CHAPTER I

INTRODUCTION

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INTRODUCTION

Protein biosynthesis or translation of messenger RNA (mRNA) is as complex as other aspects of gene expression such as DNA replication or its transcription to the corresponding RNA. Each of these processes (replication, transcription and translation) requires specific machinery. In the process of translation, the information present in the mRNA molecule is decoded to the corresponding amino acids in proteins by a machinery consisting of ribosomes, transfer RNA, very specific enzymes and protein factors. Proteins are polymers composed of large number of nitrogen containing organic monomers called amino acids. These amino acids are linearly linked together by peptide bonds. Twenty different amino acids, arranged in various sequence combinations are able to produce thousands of different proteins in living cells. This is similar to various words in the English language produced by the 26 alphabets. Free amino acids are not however used in the synthetic machinery. For an amino acid to be incorporated into a protein, it must be coupled to an adenylic moiety of an energy rich compound called adenosine triphosphate (ATP) by a process called activation. This activated amino acid is then accepted by an RNA molecule called transfer RNA (tRNA) in the presence of amino acyl-tRNA synthetase, an enzyme that catalyses the process. Characterization of the mechanism of protein biosynthesis, the hub of cellular activity, has been one of the greatest challenges in the history of Biochemistry and it continues to amaze one by its intricacy and the well-orchestrated interplay of numerous factors.

Messenger RNA (mRNA) is monocistronic in eukaryotic cells and regulation of eukaryotic translation might seem unnecessary. Yet, it provides a very rapid way to control gene expression besides the regulation which occurs at other level (promoters, RNA splicing, RNA stability and export of mRNA from
the nucleus to the cytoplasm). Translational control is defined as a change in the efficiency of mRNA translation i.e., in the number of amino acids polymerized per unit time per mRNA molecule. This control may effect a quantitative change in the overall amounts of proteins synthesized, or a qualitative change in the species of proteins produced.

Zamecnick and his colleagues have made rapid strides in protein biosynthesis research since the development of the first cell-free system and way back in the 1950s. A great deal is now known about translation, but perhaps, only a little about what is yet to be learnt. Conventionally, translation is divided into three distinct steps namely, initiation, elongation and termination (Ochoa, 1983).

This laboratory, as well as the present thesis, deals with the regulation of eukaryotic initiation factor 2 (eIF2) activity. Hence the introduction here is focused on i) brief description about the overall protein synthesis and regulation of protein synthesis to indicate the role of initiation factor 2 (eIF2) activity. As we have used baculovirus expression system in order to over produce the small or alpha subunit of human eIF2 (eIF2α) wt and mutants to further characterize the importance of eIF2α phosphorylation in the regulation of eIF2 activity, the introduction also highlights about the current information available on the advantages of the baculovirus expression system and mutants of eIF2α.

1. AN OVERVIEW OF PROTEIN BIOSYNTHESIS
   1.1. Initiation.

   This is the most complex of all the three steps, requiring a myriad of initiation factors called as eukaryotic initiation factors (eIFs). Most of the eIFs
Initiation of Protein Synthesis in Eukaryotes
(Ref. Watson et al., 1987)
are multimeric proteins and they are designed to regulate protein synthesis in meaningful ways (Hershey, 1991 and Merrick, 1992).

The formation of 80S-initiation complex and the release of eIF2.GDP binary complex occurs at the end of initiation of protein synthesis (Fig. as shown in the opposite page) The initiator tRNA, Met-tRNA, carrying the initiator methionine amino acid residue is properly positioned on the "start site" of mRNA. This complex process requires several initiation factors (eIFs) and can be divided into six sub steps as mentioned below (reviewed in Hershey, 1991; Merrick, 1992 and Rhoads, 1993).

a) Ribosomes are dissociated into their subunits at the end of protein synthesis and the subunits 40S and 60S remain separated because of the presence of anti-association factors like eIF3 and eIF4C (now called as eIF1A) and eIF6 which are found associated with the 40S subunits.

b) Formation of 43S preinitiation complex, eIF2-GTP-Met-tRNA, 40S, is the next step. This step requires the formation of a ternary complex, eIF2.GTP.Met-tRNA, which then joins 40S subunits.

c) The preinitiation complex (eIF2.GTP Met-tRNA, 40S ribosome) joins messenger RNA (mRNA) to form 48S preinitiation complex. eIF4F, a trimeric complex, consisting of eIF4E (25 kDa, previously called eIF4a), eIF4A (22 kDa) and eIF4y (also called p220) and eIF4B proteins assist the joining of mRNA to 43S initiation complex.

d) This above step involves the recognition of 'start' site on mRNA by Met-tRNA. Prokaryotic mRNAs possess distinct structural features (Shine-Dalgarno sequence) in the mRNA for it to facilitate a distinct hydrogen
bonding interaction with 16S rRNA of ribosome preceding the AUG or 'start' codon. In contrast, eukaryotic mRNA does not carry a comparable recognition sequence. The 43S complex is carrying eIF2 and Met-tRNA\_i scans the mRNA to identify the 'start' AUG codon. A purine nucleotide (A or G) in the codon preceding start AUG and G residue on the 3' site of AUG (A or GXXAUGG) are required in some mRNAs for recognition of 'start' AUG codon by the 43S preinitiation complex. Replacement of the purine residue in the 5' or 3' end of the start AUG reduces the translation. Since many mRNAs lack such purine residues before the start AUG, the mechanism by which the 'start codon' in mRNA is recognised is still not clear. However it has been observed that ATP hydrolysis is required at this step, which can facilitate the unwinding of any secondary structure present in the mRNA preceding the start site, so that the 48S complex can easily scan the 5' untranslated region and reach the start site (reviewed in Kozak 1992). 43S complex scans the mRNA and then positions itself on the 'start' codon (AUG) of the mRNA to form a 48S complex. It is envisaged that several of the eIFs including a cap binding protein are assembled in an ATP dependent process onto the mRNA to provide a conducive environment for the binding of 43S complex to the AUG start codon present on the mRNA. There are indications that eIF2 can influence the selection of initiation codons in artificially constructed mRNAs (Dasso et al., 1990).

e) Joining of 60S ribosomal subunit to 48S preinitiation complex facilitates the formation of 80S-initiation complex. This step is accompanied by the hydrolysis of GTP bound to eIF2. The enzyme GTPase, associated with eIF5 protein, catalyses the GTP hydrolysis. The resultant binary complex (eIF2. GDP) is somehow translocated to the 60S-subunit of 80S-initiation complex of polysomes (Ramaiah et al., 1992), where, it appears to interact with the guanine nucleotide exchange protein, eIF2B (Thomas et al., 1985) and also
becomes a target for eIF2a kinase (Ramaiah et al., 1992; Pavitt et al., 1998 and Mueller et al., 1998).

f) Recycling of eIF2. GDP binary complex requires a rate-limiting pentameric protein factor called eIF2B. This is because, eIF2 has a higher affinity for GDP than for GTP in the presence of physiological Mg\(^{2+}\) concentration and GDP inhibits the joining of eIF2 to Met-tRNA. Hence it is important that GDP in eIF2.GDP binary complex must be replaced by GTP. This guanine nucleotide exchange is catalysed by the largest subunit (\(\epsilon\)-subunit in mammalian systems or GCD6 in yeast) of the pentameric eIF2B protein (Fabian et al., 1997 and Pavitt et al., 1998).

