

Stansfield, I., Jones, K.M., Kushnirov, V.V., Dagakesmanskaya, A.R., Poznyakov, A.I., Paushkin, S.V., Nierras, C.R., Cox, B.S., Ter-Avanesyan, M.D., and Tuite, M.F. (1995) The products of the SUP45 (eRF1) and SUP35 genes interact to mediate translation termination in *Saccharomyces cerevisiae*. *The EMBO J.* **14**, 4365-4373.

Steitz, J.A. and Jakes, K. (1975) How ribosomes select initiator regions in mRNA: base pair formation between the 3' terminus of 16S rRNA and the mRNA during initiation of protein synthesis in *Escherichia coli. Proc. Nat. Acad. Sci. USA* **72**, 4734-4738.

Stern, D.B., Higgs, D.C., and Yang, J. (1997) Transcription and translation in chloroplasts. *Trends Plant Sci.* **2**, 308-315.

Stringer, E.A., Sarkar, P., and Maitra, U. (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**, 1739-1744.

Sussman, J.K., Simons, E.L., and Simons, R.W. (1996) *Escherichia coli* translation initiation factor 3 discriminates the initiation codon *in vivo. Mol. Microbiol*. **21**, 347-360.

Tatusova, T.A., and Madden, T.L. (1999) Blast 2 sequences - a new tool for comparing protein and nucleotide sequences. *FEMS Microbiol. Lett*. **174**, 247-250.

Thomas, A., Goumans, H., Voorma, H.O., and Benne, R. (1980) The mechanism of action of eukaryotic initiation factor 4C in protein synthesis. *Eur. J. Biochem.* **107**, 39-45.

Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**, 4673-4680.

Tomsic, J., Vitali, L.A., Daviter, T., Savelsbergh, A., Spurio, R., Striebeck, P., Wintermeyer, W., Rodnina, M.V., and Gualerzi, C.O. (2000) Late events of translation initiation in bacteria: a kinetic analysis. *The EMBO J.* **19**, 2127-2136.

Unbehaun, A., Borukhov, S.I., Hellen, C.U.T., and Pestova, T.V. (2004) Release of initiation factors from 48S complexes during ribosomal subunit joining and the link between establishment of codon-anticodon base-pairing and hydrolysis of eIF2-bound GTP. *Genes Dev*. **18**, 3078-3093.

Vambutas, A., Ackerman, S.H., and Tzagoloff, A. (1991) Mitochondrial translational-initiation and elongation factors in *Saccharomyces cerevisiae*. *Eur. J. Biochem*. **201**, 643-652.

Vornlocher, H.-P., Scheible, W.-R., Faulhammer, H.G., and Sprinzl, M. (1997) Identification and purification of translation initiation factor 2 (IF2) from *Thermus thermophilus. Eur J Biochem* **243**, 66-71.

Warner, J.R. (1999) The economics of ribosome biosynthesis in yeast. *Trends Biochem. Sci*. **24**, 437-440.

Weber, M.H., Beckering, C.L., and Marahiel, M.A. (2001) Complementation of cold shock proteins by translation initiation factor IF1 *in vivo*. *J. Bacteriol*. **183**, 7381-7386.

Wei, C.-L., Kainuma, M., and Hershey, J.W.B. (1995) Characterization of Yeast Translation Initiation Factor 1A and Cloning of Its Essential Gene. *J. Biol. Chem*. **270**, 22788-22794.

Wienk, H., Tomaselli, S., Bernard, C., Spurio, R., Picone, D., Gualerzi, C.O., and Boelens, R. (2005) Solution structure of the C1-subdomain of *Bacillus stearothermophilus* translation initiation factor IF2. *Protein Sci*. **14**, 2461-2468.

Wilson, E.B. (1928) The Cell in Development and Heredity (Macmillan, New York), pp. 738-739.

Wilson, S.A., Sieiro-Vazquez, C., Edwards, N.J., lourin, O., Byles, E.D., Kotsopoulou, E., Adamson, C.S., Kingsman, S.M., Kingsman, A.J., and Martin-Rendon, E. (1999) Cloning and characterization of hIF2, a human homologue of bacterial translation initiation factor 2, and its interaction with HIV-1 matrix. *Biochem. J*. **342**, 97-103.