1.2. Elongation.

Elongation of protein synthesis involves a cyclic process in which one amino acid residue is added to the C-terminal end of the nascent polypeptide chain per turn of the cycle (reviewed by Hershey, 1991 and Merrick, 1992). Four elongation factors (eEF's) have been characterised and each factor is known to catalyse a step in the elongation process. This process of elongation can be divided into four sub-steps, as mentioned below.

a) eEF-1\(\alpha\) catalyses the binding of the aminoacylated tRNA to the 'A' site of the ribosome, b) The ejection of eEF-1\(\alpha\) from ribosome's is accompanied by the GTP hydrolysis. The eEF-1\(\alpha\).GDP thus released is recycled by the eEF-1\(\gamma\). eEF-1\(\alpha\) is comparable to EF-Tu of prokaryotes and the eEF-1\(\beta\)/\(\gamma\), which are involved in the exchange of GTP for GDP on eEF-1\(\alpha\).GDP are comparable to EF-Ts of prokaryotes and to the initiation factor eIF2B of eukaryotes. c) The peptide transferase centre, presumably located on the 60S ribosomal subunit, catalyzes formation of peptide bond between the nascent
polypeptide and the incoming aminoacid. d) The elongation factor-2 (EF-2) hydrolyzes GTP and catalyzes the translocation of aminoacyl tRNA from the 'A' site to 'P' site on the ribosome with a concomitant movement of the message (Watson et al., 1987).

1.3. Termination.

Termination of newly made polypeptide chain occurs when the 80S complex reaches the termination codon, like UAA, UAG or UGA. Termination of the nascent polypeptide chain is aided by the releasing factor (RF). In prokaryotes, termination of protein synthesis requires the assistance of three releasing factors where as in eukaryotes, it has been observed that a single RF recognises all three-termination codons. Recognition of the termination codon by RF requires GTP. The binding of the termination factor(s) to the termination codon and to the ribosomal factor-binding site induces hydrolase activity. This results in the release of nascent polypeptide from the ribosome (Spirin, 1986).

2. REGULATION OF INITIATION OF PROTEIN SYNTHESIS

Regulation of translation can occur at various stages of protein synthesis. In such a complex sequence of reaction it is natural for the cells to exert control at the first step of initiation of protein synthesis. Two distinct types of translational controls occur. i) General control of overall protein synthesis affecting the bulk of mRNAs of the cell and ii) selective regulation of specific mRNAs or a sub set of mRNAs. The latter may arise due to structural features of specific mRNAs, especially, secondary structures, or, by mRNA binding proteins. These transacting factors may either prevent or facilitate the initiation factor binding to specific mRNAs. Regulation of protein
synthesis by phosphorylation of initiation and elongation factors and also by phosphorylation of ribosomal protein S6 has been well-documented (Hershey, 1991; Merrick, 1992; Proud, 1992 and Jefferies and Thomas, 1996).

In the initiation step of protein synthesis, phosphorylation of eIF2 and eIF4E proteins play a major role in the regulation. There is a strong correlation to enhanced eIF4E phosphorylation, that occurs, in response to growth factors, mitogens, and cytokines, to increased protein synthesis (Morley and Traugh, 1991; Kasper et al., 1990; Fredrickson et al., 1992 and Donaldson et al., 1991). eIF4E is hypo-phosphorylated during mitosis (Boneau and Sonenberg, 1987), following heat shock (Duncan et al., 1989) or infection with several viruses (Huang and Schneider, 1991; Feigenblum and Schneider, 1993) concomitant with a reduction in the translational rates. Interestingly, these conditions which reduce eIF4E phosphorylation enhance eIF2\textalpha phosphorylation. However, the connection (if any) between the phosphorylation states of these two proteins is not yet understood. It should be highlighted here that protein synthesis is one of the most complex biochemical process requiring approximately 150 different polypeptides. Seven of these polypeptides (eIF2\textalpha, eIF2\textbeta, eIF4E, eIF4G, S6, eEF1, and eEF2, have been identified as targets for regulatory pathways to date. Modification of some of these factors affects the overall rate of translation whereas modification of others affects the spectrum of mRNAs translated (Rhoads, 1999). Since the present work deals with the regulation of eIF2 activity and its interaction with eIF2B protein, the introduction here is mainly focused on the current information available on this subject.

2.1. eIF2 subunit composition, function and regulation.
Translation initiation factor-2 is a key protein involved in the initiation step of protein synthesis. It plays a central role in the translation initiation forming a ternary complex with GTP and Met- tRNAi which then joins 40S ribosomes to form 43S initiation complex. Following the joining of messenger RNA, the GTP in the ternary complex is hydrolyzed and inactive eIF2.GDP binary complex is released at the end of the initiation step in protein synthesis (reviewed in Hershey, 1991 and Rhoads, 1993).

eIF2 complex protein has been isolated from mouse fibroblasts (Levin et al., 1973), human rabbit and chicken reticulocytes (Jakubowicz et al., 1980; Chen et al., 1972; Safer et al., 1975; Benne et al., 1976; Andrews et al., 1985 and Lee, 1984), yeast (Baan et al., 1976 and Ahmad et al., 1985); Krebs ascites cells (Ranu and Wool, 1976 and Trachsel et al., 1979); pig, bovine, calf liver, rat liver and brain (Suzuki et al., 1985; Feldhoff et al., 1993, Stringer et al., 1979; Kimball et al., 1987 and Cales et al., 1985), sea urchin (Dholakia et al., 1990), frog oocytes (Carvallo et al., 1988), Ehrlich ascites tumor cells (Rowlands et al., 1988a), and embryos of wheat, Artemia and Drosophila (Treadwell et al., 1975; Spermulli et al., 1977, Benne et al., 1980., Shaikhin et al., 1992; Janaki et al., 1995; Mehta et al., 1983, 1986 and Mateu et al., 1987 and 1989).

eIF2 is a heterotrimer composed of three subunits α-(38 kDa/315 aa), β-(50 kDa/333aa) and γ-(52 kDa/472aa). All the three subunits of eIF2 from human (Ernst et al., 1987; Pathak et al., 1988 and Gaspar et al., 1994) and yeast (Cigan et al., 1989; Donahue et al., 1988 and Hannig et al., 1993) and also some of the individual subunits such as the α-subunit from Drosophila (Qu and Cavener, 1994) and bovine (Green et al., 1991) systems and the β-subunit from wheat germ (Metz and Browning, 1997) and Drosophila (Ye and Cavener, 1994) were cloned and sequenced. Sequencing information of eIF2
reveals that the subunit sequence among different organisms is well conserved to a great extent.

In the presence of physiological concentrations of $\text{Mg}^{2+}$, the protein has a 400-fold higher affinity for GDP than for GTP and forms a stable $\text{elF2.GDP}$ binary complex \textit{in vitro} (Panniers and Henshaw, 1983 and Panniers et al., 1988). The joining reaction of $\text{elF2}$ with the initiator Met-tRNA$_i$ requires GTP and is inhibited by GDP (Walton and Gill, 1976). Later studies have isolated an heteropentameric initiation factor with guanine nucleotide exchange activity from a variety of mammalian systems and was variously designated as GEF (Panniers and Henshaw, 1983), anti-HRI (Amesz et al., 1979), SP (Siekierka et al., 1981), RF or reversing factor (Siekierka et al, 1981; Matts et al., 1983 and Grace et al., 1982) and or $\text{elF2B}$ (Konieczny and Safer, 1983). It is now referred as $\text{elF2B}$. The guanine nucleotide exchange activity of $\text{elF2B}$ is estimated from the rate of displacement of labeled $[^3\text{H}]$ or $[^32\text{P}]$ GDP from the preformed $\text{elF2.GDP}$ binary complex on incubation with labeled GDP or GTP. The $\text{elF2}$ complex with guanine nucleotides bind to the nitrocellulose filters and is stable in the presence of $\text{Mg}^{2+}$. In the presence of active $\text{elF2B}$, labeled GDP is exchanged for unlabelled GDP or GTP present in the reaction mixture. Several mechanisms have been proposed to explain the ability of $\text{elF2B}$ to dissociate GDP bound $\text{elF2}$ (Reviewed in Pain, 1983 and Manchester, 1987). Two of the models were studied in detail which unfortunately do not agree with each other. One group (Rowlands et al., 1988) proposed an enzyme displacement or substituted mechanism using the nitrocellulose filter binding assay and $\text{elF2B/elF2}$ complex as the source of $\text{elF2B}$. This complex $\text{elF2B}$ is more stable than free $\text{elF2B}$. According to this mechanism, $\text{elF2.GDP}$ joins the enzyme, $\text{elF2B}$. This is followed by the dissociation of GDP, binding of GTP and then the release of the enzyme from $\text{elF2.GTP}$. The reaction thus proceeds by way of $\text{elF2.GDP}$ or $\text{elF2.GTP}$.
binary complexes with an enzyme (elF2B) intermediate which, free from either substrate, carries the group to be transferred. Another group (Dholakia et al., 1989) used elF2B free from elF2 and studied the displacement of labeled GDP and fluorescent GDP derivatives from elF2 by elF2B and observed that there was no displacement of labeled GDP unless GTP was present. Hence, they proposed a sequential mechanism which involves a (GTP).elF2B.(elF2.GDP) complex. Consistent with their hypothesis, it was observed that the 40 kDa subunit of elF2B could bind GTP (Dholakia et al., 1989b).

There is a general agreement that all eIF2 preparations have a higher affinity for GDP than for GTP in the presence of physiological concentrations of Mg\(^{2+}\) and the guanine nucleotide exchange on eIF2 requires eIF2B like protein except in the dormant embryos of wheat, Drosophila and Artemia. The eIF2.GDP binary complexes prepared from these embryos could exchange bound GDP readily with free GTP or GDP present in the reaction mixtures in the absence of any factor like eIF2B when the reactions were carried out at 30 °C (Shaikin et al., 1992; Mehta et al., 1983; Janaki et al., 1995 and Krishna et al., 1997). In contrast, at low (10 °C) temperatures, it was shown that the GDP/GTP exchange on Artemia eIF2 can occur in the presence of mammalian eIF2B protein (Mateu and Sierra, 1987 and Mateu et al., 89). However, no eIF2B-like protein is yet identified or purified from these embryos.

Both gamma and beta subunits can be cross linked to guanine nucleotides and to Met-tRNA\textsubscript{i} (Bommer et al., 1991 and reviewed in Merrick, 1992). The current understanding however is that the N-terminus region of the y-subunit of eIF2, rather than the \(\beta\)-subunit, binds GDP based on the fact that the y-subunit has all the three consensus guanine nucleotide binding domains in
close proximity to each other and mutations in the guanine nucleotide binding domains in human and yeast protein (Naranda et al., 1995 and Erickson et al., 1997) decrease significantly its ability to bind GDP.

The beta subunit has two features which appear to be important in its interaction with nucleic acids and also with other initiation factors such as elf5 which hydrolyzes elf2.GTP to elf2.GDP and or elf2B, the guanine nucleotide exchange factor. These are, a) three runs of seven lysine residues in the amino-terminal half of the subunit which are conserved in yeast, human and Drosophila (Donahue et al., 1988; Pathak et al., 1988 and Ye and Cavener, 1994) and b) a C2-C2 motif reminiscent of a potential zinc finger structure. However there are no reports that zinc is found on purified elf2 or required for the elf2 activity. Mutational analysis of yeast elf2δ suggests that the lysine repeats and the C2-C2 motif present in the amino and carboxy-terminal regions of the protein are important for interaction with mRNA. (Laurino et al., 1999). The γ-subunit of yeast elf2 contains homologous sequences that is found in the elongation factor Tu (EF.Tu) of eubacteria. These sequences have been found important for this factor to bind tRNA (Hannig et al., 1993). Hence it is suggested that it may be involved in initiator tRNA binding. Also, elf2 containing a suppressor p-subunit binds the Met-tRNAi with lower affinity than the wild type elf2 complex, thereby suggesting that elf2i may be involved in interacting with initiator tRNA (Donahue et al., 1988). Recent information suggests that the binding domain for mammalian and also for yeast elf5 resides in the N-terminal half of elf2p and includes the second of the three lysine boxes or all of them respectively (Das et al., 1997 and Asano et al., 1999). In addition, rat liver and yeast elf2[i-subunit are shown to interact with the 5- and e-subunits of their respective elf2B proteins (Kimball et al., 1998 and Asano et al, 1999). The interaction requires the N-terminus lysine boxes in the case
of yeast elF2β whereas, the binding site on rat liver elF2β has been shown located within approximately 70 amino acids from the C-terminus. In yeast, through mutational analysis, it is suggested that the lysine boxes in elF2β are required for a tight binding of elF2B with its substrate elF2 and the interaction is also dependent on the bipartite motifs rich in aromatic and acidic residues which are conserved at the C-termini of the catalytic subunit of elF2B (elF2Bc) (Asano et al., 1999). Based on the unpublished observations that archea lack all the five subunits of elF2B and archeal elF2β is devoid of the lysine boxes (Asano et al., 1999), Hinnebusch's laboratory suggested that during evolution, elF5 and elF2B acquired domains containing the bipartite motifs, whereas, their common substrate elF2 acquired polylysine residues.

2.2. Phosphorylation of elF2 and physiological significance.

Both the α and β-subunits of elF2 are accessible for phosphorylation by several kinases in vivo and vitro. Phosphorylation of the ser^7 and ser^67 in the β-subunit occurs by CK-II, where as, ser^13 and ser^218 have been shown to undergo phosphorylation in the presence of PKC and PKA (Clark et al., 1989 and Welsh et al., 1994). It is not clear if this phosphorylation has any regulatory significance. However, Singh et al (1994) has reported that dephosphorylation of the (i-subunit stimulates the ability of elF2 to bind GDP. In addition it was shown that phosphorylation of C-terminal portion of this subunit by protein kinase A, increased the guanine nucleotide exchange activity of elF2B, whereas, the phosphorylation in the N-terminal region by CK-II did not cause a similar effect (Kimball et al., 1998). These studies are consistent with the idea that p-subunit of elF2 interacts with elF2B and suggest that probably phosphorylation of this subunit in the C-terminus may also alter this interprotein interaction. The elF2 protein is partly ribosome
bound. It is isolated from both ribosome and non-ribosome fractions of cytoplasmic extracts.

One of the most important ways through which the recycling of eIF2 and regulation of protein synthesis occurs is through phosphorylation of the small or alpha subunit in eIF2 (eIF2a) (reviewed in Jackson, 1991; Webb and Proud, 1997 and Clemens 1996). Phosphorylation of eIF2a is now clearly recognised as a major mechanism in the regulation of initiation step of eukaryotic protein synthesis.