Wintermeyer, W., and Gualerzi, C. (1983) Effect of *Escherichia coli* initiation factors on the kinetics of *N*-AcPhe-tRNA^{Phe} binding to 30S ribosomal subunits. A fluorescence stopped-flow study. *Biochemistry* **22**, 690-694.

Wu, X.-Q., and RajBhandary, U.L. (1997) Effect of the amino acid attached to *Escherichia coli* initiator tRNA on its affinity for the initiation factor IF2 and on the IF2 dependence of its binding to the ribosome. *J. Biol. Chem.* **272**, 1891-1895.

Wu, X.Q., Iyengar, P., and RajBhandary, U.L. (1996) Ribosome-initiator tRNA complex as an intermediate in translation initiation in *Escherichia coli* revealed by use of mutant initiator tRNAs and specialized ribosomes. *The EMBO J.* **15**, 4734–4739.

Yusupova, G., Reinbolt, J., Wakao, H., Laalami, S., Grunberg-Manago, M., Romby, P., Ehresmann, B., and Ehresmann, C. (1996) Topography of the *Escherichia coli* initiation factor 2/fMet-tRNA$_f^{Met}$ complex as studied by cross-linking. *Biochemistry* **35**, 2978–2984.

Yusupova, G., Yusupov, M., Cate, J.H.D., and Noller, H.F. (2001) The Path of Messenger RNA Through the Ribosome. *Cell* **106**, 233-241.

Zhang, S., Welch, E.M., Hogan, K., Brown, A.H., Peltz, S.W., and Jacobson, A. (1997) Polysome-associated mRNAs are substrates for the nonsense-mediated mRNA decay pathway in *Saccharomyces cerevisiae*. *RNA* **3**, 234-244.

Zhouravleva, G. Frolova, L., LeGoff, X., LeGuellec, R., Inge-Vechtomov, S., Kisselev, L. and Phillippe, M. (1995) Termination of translation in eukaryotes is governed by two interacting polypeptide chain release factors, eRF1 and eRF3. *The EMBO J.* **14**, 4065-4072.

Zucker, F.H., and Hershey, J.W.B. (1986) Binding of *Escherichia coli* protein synthesis initiation factor IF1 to 30S ribosomal subunits by fluorescence polarization. *Biochemistry* **25**, 3682-3690.

CURRICULUM VITAE

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Research interest:

Always interested in research that carries some exiting challenges and novel thoughts. Specifically find interest in studies related to host-pathogen interactions and the defense provided by the immune system towards the pathogenic invasion. Exploring the down-stream cellular signaling pathways is exciting.

Academic Qualifications:

Ph.D. (July, 2001 till date)
Centre for DNA Fingerprinting and Diagnostics, Hyderabad, INDIA
(Registered at the Department of Biochemistry, University of Hyderabad, INDIA)

Title of thesis: Identification and characterization of a novel eIF5B-like protein from *Pisum sativum* involved in translation process.

Thesis supervisor: Prof. Seyed E. Hasnain, Vice-Chancellor, University of Hyderabad, Hyderabad.

Master of Science (M.Sc.), 2001
Department of Biotechnology,
Aligarh Muslim University, Aligarh, INDIA
Specialization: Biotechnology
First Division with 68.2% marks

Bachelor of Science (B.Sc.) (Honors), 1999
Department of Biochemistry,
Aligarh Muslim University, Aligarh, INDIA
Subjects: Main-Biochemistry, Subsidary-Chemistry and Botany
First Division with 80.4% marks

Higher Secondary School (10+2), 1996
Aligarh Muslim University, Aligarh, INDIA
Subjects: Biology, Physics, Chemistry, English
First Division with 76.4% marks

Secondary School (10), 1994
Indian Council of Secondary Education (ICSE), New Delhi, INDIA
Subjects: Physics, Chemistry, Mathematics, Biology, English, Social Sciences, Hindi, Arts
First Division with 83.5% marks

Awards/Honours:

Certificate awarded in appreciation of witnessing the Republic Day Parade, 2000 from the Prime Minister's Box on Rajpath, New Delhi as a Guest of the Hon'ble Prime Minister of India in recognition of **'Excellent Academic Performance'**.

Dr.Mrs.J.D.Dheer Science Award for **'Excellence in Science'** for the session 1998-99 at Women's College, Aligarh Muslim University, Aligarh, INDIA

Qualified for **award of Junior/Senior Research Fellowship** (for a period of five years) in subject LIFE SCIENCES under the Council of Scientific and Industrial Research fellowship scheme-2000 for pursuing a career in research.

Qualified in Graduate Aptitude Test In Engineering-2001 with a percentile score of 90.93.

Stood II for the poster entitled 'Tissue Engineering-A Revolution in Medical Science' in the poster presentation during the National Science Day Programme-2001, sponsored by the Department of Biotechnology, Ministry of Science and Technology, Govt. of India, New Delhi.

Awarded the Junior/Senior Research Fellowship in Life Sciences/Social Science under the Indian Council of Medical Research fellowship scheme-2001.

Research Experience (Please see Appendix for details):

Project 1: Identification and characterization of a novel eIF5B-like protein from *Pisum sativum* involved in translation process.
[Work done for PhD thesis: Publications 1, 2 and 3]

Project 2: Deciphering the cross talk between host and viral factors in driving transcription from the baculoviral very late polyhedrin gene promoter.
[Additional work done during Ph.D. tenure: Publication 4]

Project 3: Biophysical characterization involving ligand-binding and stability studies of recombinant late expression factor 4 subunit of *Autographa californica* nuclear polyhedrosis virus RNA polymerase.
[Additional work done during Ph.D. tenure: Publications 5 and 6]

Project 4: Folding pathway studies and biochemical characterization of stem bromelain, a thiol protease, from *Ananas comosus.*
[M.Sc final year project, in partial fulfillment of the degree of Master of Science: Publications 7, 8, 9 and 10]

Project 5: Cloning and partial screening of gene encoding 71kDa cell wall associated protein of *Mycobacterium tuberculosis* H_{37}Ra.
[Work done during short-term training program (May-July, 2000) at Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, INDIA]

Technical Expertise:

Molecular biology techniques including Polymerase Chain Reaction (PCR); transformation and plasmid isolation from bacterial cells; cloning and expression of recombinant proteins in *Escherichia coli*.

Generating recombinant baculoviruses and expressing foreign proteins in baculovirus expression system.

Basic tissue culture techniques including maintenance of cell lines; transfection in insect and mammalian cell lines; pulse chase experiments.

Blotting techniques: Northern, Western, Southern, South-Western, dot-blot analysis.

Biophysical techniques involving UV-visible spectroscopy, circular dichroism, fluorescence spectroscopy.

Enzymatic assays: colorimetric and luciferase reporter gene assay.

Protein fractionation techniques including gel-filtration, ion-exchange chromatography, affinity chromatography. Familiar with Acta-Prime (Amersham Pharmesia), BioRad instrument etc.

Genetic approaches towards complementation in *Escherichia coli.*

Protein-protein and protein-DNA interaction techniques like Gel Retardation Assay, GST-pulldown, immuno-precipitation, south-western blotting.

Raising polyclonal antibodies in rabbits; ELISA; preparing nuclear extract from plant tissues, insect and mammalian cells; handling radioactive materials, fluorescence imaging.

Computer knowledge: MS-office (Power point, MS-excel, MS-word); Adobe Photoshop; primer designing; gene and protein sequence alignment and phylogenetic tree building using softwares like Laser Gene Navigator and Genchek (Ocimum Biosciences).

Communication skills: Preparation of scientific manuscripts, scientific data analysis, literature review and presentation of scientific work at various national and international meetings.

Publications:

Manuscripts published:

1.Viswanathan, P., Venkaiah, B., Kumar, M.S., **Rasheedi, S**., Vrati, S., Bashyam, M.D., Hasnain, S.E. (2003) The homologous region sequence (hr1) of *Autographa californica* multinucleocapsid polyhedrosis virus can enhance transcription from non-baculoviral promoters in mammalian cells. ***J. Biol. Chem***. 278, 52564-52571.

2. Ghosh, S., **Rasheedi, S**., Rahim, S.S., Banerjee, S., Choudhary, R.K., Chakhaiyar, P., Ehtesham, N.Z., Mukhopadhyay, S., and Hasnain, S.E. (2004) Method for enhancing solubility of the expressed recombinant proteins in *E. coli*. ***BioTechniques*** 37, 2-6.