Phosphorylation of the α-subunit in eIF2 occurs in cells or in cell-free translational systems in response to a variety of stimuli such as heme-deficiency (Levin et al., 1976; Kramer et al., 1976; Farrell et al., 1977 and Surolia et al., 1991), viral infection or low levels of double-stranded RNA (Farrel et al., 1997 and Levin et al., 1978), amino acid and nutrient starvation (reviewed in Pain, 1994; Dever et al., 1992; Scorsone et al., 1987; Clemens et al., 1987 and Alcazar et al., 1995), purine limitation (Rolfes et al., 1993), serum and growth factor deprivation (Duncan et al., 1985; Montine et al., 1987 and Ito et al., 1994), transient transfection of certain plasmids (Kaufman et al., 1989), during cerebral ischemia (Burda et al., 1998) exercise (Menon et al., 1995), heat shock (Duncan et al., 1984 and De Benedetti et al., 1986), heavy metals (Hurst et al., 1987; Matts et al., 1991 and Alirezaei et al., 1999), release of calcium from the endoplasmic reticulum (ER) or ER-stress (Prostko et al., 1992; Prostko et al., 1993; Prostko et al., 1995; Aktas et al., 1998 and Laitusis et al., 1999), oxidising agents such as oxidised glutathione (Ernst et al., 1979 and Kan et al., 1988), pyrroloquinoline quinone (Ramaiah et al., 1997), sodium arsenite (Laitusis et al, 1999), denatured proteins (Matts et al., 1993) and nitric oxide (Kim et al., 1998) etc.
Phosphorylation is correlated with a global inhibition of protein synthesis in cell-free translational systems obtained from reticulocyte lysates, or, selective stimulation of certain mRNAs over the others as in the case of yeast subjected to amino acid starvation (reviewed in London et al., 1987; Jackson, 1991 and Hinnebusch, 1993). Increased eIF2a phosphorylation is correlated to enhanced synthesis of GCN4 in yeast. GCN4 is a transcriptional factor and stimulates the synthesis of several mRNAs that encode proteins which are required in the various amino acid biosynthetic pathways. Recent studies suggest that eIF2a phosphorylation plays an important role in growth and development and in apoptosis (Donze et al., 1995; Qu et al., 1997; Der et al., 1997; Srivastava et al., 1998 and Alcazar et al., 1995). Abrogation of eIF2 phosphorylation by expressing non-phosphorylatable mutant eIF2a or a mutant eIF2a kinase like PKR can lead to malignancy (Donze et al., 1995; Koromilas et al., 1992). Also, a recent study reported that eIF2α was cleaved in apoptotic Saos-2 cells on treatment with poly(I),poly(C) or tumor necrosis factor α and in the presence of caspase-3 in vitro. By site directed mutagenesis, the cleavage site was mapped to an Ala-Glu-Val-Asp^{300}↓Gly^{301} sequence located in the C-terminal protein of eIF2α. (Satoh et al., 1999)

Recent studies demonstrated the presence of eIF2 in the nucleus, interaction of eIF2 with DNA-dependent protein kinase (DNA-PK) and the phosphorylation of the β-subunit of eIF2 by DNA-PK (Ting et al., 1998). In addition, eIF2α and eIF4E expression is increased in response to growth induction by c-myc (Rosenwald et al., 1993). These findings suggest that probably the role of eIF2 is not limited to translational initiation but it may as well be associated with DNA repair, apoptosis and in malignancy.

2.3. eIF2α kinases, their activation and regulation.
As of date, at least half a dozen $\text{eIF2}_\alpha$ kinases have been characterised which are all known to phosphorylate serine 51 residue in the $\text{eIF2a}$ subunit. However their regulation is different. For example, heme-deficiency activates heme-regulated kinase, HRI. Added hemin inhibits the activation of this kinase (reviewed in Chen and London, 1995). Similarly an $\text{eIF2}_\alpha$ kinase found in malarial parasite, called PfPK4, is regulated by hemin (Mohrle et al., 1997). In contrast, double stranded RNA activates another $\text{eIF2}_\alpha$ kinase called PKR (Meurs et al., 1990 and reviewed in Clemens and Ellia 1997). Low concentrations of dsRNA stimulates the kinase activity and high concentrations inhibit it (Hunter et al., 1975). In Sachharomyces cerevisiae, Drosophila melanogaster and in Neurospora crassa, amino acid starvation leads to the activation of yet another $\text{eIF2}_\alpha$ kinase called GCN2 (Dever et al., 1992; reviewed in Wek, 1994 and Santoyo et al., 1997). A mammalian homologue of GCN2 has also been found and characterised recently (Sood et al., 2000a and Berlanga et al., 1999). In addition, two more $\text{eIF2}_\alpha$ kinases have been recently characterised which are different from HRI, PKR and GCN2. These are PEK, a pancreatic kinase, PERK, an endoplasmic reticulum resident kinase (Shi. et al., 1998 and Harding et al., 1999). Both of them are activated in response to endoplasmic stress and the mammalian homologue of PEK is also found Drosophila and Caenorhabditis elegans (Sood et al., 2000b). It is not clear if there are any other $\text{eIF2}_\alpha$ kinases that are responsible for the enhanced $\text{eIF2}_\alpha$ phosphorylation that is observed in other physiological conditions such as heat shock and heavy metal stress.

From a variety of studies such as amino acid sequencing (Colthrust et al., 1987), site specific mutagenesis (Pathak et al., 1988), genetic studies (Vazquez de Aldana et al., 1993) and through the over expression of mutants of $\text{eIF2}(x$ (Kaufman et al., 1989; Choi et al., 1992 and Murtha-Riel et al., 1993) which can overcome the inhibitory effects caused by endogenous
elF2α phosphorylation, it is well established now that serine 51 residue is the only phosphorylation site in mammalian elF2α. However, in some systems like in yeast, the elF2α contains three more additional sites for phosphorylation in the highly acidic C-terminal region which can be phosphorylated by CK-II (van den Heuvel et al., 1995). Similarly elF2α, the p41-42 doublet, in wheat germ is accessible for phosphorylation both by reticulocyte elF2α kinases and CK-II as has been reported by us earlier (Janaki et al., 1995). In addition, the elF2α from sea urchin, Brine shrimp and Artemia embryos is phosphorylated by CK-II suggesting the presence of a second site of phosphorylation in the alpha-subunit (Dholakia et al., 1990 and Mehta et al., 1986). The physiological significance of these additional phosphorylation sites in elF2α is however not clear. Purified mammalian elF2 alpha kinases from one source can phosphorylate elF2 from other sources as well (Dever et al., 1993 and Janaki et al., 1995).

2.4. elF2B and its regulation.

Phosphorylation of a small portion of elF2α subunit (20-30%) in the trimeric elF2 decreases protein synthesis globally in reticulocytes and their lysates during heme-deficiency and double-stranded viral infection or selectively in yeast during amino acid deprivation (reviewed in London et al., 1987; Jackson, 1991 and Hinnebusch, 1994). Since phosphorylation of only a portion of elF2α suffices to inhibit protein synthesis completely (Leroux and London, 1982), it is suggested that there must be yet another rate-limiting initiation factor which must be regulating elF2 activity. Subsequent studies lead to the identification of elF2B, a rate-limiting factor that regulates elF2 recycling when the α-subunit in elF2 is phosphorylated. This is because i) addition of purified elF2B restores the inhibition of protein synthesis caused by elF2α phosphorylation in cell-free translational systems (Amesz et al,
1979; Grace et al., 1982 and Matts et al., 1983) and ii) phosphorylation of eIF2a is associated with the inhibition in the guanine nucleotide exchange (GNE) activity of eIF2B in vitro (Clemens et al., 1982 and Matts et al., 1983) and in translating extracts (Matts et al., 1984 and Rowlands et al., 1988). Later studies have shown that eIF2B activity, measured in small amounts of translating lysates, decreases specifically whenever eIF2a is phosphorylated but is not affected by general inhibition in protein synthesis caused by inhibitors such as cycloheximide, puromycin and pactamycin that do not affect eIF2 phosphorylation (Babu and Ramaiah, 1996). In reticulocyte lysates, eIF2B pool is estimated to be 1.5% on a molar basis of the eIF2 pool. Hence low levels (20-25%) of eIF2α phosphorylation in reticulocyte lysates completely inhibit the eIF2B activity and leads to the shut down in protein synthesis. In contrast, a lower ratio of eIF2 to 2B, as has been observed in Ehrlich ascites cells, requires a higher level of eIF2α phosphorylation (Rowlands et al., 1988).