3. Haq, S.K., **Rasheedi, S.,** and Khan, R.H. (2002) Characterization of a partially folded intermediate of stem bromelain at low pH. ***Eur. J. Biochem***. 269, 47-52.

4. Haq, S.K., **Rasheedi, S**., Sharma, P., Ahmed, B., and Khan, R.H. (2005) Influence of salts and alcohols on the conformation of partially folded intermediate of stem bromelain at low pH. ***The International Journal of Biochemistry and Cell Biology*** 37, 361-374.

5. **Rasheedi, S**., Haq, S.K., and Khan, R.H. (2003) Guanidine Hydrochloride denaturation of glycosylated and deglycosylated stem Bromelain. ***Biochemistry (Moscow)*** 68, 1097-1100.

6. Khan, R.H., **Rasheedi, S**., and Haq, S.K. (2003) Effect of pH, temperature and alcohols on the stability of glycosylated and deglycosylated stem Bromelain. ***J. Biosciences*** 28, 709-714.

Manuscripts under revision/communicated:

1. **Rasheedi, S**., Ghosh, S., Suragani, M., Tuteja, N., Sopory, S.K., Hasnain, S.E., and Ehtesham, N.Z. *Pisum sativum* contains a factor with strong homology to eIF5B. (*under revision*).

2. **Rasheedi, S**., Ramachandran, A., Ehtesham, N.Z., and Hasnain, S.E. Biochemical characterization of *Sf*9 Sp family-like protein factors reveals interesting features. (*under revision*).

3. **Rasheedi, S**., Suragani, M., Haq, S.K., Ghosh, S., Ehtesham, N.Z., and Hasnain, S.E. Biophysical characterization and unfolding of recombinant late expression factor 4 subunit of *Autographa californica* nuclear polyhedrosis virus RNA polymerase. *(communicated).*

Manuscripts under preparation:

1. **Rasheedi, S**., Suragani, M., Haq, S.K., Joshi, D., Ghosh, S., Mande, S.C., Hasnain, S.E., and Ehtesham, N.Z. Biophysical characterization and structural modelling of translation initiation factor eIF5B from *Pisum sativum. (manuscript under preparation)*.

2. **Rasheedi, S**., Suragani, M., Ghosh, S., Rajasekhar, S.N.V.S., Ramaiah, K.V.A., Hasnain, S.E., and Ehtesham, N.Z. A novel eIF5B-like protein from *Pisum sativum* is involved in translation process. *(manuscript under preparation)*.

3. **Rasheedi, S**., Suragani, M., Haq, S.K., Ghosh, S., Ehtesham, N.Z., and Hasnain, S.E. Characterization of LEF4 ligand-binding property in lieu of its proposed function and interaction with the viral transcription machinery. *(manuscript under preparation)*.

Conferences/Symposia attended:

1. Awarded a waiver to present a poster entitled **"Transcription from *Ac*NPV very late gene polyhedrin promoter involves Sp-like protein factors, TFIID complex and viral RNA polymerase"** authored by **Rasheedi, S.**, Ramachandran, A., and Hasnain, S.E. at the meeting on **"Mechanism of Eukaryotic Transcription" at Cold Spring Harbor Laboratory, New York**, held during August 31- September 4, 2005.

2. Presented a poster entitled **"Novel insect Sp family proteins play important role during transcription from A*c*MNPV *polh* gene promoter"** authored by **Rasheedi, S**., Ramachandran, A., and Hasnain, S.E. at the **VIII Asian Conference on Transcription (ACT-VIII), held at Chulabhorn Research Institute, Bangkok, Thailand** during November 16-19, 2004.

3. Presented a poster entitled **"Characterization of Sp-like Factors from *Sf*9 cells"** by **Rasheedi, S**., Ramachandran, A., and Hasnain, S.E. ***In* XXVII All India Cell Biology Conference & International Symposium on "Frontiers In Biomedical Research & Technologies"**, January 7-10, 2004, organized by IBB & Zoology Dept., University of Pune, Pune.

4. Presented a poster entitled **"A Novel Translation Initiation Factor from Pea"** by **Rasheedi, S**., Ghosh, S., Rajasekhar, S.N.V.S., Ramaiah, K.V.A., Hasnain, S.E., and Ehtesham, N.Z. ***In* The Satellite Symposium on "Molecular Aspects of Cellular Signaling"**, December 3-4, 2003, organized by Deparment of Biochemistry, University of Hyderabad, Hyderabad.

5. Participated in "**International Symposium on Insect Genetics and Genomics**", 9-11 January, 2006, organized by Centre for DNA Fingerprinting and Diagnostics, Hyderabad.

6. Participated in the **"Third Indo-Australian Conference on Biotechnology"** on "**Vaccines for Cancer, Infectious Diseases, Lifestyle & Degenerative Diseases**", 6-8 March, 2006, organized by Centre for DNA Fingerprinting and Diagnostics, Hyderabad and Queensland Institute of Medical Research, Australia.

7. Participated in the **National Symposium on "Stability and Stabilization of Biomolecules"**, March 13-14, 2001, organized by Interdisciplinary Biotechnology Unit, Aligarh Muslim University, Aligarh.

8. Attended the **70th Annual Meeting-Society of Biological Chemists (INDIA)**, 27-29 December, 2001, organized by Department of Biochemistry, Osmania University, Hyderabad.

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References:

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Declaration:

I hereby declare that the above details are true to the best of my knowledge.

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Appendix

RESEARCH EXPERIENCE

Project 1:

Identification and characterization of a novel eIF5B-like protein from *Pisum sativum* involved in translation process.
[Work done for PhD thesis at the Centre for DNA Fingerprinting and Diagnostics, Hyderabad, India]

During translation initiation, ribosomes loaded with charged initiator tRNA position themselves at the start codon on the messenger RNA in the presence of several initiation factors and start the process of polypeptide chain synthesis. In prokaryotes mainly three initiation factors are involved, namely, IF1, IF2 and IF3. Protein synthesis in eukaryotic organisms is a rather complex process with several factors involved. Amino acid sequence comparisons of the translation initiation factors in prokaryotes, archaea and eukaryotes reveal that IF1/eIF1A and IF2/eIF5B form a pair of universally conserved translation initiation factors. Bacterial IF2 has its sequence homologue in eukaryotes (eIF5B) and archaea (aIF5B). Deletion of the *FUN12* gene, encoding eIF5B factor in yeast, is reported to hamper the growth that can be complemented by recombinant yeast eIF5B. eIF5B is shown to be necessary for Met-$tRNA_i^{Met}$ stabilization on the ribosome and ribosomal subunit joining. It also harbors ribosome-dependent GTPase activity required during dissociation of eIF5B for its recycling. Till date, among eukaryotes, full-length eIF5B protein sequences have been characterized from *Saccharomyces cerevisiae, Homo sapiens* and *Drosophila melanogaster*.

We identified, for the first time, an eIF5B-like factor in plant system and characterized it for the better understanding of the mechanism of translation initiation in plants. Immunoscreening of a cDNA expression library, prepared from seven days old young shoots of pea (*Pisum sativum*), identified a novel gene comprising of 2586 bp ORF with 381bp and 532bp 5' and 3' UTRs, respectively. Sequence analysis of this gene, termed as *PeIF5B*, revealed striking homology to eukaryotic translation initiation factor eIF5B - a sequence homologue of prokaryotic translation initiation factor IF2. Northern blot hybridization revealed the presence of a single transcript of *PeIF5B*. *In vitro* translation

using rabbit reticulocyte lysate system yielded a protein corresponding to 116kDa which was higher than the calculated value of 96kDa – a reflection of the highly charged N-terminal end. Phylogenetic analysis of PeIF5B placed it closer to eIF5B from yeast, human and *Drosophila*. Pfam domain search analysis pointed to its likely role as a translation initiation factor.

The gene coding for pea eIF5B was expressed and purified in *E.coli* and detailed study was employed to get an insight into the conformational changes following ligand-binding to this translation initiation factor. Fluorescence spectroscopy and circular dichroism were employed to study the structural details of this novel, lately reported translation initiation factor eIF5B from *Pisum sativum.* It was shown that Mg^{2+} binding leads to minimal changes to the protein tertiary structure whereas on GTP binding, the protein undergoes rapid and drastic conformational changes. Also it was observed that the metal ion provides some stability to the protein against GTP-induced structural changes. Initiator tRNA binding to PeIF5B was also studied. Intrinsic fluorescence and far-UV CD studies showed that PeIF5B is extremely stable towards temperature denaturation.