Several investigations were carried out to understand the mechanism by which phosphorylated eIF2α inhibits the GNE activity of eIF2B. It was shown that phosphorylation of eIF2α sequesters eIF2B activity into a 15S complex, eIF2α(P).eIF2B, in which eIF2B becomes inactive (Thomas et al., 1985). Rowlands et al (1988b) explained the apparent stoichiometric sequestration of eIF2B by eIF2α(P) as has been demonstrated by Thomas et al., is due to the differences in affinities of eIF2B for eIF2α(P).GDP and eIF2.GDP. This is because eIF2α(P).GDP is not a substrate but is a competitive inhibitor of eIF2B competing with eIF2.GDP for binding to eIF2B and that eIF2B has higher affinity for the inhibitor eIF2α(P) than for the substrate, eIF2.GDP (Goss et al., 1984 and Rowlands et al., 1988). The difference in the dissociation constants, $K_D$, of the eIF2B.eIF2 and eIF2B.eIF2α(P) estimated by fluorescence anisotropy was only two fold in
the presence of GDP (Goss et al., 1984) rather than the 150-fold estimated by kinetic measurements (Rowlands et al., 1988b). Recent studies using polyhistidine tagged yeast eIF2, it has been further demonstrated that binding of all the five subunits of over expressed yeast eIF2B to eIF2a(P) was two fold higher than to unphosphorylated eIF2 and ten fold higher than the background level of binding observed in the presence of eIF2 (Pavitt et al., 1998). In mammalian systems, it has been hypothesized that serine 48 residue is required for the interaction to occur between phosphorylated eIF2a(P) and eIF2B (Ramaiah et al., 1994) and is consistent with the observations that over expression of this 48A mutant eIF2a (in which 48 serine residue is replaced by alanine and the mutant can still be phosphorylated on its 51 serine residue) escapes the inhibition of protein synthesis and eIF2B activity caused by phosphorylation of endogenous eIF2α (Choi et al., 1992; Murtha-Riel et al., 1993 and Ramaiah et al., 1994).

In related studies, point mutations in the α, β- and δ-subunits of the enzyme eIF2B resulted in a phenotype in yeast that is insensitive to eIF2α phosphorylation (Vazquez de Aldana and Hinnebusch, 1994). These above studies suggest that probably eIF2α subunit may be involved in binding one or more of the above subunits of eIF2B. However, results of a recent study (Kimball et al., 1998a) indicate that eIF2B is shown to bind only to the p-subunit of eIF2 by far-western analysis. Since phosphorylation of the α-subunit in the trimeric eIF2 complex increases its interaction with eIF2B, it is suggested that phosphorylation of eIF2α results in a conformational change in the eIF2 holoprotein that alters the affinity of eIF2B for eIF2α (Kimball et al., 1998a).

Unlike other guanine-nucleotide exchange factors which are generally monomers, eIF2B involved in the initiation of protein synthesis is a
heteropentameric protein consisting of \(\alpha\), \(\beta\), \(\gamma\), \(\delta\), and \(\varepsilon\) and the yeast equivalents are designated as GCN3, GCD7, GCD1, GCD2, and GCD6. In order to understand the subunit structure, function and interaction with other subunits of the protein and also with other proteins, the yeast and mammalian elF2B subunits are cloned (Hannig and Hinnebusch, 1988; Price et al., 1994 & 96a and b; Asuru et al., 1996; Flowers et al., 1995 and 1996) and the coding regions of these subunits in both systems show considerable homology (Bushman et al., 1993; Price et al., 1996a & b and Pavitt et al., 1997). Interestingly, \(\alpha\) (GCN3), \(\beta\) (GCD7), and the carboxy-terminal half of \(\delta\) (GCD2) subunits share sequence similarity (Paddon et al., 1989 and Bushman et al., 1993a), form a stable complex \(\textit{in vivo}\) and the over expressed subunits can partially suppress the toxic effects of elF2\(\alpha\)(P) in yeast cells (Yang and Hinnebusch, 1996). This suggests that the homologous regions may be devoted to this regulatory function i.e in recognizing the phosphorylation status of elF2\(\alpha\). Also, the \(\gamma\), and \(\varepsilon\)-subunits share sequence similarity and can form a complex together (Pavitt et al., 1998). This second subcomplex however is unable to overcome the growth inhibitory effects caused by elF2\(\alpha\) phosphorylation in yeast cells (Yang and Hinnebusch, 1996). Further, the first subcomplex, that is formed by \(\alpha\), \(\beta\) and \(\delta\) subunits, does not carry any guanine nucleotide exchange activity and it binds elF2\(\alpha\)(P) at least 10-fold above background and three fold higher than elF2 as has been demonstrated (Pavitt et al., 1998) in yeast in a pull-down assay with extracts over expressing this subcomplex and purified his-tagged elF2. In contrast, the second sub complex formed by the \(\gamma\)- and \(\varepsilon\)-subunits has higher GNE activity than wild type 5-subunit elF2B of this subcomplex (\(\gamma\) and \(\varepsilon\)-subunits). The GNE activity or its interaction with elF2 is not affected by elF2\(\alpha\) phosphorylation. The \(\textit{in vitro}\) biochemical assays suggest that the \(\iota\)-subunit of yeast and mammalian elF2B actually carries the catalytic or GNE activity (Fabian et al., 1997 and Pavitt et al., 1998) and is enhanced by the
presence of the γ-subunit of eIF2B (Pavitt et al., 1998). Further, inhibition of eIF2B activity that occurs due to eIF2α phosphorylation under amino acid starvation conditions (Pavitt et al., 1998; Dever et al., 1992) stimulates GCN4 translation (reviewed in Hinnebusch, 1997). As has been mentioned earlier, the deletion of the α-subunit of eIF2B or point mutations in this subunit inhibits the induction of GCN4 mRNA translation under amino acid starvation conditions (Hinnebusch and Fink, 1983; Hannig and Hinnebusch, 1988 and Pavitt et al., 1997). Moreover, deletion of eIF2Ba reduces the growth inhibitory effect of high level eIF2α phosphorylation induced by over expression of the human double-stranded RNA dependent eIF2a kinase (Dever et al., 1993). Consistent with these findings, biochemical studies in vitro indicate that both human and yeast eIF2B devoid of the α-subunit are found to be insensitive to eIF2α phosphorylation (Pavitt et al., 1998; Fabian et al., 1997 and Kimball et al., 1998). However, eIF2B lacking the α-subunit has showed a higher affinity binding of eIF2α(P) versus eIF2 characteristic of wild-type eIF2B (Pavitt et al., 1998 and Kimball et al., 1998). This information suggests that the mutant eIF2B devoid of the α-subunit can accept eIF2α(P).GDP as a substrate and the mutation or deletion of this subunit does not affect the GNE activity of eIF2B (Fabian et al., 1997; Pavitt et al., 1998 and Kimball et al., 1998) or simply reduce the binding affinity between eIF2B for the inhibitor eIF2α(P) versus the substrate as has been hypothesized earlier (Hinnebusch, 1994). These findings summarize that the α-subunit of eIF2B is not required for the catalytic activity but is needed for inhibition by eIF2α(P) (Pavitt et al., 1998 and Fabian et al., 1997). Interaction of the regulatory subcomplex but not the catalytic complex, with eIF2 is significantly increased when the 51 serine residue in eIF2α is phosphorylated as has been demonstrated (Pavitt et al., 1998). These observations also correlate with the ability of the regulatory complex to overcome partially the growth inhibitory effects in yeast caused by eIF2a
activity (and indirectly eIF2 activity as well) is modulated by signalling pathways (reviewed in Rhoads, 1999). eIF2B is reported to be associated with NADPH (Dholakia et al., 1986) and its activity is decreased when there is an increase in NADP/NADPH ratio (Proud et al., 1992; Dholakia et al., 1986 and Akkaraju et al., 1991). Also redox factors like pyrroloquinoline quinone (Ramaiah et al., 1997), oxidised glutathione and low concentrations of sugar phosphates (Kan et al., 1988) can alter eIF2B activity. Reducing conditions stimulate the GNE activity of eIF2B while oxidising conditions are found to inhibit its activity. In addition, higher concentrations of sugar phosphates and nucleotides like ATP and inositol phosphate are shown to modulate eIF2B activity by allosteric means (Singh and Wahba, 1995 and Kimball and Jefferson, 1995).