Further functional studies were performed on PeIF5B. It was observed that there was comprehensive interaction between charged initiator tRNA and this factor. Also in the presence of divalent ions like Mg^{2+} and GDP/GTP, PeIF5B protein showed a trend similar to what is expected out of a prokaryotic translation initiation factor. UV crosslinking experiments showed that this novel factor from *Pisum sativum* binds to GTP as is predicted by *in silico* study and also performs metal ion-dependent GTPase activity. Polyclonal antibodies were raised against the purified recombinant protein in rabbit and immuno-precipitation performed with ribosomal fraction. As expected PeIF5B co-immuno-precipitated with ribosomes. Adding to the novelity of this transcription factor, it showed phosphorylation by protein kinases- PKC and PKA. This study is about a novel player of the translation machinery from plant system.

Project 2:

Deciphering the cross talk between host and viral factors in driving transcription from the baculoviral very late polyhedrin gene promoter.
[Additional work done during Ph.D. tenure at the Centre for DNA Fingerprinting and Diagnostics, Hyderabad, India]

Autographa californica nuclear polyhedrosis virus (*Ac*NPV) is a well known vector for expression of foreign genes driven by the viral polyhedrin (polh) gene promoter in *Spodoptera frugiperda* (*Sf*9) insect cells. The virus uses a combination of host and viral factors for polh transcription. We have been investigating the DNA elements and transcription factors responsible for the expression from the *Ac*NPV polyhedrin promoter. We earlier documented the involvement of novel Sp family-like protein factors. Further, gel retardation assays pointed to a possible crosstalk between *Sf*9 Sp-like factors and TFIID complex that may result in the recruitment of the virus-encoded RNA polymerase to this TATA-less promoter more efficiently. In complementary experiments, specific replacements of the *Sf*9 Sp-like factor binding motifs (AcSp) with TATA-like elements resulted in expression of luciferase reporter gene to almost the same level as wild type native construct. Our study points to a possibility of the involvement of TFIID and *Sf*9 Sp protein interaction in transcription from the baculovirus polyhedrin promoter.

The viral RNA polymerase reportedly consists of four subunits: LEF4, LEF8, LEF9 and P47. LEF8 and LEF9 are hypothesized to constitute the catalytic core of RNA polymerase since they possess amino acid sequence motifs homologous to other polymerases. The role of P47 protein is still unknown. LEF4 is shown to be part of the 5' capping apparatus of the enzyme. To study the interaction between the subunits and their interaction with rest of the transcription machinery and the promoter, we cloned and purified recombinant LEF4, LEF9 and P47 to homogeneity. We found through GST-pull-down method that LEF4 interacts with LEF9 and also P47 interacts with LEF9. These interactions involve different domains since there was no competition. Gel retardation assay showed that LEF4 subunit of viral RNA polymerase shows binding to the polh promoter. Fine dissection of the polh promoter shows that it is the A region within polh promoter that showed maximum binding to LEF4.

Project 3:

Biophysical characterization involving ligand-binding and stability studies of recombinant late expression factor 4 subunit of *Autographa californica* nuclear polyhedrosis virus RNA polymerase.
[Additional work done during Ph.D. tenure at the Centre for DNA Fingerprinting and Diagnostics, Hyderabad, India]

Late expression factor 4 (*lef4*) encodes one of the four subunits of *Ac*NPV RNA polymerase that drives transcription from the late and very late viral gene promoters. Structural characterization, guanidine hydrochloride (GnHCl)- and urea- induced unfolding of this subunit of baculovirus-encoded RNA polymerase was investigated using intrinsic fluorescence, hydrophobic dye binding, fluorescence quenching and circular dichroism (CD) techniques. Circular dichroism spectral analysis revealed that LEF4 polypeptide folds into α-helices and β-sheets with major portion as random coils. An intermediate is detected at around 2M GnHCl and around 4M urea during unfolding. Steady-state fluorescence and far-UV CD showed that while most of the tertiary structure is lost, the secondary structure of LEF4 subunit of baculovirus RNA polymerase remains intact at 2M GnHCl and a wide range of urea concentration. Having shown that LEF4 protein at 2M GnHCl and 4M urea has native-like secondary structure, these two intermediate states were, however, found to be functionally inactive. Data from acrylamide quenching showed that the two intermediate states at 2M GnHCl and 4M urea do share some common structural features and hence may lie on the same pathway of protein folding. LEF4 also resists temperature-induced unfolding. The robust nature of this protein might reflect one of the many tools adapted by the virus to survive in the very adverse conditions.