Previous studies have shown that expression of eIF2α mutants in which the putative phosphorylation sites, that is, the serine residues at 48 and 51 were changed to alanine, can relieve the inhibition of protein synthesis (Choi et al., 1992 and Murtha-Riel et al., 1993) and mitigate the inhibition in the GNE activity of eIF2B (Ramaiah et al., 1994) that is caused by eIF2α phosphorylation in cultured mammalian cells. While the 51A mutant is not phosphorylated, the 48A mutant is found to be substrate for phosphorylation in these studies. In contrast, expression of 51 aspartic acid mutant (51D) causes inhibition of protein synthesis. These eIF2α mutants are useful in resolving the phosphorylation sites in mammalian eIF2α (Pathak et al., 1988 and Choi et al., 1992), in localizing protein synthesis defects that occur due to eIF2α phosphorylation in such cases as heat shock and calcium sequestration (Murtha-Riel et al., 1993 and Srivastava et al., 1995), in determining the importance of eIF2α phosphorylation in growth and development and in apoptosis (Donze et al., 1995 and Srivastava et al.,
1998), and also in the expression of \(\text{eIF}2\alpha\) kinases that are inhibitory for protein synthesis (Chefalo et al., 1994).

2.5. \(\text{eIF}2\alpha\) Phosphatases.

So far, no protein phosphatase has been shown to rescue protein synthesis inhibition caused by \(\text{eIF}2\alpha\) phosphorylation, although both type 1 and 2 protein phosphatases are found to dephosphorylate \(\text{eIF}2\alpha(P)\) \textit{in vitro} (Pato et al., 1983a and b and Redpath and Proud, 1990). There was no such indication however that such preparations could restore protein synthesis inhibition or \(\text{eIF}2\beta\) activity caused by \(\text{eIF}2\alpha\) phosphorylation in translating lysates. With the help of inhibitors of type 1 and 2 phosphatases, this laboratory suggested that probably a type 1 phosphatase may be involved in the dephosphorylation \(\text{eIF}2\alpha(P)\) and restoration of \(\text{eIF}2\beta\) activity in heme-deficient inhibited lysates rescued by the delayed addition of hemin. These observations are consistent with the earlier observations that addition of an inhibitor of type 1 phosphatase stimulates \(\text{eIF}2\alpha\) phosphorylation and inhibits protein synthesis in hemin-supplemented reticulocyte lysates (Ernst et al., 1982) and involvement of a type 1 phosphatase in controlling the extent of \(\text{eIF}2\alpha\) phosphorylation in yeast (Wek et al., 1992). Also, a type 1 protein phosphatase is shown to form a complex with the viral gene product, \(\gamma34.5\) protein in herpes simplex virus-infected cells and blocks the shutoff of host protein synthesis that occurs due to the activation of double stranded RNA dependent \(\text{eIF}2\alpha\) kinase by directing the protein phosphatase 1a to dephosphorylate \(\text{eIF}2\alpha\) (He et al., 1997).

2.6. Physiological Inhibitors of \(\text{eIF}2\alpha\) Phosphorylation.
The rate of protein synthesis is tightly correlated with the growth state of the cell. Since small changes in eIF2α phosphorylation influences dramatically the rate of initiation of protein synthesis, cells have evolved different strategies to regulate their eIF2α kinases and the level of eIF2α phosphorylation. Heme, an iron protoporphyrin compound is known to inhibit the activation of HRI kinase by promoting disulfide bonds in the protein (Chen et al., 1989). However HRI kinase as mentioned above appears to be present mainly in erythroid tissues and is activated during heme-deficiency (Crossby et al., 1994). Unlike HRI, PKR is ubiquitously found in most of the cells. This protein kinase activation occurs not only in the presence low concentrations of double stranded viral RNA or by viral infection, but also by various stress conditions such as heat shock, growth factor deprivation, treatment with tumor necrosis factor α, and release of calcium ions from the endoplasmic reticulum. In addition, other regulatory molecules such as polyanions like heparin and dextran sulfate also cause activation (reviewed in Clemens and Elia 1997). In view of this wide ranging conditions that stimulate PKR activation, cells and viruses have evolved various mechanisms to check or stimulate the activation and activity of PKR. Several cellular and viral proteins and also the viral RNAs as mentioned below are known to regulate PKR activation and eIF2α phosphorylation. These products are produced as part of cellular or viral defense mechanisms. These regulatory molecules inhibit or activate eIF2α phosphorylation through multiple mechanisms as mentioned in here. 1) A block in eIF2α phosphorylation by active HRI and PKR occurs as in the case of a cellular glycosylated protein like p67 (Chakraborty et al., 1994). 2) Inhibition of the activation of PKR enzyme occurs by RNA transcripts produced by the interferon-resistant viruses such as adenovirus small RNA VA1, EBER-1 of Epstein-Barr Virus RNA, the TAR-RNA of HIV-1 and the Hepatitis C Virus envelope protein E2, through competition and induction of a conformational change in PKR protein.
that disrupts the association of PKR with ribosome at all the sites (reviewed in Clemens and Elia, 1997 and Taylor et al., 1999). 3) DsRNA molecules are sequestered by viral proteins like E3L and sigma 3 proteins coded by Vaccinia and Reovirus so that PKR activation does not take place (Chang et al., 1992 and Giantini et al., 1989). 4) The viral proteins serve as pseudo substrates or mimick eIF2α as in the case of K3L and PK2 proteins produced by Vaccinia and Baculovirus (Davies et al., 1993 and Dever et al., 1998). 5) Herpes Simplex Virus products, the γ134.5 gene product which is homologous to the mammalian protein known as GAAD34 (growth arrest and DNA damage protein 34) and a ribosome-associated RNA-binding protein, (Us11) blocks the inhibition of protein synthesis caused by PKR activation by directing the protein phosphatase 1α with which it is associated to dephosphorylate eIF2α(P) as in the case of γ134.5 gene product (He et al., 1997, 1998 and Mulvey et al., 1999) or, by blocking PKR activation as in the case of Us11 as has been postulated Mulvey et al., (1999). 6) Activation of a cellular inhibitor, p58, as identified originally in cells infected with influenza virus inhibits the dimerization of PKR (Lee et al., 1994). 7) L18, one of the proteins of the 60S subunits competes with dsRNA for binding to PKR and prevents PKR activation by dsRNA while PKR is associated with the ribosome (Kumar et al., 1999). In addition to these inhibitors, there are other cellular inhibitors of PKR kinase and eIF2α phosphorylation whose mechanism of action is not yet well characterized. These include a) TAR RNA binding protein of Human immunodeficiency virus whose over expression induces a transformed phenotype in NIH3T3 cells (Park et al., 1994; Benkirane et al., 1997 and Cosentino et al., 1995) b) a 100 kDa cellular protein, induced in NIH3T3 cells in response to the expression of the transforming Harvey ras oncogene (Mundschau and Faller 1992) c) a 15 kDa protein, produced in Murine 3T3F442α cells that are induced to differentiate into adipocytes (Judaware and Petryshyn 1992) d) the La antigen which probably inhibits
PKR by sequestering and unwinding dsRNA (Xiao et al., 1994) e) inactivation of PKR by Alu RNA (Chu et al., 1998) and f) Simian virus 40 large-T antigen rescues translational inhibition without affecting PKR activation and eIF2α phosphorylation probably at a step downstream of eIF2α phosphorylation (Swaminathan et al., 1996).

3. BACULOVIRUS

Baculoviruses or Nuclear polyhedrosis viruses are a family of insect viruses. They infect mostly Lepidopterans (butterflies and moths), Hymenoptera (sawflies) and Coleoptera (butterflies). They are also found to infect Crustaceans. The baculo portion of the name refers to the rod shaped capsids of the virus particles. Within the capsid, the DNA is condensed into a nucleoprotein structure known as the core. The DNA of baculoviruses is double stranded, covalently closed circular structure and is 130-200 kb in length. In nature, baculoviruses occur as virions that are occluded within the proteinaceous crystals known as polyhedra on plant leaves, plant debris and soil. Viral occlusion bodies are formed in the nucleus. Polyhedral occlusion bodies of nuclear polyhedrosis viruses (NPV) are known as polyhedra, occluded viruses (OV), or polyhedral inclusion bodies. The word nuclear in the NPV is used to distinguish it from the nuclear polyhedrin protein from the matrix polyhedrin protein characteristic of cytoplasmic polyhedrosis viruses which are members of reoviridae. Among the various viruses of baculoviridae, Autographa californica nuclear polyhedrosis virus and Bombyx mori nuclear polyhedrosis virus are the most well characterised. Recently, the genome of another baculovirus, Lymantria dispar nuclear polyhedrosis virus has been sequenced and is composed of 161 kb (Kuzio et al, 1999).
Baculoviruses have proven to be the most powerful and versatile eukaryotic expression vectors available. Although several mammalian viruses like SV40, adenoviruses, herpes simplex, vaccinia virus, cytomegalovirus and mammary tumour virus etc., have been used to express foreign proteins very successfully, most of these viruses have their own limitations in terms of safety, biological containment, level of expression, proper post translational modifications and proper processing. The baculovirus expression system bypasses most of these limitations. In addition, they have been recognised as eco-friendly and possessing the ability to develop into potential biopesticides. Baculoviruses have recently been found to infect mammalian cells, but they fail to replicate, thereby contributing to the additional application of baculoviruses as vectors for the delivery of foreign genes in mammalian systems (Hofman et al., 1995; Boyce and Bucher 1996 and Sandig et al., 1996). The p29 and p10 promoters of baculovirus system are simple in architecture but they are powerful in driving foreign gene expression to spectacular levels (400 μg/ml). However, several factors influence the level of expression (Hasnain et al., 1994 and Ranjan and Hasnain, 1995a and b).

The baculovirus expression vector system is a helper virus-independent system, which has been used to express foreign genes from many different sources: eukaryotes, fungi, plants, bacteria and viruses. Recombinant proteins have been produced as fusion or nonfusion proteins at levels ranging between 0.1% and 50% of total insect cell protein.

The most extensively studied baculovirus strain is Autographa californica nuclear polyhedrosis virus (AcNPV). During recent years its entire genome is mapped and sequenced (O'Reilly et al., 1992 and Ayres et al., 1994). Although, AcNPV was first isolated from the alfalfa looper (Autographa californica), it multiplies readily in cell lines derived from the fall armyworm
(Spodoptera frugiperda) and the cabbage looper (Trichoplusia ni). Though most of the expression vectors harness AcNPV infection of Spodoptera frugiperda cells, heterologous proteins also produced in silkworm larvae (Bombyx mori) by infecting them with recombinant BmNPV (Maeda, 1989). Recombinant virus construction is based on the homologous recombination between the plasmid carrying the foreign gene and viral genome.

3.1. The infection process.

Baculoviruses are further classified into two subfamilies, the Eubacteriovirinae (occluded baculovirus) that infect the larvae of lepidoptera, coleoptera, diptera, etc. and the Nudibaculovirinae, non-occluded baculoviruses. Two biochemically and morphologically distinct virus forms characterise baculovirus infection: a) extracellular or dubbed virion (BV) and b) occluded virion (OV) or the polyhedral derived virion (PDV). In the latter form, the occlusion bodies are embedded in crystalline polyhedrin protein matrix and are responsible for primary infection.

The infection cycle is divided into three phases: early, late and very late. During the early phase, there is transcription of genes, whose product is essential for viral DNA replication, and these genes are transcribed by the RNA polymerase encoded by the host (Grula et al., 1981) This phase continues up to 5 to 6h post- infection (h p.i). Between 5 and 18h p.i, late phase genes are transcribed which encode structural proteins and budding of nucleocapsid. The very late phase starts from around 20h p.i and is characterised by the transcription of occlusion-specific genes (polyhedrin gene, p10 gene) involved in viral occlusion process. The promoters of these genes are so strong that these genes continue to be overexposed such that 50-75% of the total protein in an infected cell is polyhedrin protein. The late
phase and very late phase genes are transcribed by virus-encoded or virus-modified host RNA polymerase (Huh et al., 1990). The Autographa californica nuclear polyhedrosis virus polyhedrin gene is transcribed at high levels very late in the lifecycle and involves a virus-specific or a virus modified host RNA polymerase. Reversible phosphorylation is an important posttranscriptinal modification that can modulate the functions of many cellular enzymes and transcription factors, control protein synthesis and macromolecular assembly, and regulate cell cycle propagation and signal transduction pathways (reviewed in references Boulikas, 1995; Edelman et al., 1987; Hunter, 1995 and Hunter et al., 1992). The activation of many viral proteins also can be regulated by phosphorylation, and this can be an important factor in viral replication (reviewed in references (Leader, 1988 and Prives, 1990).

AcNPV infection produces two types of viral progeny which are different structurally and functionally. The occluded or polyhedra-derived viral forms (PDV) are responsible for primary infection and embedded within a matrix of proteinaceous structures called occlusion bodies (OBs). During natural infection, the larvae ingest PDV containing OBs that contaminate their food. Then in the midgut of the larvae, the polyhedra are dissolved due to the presence of an alkaline environment. This process releases the embedded virions. The liberated PDV infect the midgut columnar epithelial cells by receptor mediated membrane fusion (Horton and Burand, 1993). The first viral occlusion bodies of wt AcNPV develop 2 days p.i, but continue to accumulate and reach a maximum between 5-6 days p.i. Occlusion bodies are visible under light microscope as dark, polygonal shaped bodies filling the entire nucleus of the infected cell These infected cells produce the BV or extracellular viruses. The budded virus form (BV) transmits infection from cell to cell during secondary infection. ODV infects gut cells by fusion of the viral
envelope with the columnar cell microvillar membranes, whereas BV entry of other cells occurs by Adsorptive endocytosis (Volkman and Goldsmith, 1985). Once the viral DNA is released the host nucleus becomes enlarged and forms distinct electron dense granular structure, called the virogenic stroma (Fraser 1986). Around 12h, progeny BVs are formed and are released to the extracellular compartment. Polyhedra soon begin to be formed thereafter and mature PDVs surrounded by envelopes become occluded. PDV and BV differ significantly in their ability to infect the insect through the gut and, viral envelopes and nucleocapsids of the two viral forms contain different proteins, or proteins that are processed differently (Braunagel et al., 1988). The most distinctive difference observed to date is the presence of viral encoded glycoprotein, gp64, which is found in BV but not in PDV. During secondary infection, gp64 is intimately involved in virus entry into the cells via the process of Adsorptive endocytosis. Other differences include the presence of an O-glycosylated protein, gp41 and the protein p74 in PDV, but not in BV.