Late expression factor 4 also harbors 5’ mRNA capping activity that includes RNA 5’-triphosphatase, nucleoside triphosphatase and guanylyltransferase activities. Hydrolysis of 5’ triphosphate RNA and free NTPs is metal ion dependent property of the protein. We described the structural changes in the conformation of LEF4 protein following ligand binding. It is found that GTP and divalent cation cofactor produce some prominent changes in the secondary and tertiary structures of the protein.

Project 4:

Folding pathway studies and biochemical characterization of stem bromelain, a thiol protease, from *Ananas comosus.*
[M.Sc final year project, in partial fulfillment of the degree of Master of Science]

Bromelain, a thiol protease, was used in our study as a model to investigate the role of glycosylation of proteins. Various studies had been made in the past regarding the influence of covalently linked carbohydrate chains on the stability of both small single-domain and large oligomeric glycoproteins and controversial results were obtained. Experiments carried out with the external glycosylated yeast invertase and its internal non-glycosylated counterpart have led to varied conclusions; some stating the role of carbohydrate moiety in providing stability and potential to reactivate after folding while others disproving their involvement. Bromelain is a glycoprotein with a single, N-glycosylated polypeptide chain. In the following study, periodate oxidation was used to obtain the deglycosylated form of the enzyme. The stability of the two forms of enzyme was compared in the presence of guanidine hydrochloride (Gn.HCl), temperature, pH using various probes such as loss of enzymatic activity and by the changes in fluorescence and CD spectra. The glycosylated stem bromelain was found to be stabilized by 1.9 kcal/mole as compared to the deglycosylated one. Fraction denatured of native protein, at a given concentration of denaturant, was higher in case of deglycosylated as compared to glycosylated stem bromelain. It indicates the contribution of the carbohydrate part of the glycoprotein to the stability and function of the enzyme bromelain. In addition to this, a comparative study of their activities in different organic solvents showed a marked decrease in the case of deglycosylated form of the enzyme.

We had performed equilibrium studies on the acid-induced denaturation of stem bromelain (EC 3.4.22.32) using circular dichroism(CD) spectroscopy, fluorescence emission spectroscopy and binding of the hydrophobic dye, 1- anilino 8-naphthalene sulfonic acid (ANS). At pH 2.0 a partially folded intermediate was found that was characterized with lose of all its tertiary interactions but retention of about 42.2% of the native state secondary structure. Enhanced binding of ANS was observed in this state as compared to the native folded state at neutral pH or completely unfolded state in the presence of 6M Gn.HCl. Acrylamide quenching of the intrinsic tryptophan residues in the protein molecule showed that at pH 2.0 the protein is in an unfolded conformation with more tryptophan residues exposed to the solvent as compared to the native conformation

at neutral pH. Hence, existence of partially folded intermediate states on the folding pathway of stem bromelain was suggested.

We also studied the effect of various alcohols and salts on the partially folded state of stem bromelain at pH 2.0 as monitored by far-UV circular dichroism, intrinsic fluorescence and binding of the hydrophobic dye, ANS. We found by far-UV CD studies that alcohols induce helical structure in the partially folded intermediate state of bromelain. A corresponding increase in intrinsic fluorescence intensity as well as increase in ANS binding was also observed in stem bromelain at pH 2.0 on the addition of alcohols. Stem bromelain at pH 2.0 in the presence of various salts was also found to acquire helical structure as seen by ellipticity measurements at 222nm. Intrinsic fluorescence intensity of stem bromelain at pH 2.0 in the presence of salts was found to increase with the addition of salts. However, ANS fluorescence data showed a decrease in ANS binding on the addition of salts. Thus, both alcohols and salts led to conformational changes in the partially folded intermediate of stem bromelain at pH 2.0 where salt-induced state is different from the alcohol-induced state.