The polyhedrin protein is essential for in vivo function but is functionally dispensable during infection of cells in culture. Hence most baculovirus vectors exploit this phenomenon by substituting its coding sequence.

3.2. The advantages of baculovirus expression system.

Two important features of the baculovirus account for the success of this virus as an expression vector. First, the virus contains at least two non-essential regions that can be replaced by foreign genes. Second, many of these genes especially the late genes are under the control of very strong promoters that allow hyper expression of the foreign genes. Most of these transfer vectors make use of p29 and p10 promoters along with
neighbouring sequences to allow homologous recombination. Both p29 and p10 are nonessential genes and deletion of these genes does not affect replication of virus in cell culture (Smith et al, 1983 and Were et al, 1989).

Choosing a eukaryotic system for the expression of a eukaryotic gene can be particularly important in obtaining biologically active recombinant protein. Several unique features of the baculovirus expression system have made the system of choice for many applications. These are i) expression of functional recombinant proteins with proper folding, ii) post-translational modifications iii) high level of expression, iv) capacity for large insertions, v) ability to express unspliced genes, vi) simplicity in the technology due to the availability of kits, vii) correct targeting mechanisms, viii) facility for the simultaneous expression of multiple genes, ix) potential ability to serve as biopesticides and their x) ecofriendly nature.

Since the baculovirus gene is too large to manipulate in vitro to produce recombinant virus, transfer vector or transplacement vectors are widely used (Luckow and Summer, 1989). Although, there are several reports describing direct insertion of foreign genes into the genome via enzymatic ligation (Peakman et al., 1992), through the use of large bacterial plasmids and transposon element (Luckow et al., 1993) or inserting a yeast replicon element (Patel et al., 1992). However, these methods are cumbersome. The transfer vectors contain a bacterial plasmid, a portion of the baculoviral genome encompassing a gene promoter and a transcriptional terminator. Two regions of AcNPV have been used to construct transfer vectors. These are 7.3 Kb EcoRI fragment containing the polyhedrin gene and 2.0 Kb EcoRI-P fragment containing the p10 gene. Earlier transfer vectors used to have too long flanking sequences but now the size of these vectors have been significantly reduced to accommodate larger insert. A number of improved
methods are now available to simplify the tedious process of identifying the polyhedra negative recombinant viruses. These include methods wherein linearized baculovirus DNA (Kitts et al., 1990; Kitts and Possee 1993) were used to increase the number of recombinant viruses obtained after cotransfection. An engineered baculovirus was constructed such that two sites for Bsu36l were introduced in the flanking sequences upstream to the promoter and in the downstream orf129 encoding an essential gene, the viral replicase. This linearized modified viral genome is cotransfected with the plasmid carrying a gene of interest under the polyhedrin promoter and deleted portion of the viral genome. Bsu361 digested viral genome lacking the portion of the essential downstream gene, even after in vivo repair and recircularization is unable to produce viable viruses. Only when it recombines with the transfer vector carrying the missing segment along with the gene of interest it can form viable progeny virus. This approach results in assured recombination and results in >90% recombinants. Clontech markets this system under the trade name of BacPAK and is marketed by Pharmingen as 'baculogold'.

Expression of foreign genes prior to very late phase, i.e. in the late phase may be advantageous for efficient post-transcriptional modifications. Since, most of the late genes serve as structural genes, they cannot be substituted for insertion of foreign genes. This problem has been circumvented by employing polyhedrin locus as a site for adding a copy of the preferred gene under the late promoter. Several vectors of this category have been constructed and used successfully (Thiem and Muller, 1990; Hill-Perkin and Possee, 1990).

4. OBJECTIVES
This laboratory is interested in studying the regulation of eukaryotic initiation factor quite for sometime now. Some of the initial studies of this laboratory were focused on eIF2 recycling, dephosphorylation of eIF2 by protein phosphatases and the effects of some newly made metal-sugar complexes, redox agents and purified plant lectins on the translational ability of lysates and on eIF2 phosphorylation (Babu and Ramaiah, 1996; Janaki et al., 1995; Krishna et al., 1997; Ramaiah et al., 1997 and Krishnamoorthy et al., 1998). In addition, at present this laboratory has also been involved in understanding the difference in the translational ability of different recombinant chimeric RNAs and the role of eIF2 alpha phosphorylation in the regulation of the plant protein synthesis. A recent study by Dr. Ramaiah and his colleagues (Pavitt et al., 1998) has shown that phosphorylated yeast histidine tagged eIF2 can form a much more tight complex with eIF2B in vitro.

The availability of site-specific mutants of eIF2α like the 48A or 51A in which the serine residues in the respective positions of eIF2α have been replaced by alanine, has advanced our understanding in identifying that, a) serine 51 residue in eIF2α is the only site for phosphorylation in mammalian eIF2α (Pathak et al., 1988), b) the translational block caused by adenoviral mRNAs, plasmid derived mRNAs, heat shock or calcium release from the endoplasmic reticulum is due to increased eIF2α phosphorylation (Kaufman et al., 1989; Choi et al., 1992; Murtha-Riel et al., 1993 and Srivastava et al., 1995) or in localising the translational inhibition caused by eIF2α phosphorylation, and c) phosphorylation of eIF2α plays critical role in cell proliferation and development (Donze et al., 1995 and Qu et al., 1997). In addition, the coexpression of a mutant eIF2α (51A) which cannot be phosphorylated has facilitated the expression of mammalian eIF2α kinases like the heme-regulated kinase in insect cells (Chefalo et al., 1994).
In order to obtain a good amount of purified wt and mutants of eIF2α for a variety of biochemical characterizations to determine protein-protein interaction and eventually express the other subunits of initiation factor and other initiation factors, it is felt that we should have a system that is relatively easy to handle and maintain. In this context we chose to express eIF2α subunit and mutants of eIF2α in Spodoptera frugiperda insect cells using baculovirus expression system. The following site-specific mutations of eIF2α were cloned into baculovirus vector and expressed.

The mammalian cells were unable to express 51D mutant (aspartic acid mutant) of eIF2α efficiently and the cells expressing this mutant were killed. This is probably because the mutation created to mimic the charge of a phosphorylated serine and the mutant behaves like phosphorylated species. One of the objectives of this thesis is also to see if it is possible to express 51D mutant protein in insect cells using baculovirus expression system.

a) 51A is a mutant eIF2α in which the 51 serine residue is replaced by an alanine. Its characteristic feature is that it cannot be phosphorylated by kinases in vitro suggesting that 51 serine residue is the only phosphorylation site (Pathak et al., 1989).

b) 51D is a mutant eIF2α in which the 51 serine residue is replaced by an aspartic acid. The expression of this mutant eIF2α inhibits the protein synthesis of mammalian cells and the cells are unable to survive, suggesting that it is behaving like a phosphorylated form (Choi et al., 1992 and Kaufman et al., 1989).

c) 48A is another mutant in which 48 serine residue is replaced by an alanine. This mutant is phosphorylated on its 51-serine residue. However
over expression of this mutant escapes the inhibition in protein synthesis and mitigates the inhibition in eIF2B activity caused by endogenous eIF2a phosphorylation (Kaufman et al., 1989; Choi et al., 1992, Murtha Riel et al., 1993 and Ramaiah et al., 1994).