1.0 INTRODUCTION

Information for the synthesis of proteins is located in the nucleotide sequences of messenger ribonucleic acid (mRNA) molecules which are in turn derived from their corresponding genes or deoxy ribonucleic acid (DNA) sequences. The information in the template molecules, be that, RNA or DNA, is decoded by a complex cellular machinery. The process that facilitates the synthesis of protein from mRNA is called translation and the synthesis of RNA from a DNA template is called transcription. In addition to transcription, it has become increasingly clear in recent years that mRNA translation represents an important control point in gene expression in many animal systems studied to date. The process of translation, for convenience, is divided into three stages, namely, initiation, elongation and termination. Each of these stages is mediated by protein factors, termed initiation, elongation, and termination factors (Ochoa, 1983 and Watson, 1987). Since protein synthesis consumes a significant proportion of the available energy of eukaryotic cells, it is natural for the cells to exert control at the initiation step of protein synthesis. Protein synthesis in eukaryotes is regulated in most cases at the initiation step by changes, either in the cellular concentration, or phosphorylation status of *only a few* initiation factors. Two important rate-limiting factors, whose phosphorylation is known to effect the regulation of protein synthesis, have been well characterized in mammalian systems. These are initiation factors -2 and -4E (eIF-2 and eIF-4E). While enhanced phosphorylation of eIF-4E increases translation of several mRNA's, increase in the phosphorylation of the small or alpha-subunit of initiation factor-2 (eIF-2a) down regulates protein synthesis in many animal systems studied to date (Hershey, 1989).

Since the present work is concerned with the regulation of protein synthesis, particularly mediated by changes in the phosphorylation of eIF-2 subunits in plant cells, the current information available on the protein synthesis regulation due to changes in the eIF-2 activity in animal (see section 1.3) and plant systems (see section 1.5) has been focused here. Preceeding these sections, various steps in protein synthesis and the role of several factors regulating the

translational initiation, elongation and termination steps has also been presented here briefly.

1.1 Bird's eye view of eukaryoticprotein synthesis.

1.1.1 Initiation of protein synthesis.

The initiation phase of protein synthesis is marked by the formation of 80S initiation complexes and the release of eIF-2.GDP binary complex from ribosomes. However, the process is very complicated and is divided into six sub-steps as mentioned below:

a) Dissociation of SOS ribosomes into their subunits, 40S and 60S. The joining of these subunits is prevented by the attachment of anti-association factors like eIF-3, eIF-4C (now called eIF-1A) and eIF-6 to the dissociated subunits (Russel and Spermulli, 1979; Peterson *et ah,* 1979; Goumans *et ai,* 1980; Raychaudhri *et al.,* 1985).

b) Formation of 43S preinitiation complex (40S.eIF-2.GTP.Met.tRNA$_i$) is mediated by initiation factor-2. eIF-2 joins first to Met.tRNA$_i$ (initiator tRNA) in the presence of GTP and forms the ternary complex, eIF-2.GTP.Met.tRNAj (Trachsel *et ai,* 1977; Benne and Hershey, 1978). The resulting ternary complex then joins the 40S ribosomes to form the 43 S preinitiation complex.

c) Joining of mRNA to the 43S preinitiation complex requires several eukaryotic initiation factors and are termed as eIF-4's. The resulting 48S preinitiation complex, which forms upon joining of mRNA to 43S preinitaion complex, has been identified and well characterized (Rhoads, 1988; Sonenberg, 1988; Hershey, 1991). As judged by cross-linking experiments, four of the eIF-4 proteins can associate with the 5'end of cytoplasmic eukaryotic mRNAs, which have a modified guanosine moiety at the 5'end (7-methyl guanosine), termed the 5' cap, which is linked to the next nucleotide by 5'-5' triphosphate loop. The various eIF-4 proteins that help in the joining of mRNA to the 43S initiation complex, are eIF-4F and eIF-4B. eIF-4F is a complex of three proteins, namely, eIF-4E (25 kDa,

previously called as eIF-4α), eIF-4A (45 kDa), eIF-4γ (220 kDa, also called as p220). eIF-4E, also called elF-4a, is the only protein that binds to the cap In normal physiological state, however, this protein is associated with eIF-4A and p220 to form eIF-4F complex. While eIF-4A carries the RNA-dependent ATPase and helicase activities, the integrity of p220 is required for eIF-4F activity in cap-dependent translation. The cleavage of p220 following poliovirus infection results in the shut down of host protein synthesis. In addition to the various proteins in eIF-4F complex, eIF-4B protein is required probably in recycling eIF-4E from eIF-4F complex and in stimulating the RNA-dependent ATPase and helicase activities of eIF-4A (Reviewed in Sonenberg, 1988; Hershey, 1991; Merrick, 1992; Rhoads, 1993).

d) Identification of 'start' codon AUG in eukaryotic mRNA by 48S preinitiation complex is the next step in protein synthesis. Unlike in prokaryotes, which possess a distinct structural feature (Shine Dalgerno sequence) in the mRNA for it to facilitate a direct hydrogen bonding interaction with the 16S rRNA of ribosomes, eukaryotic mRNA's do not carry a comparable recognition sequence.

Among several models proposed for the pathway by which mRNA and 43S preinitiation complex associate, a consensus model which probably accounts for most of the cellular mRNA's is that, the 5'cap structure is first recognized by eIF-4F complex. Subsequently, the secondary structures within the 5'UTR (untranslated regions in mRNA) are melted due to the activity of eIF-4A and ATP. This facilitates ribosomes to scan the mRNA until the 'start' AUG codon is encountered (scanning model). Alternatively, the 43S complex enters directly at the internal site within the 5'UTR (internal initiation model) (Hershey, 1991).

e) The joining of 60S ribosomal subunit to the 48S preinitiation complex results in the formation of 80S initiation complex and this event is stimulated by eIF-5 protein and the release of anti-association factors. The joining reaction requires the hydrolysis of GTP, a function promoted by eIF-5 in the absence of 60S subunit, and results in the release of eIF-2.GDP along with other bound factors such as

eIF-4C and eIF-3 (Benne and Hershey, 1978). Recent findings however, indicate that eIF-2 is translocated from the 40S subunits to the 60S subunits of the 80S initiation complexes (Ramaiah *et al,* 1992).

f) The recycling of eIF-2.GDP that occurs at the end of initiation step requires the exchange of GTP for GDP in elF-2.GDP binary complex. This is critical for eIF-2 to join initiator tRNA (Met.tRNA;), since, GDP inhibits the joining of eIF-2 to $Met.tRNA_i$. The rate of guanine nucleotide exchange being slow at the physiological concentration of Mg^{2+} in mammals and since the binding affinity of GDP is 400 times higher than GTP (Rowlands *et al,* 1988a), a catalyst is known to promote the exchange of GTP for GDP in eIF-2 GDP binary complex. This catalytic factor, referred to here as eIF-2B, has enjoyed several names in the literature previously, namely, co-eIF-2C (Das *et al.,* 1979), guanine nucleotide exchange factor (GEF, Panniers and Henshaw, 1983), reversing factor (RF, Siekierka *et al,* 1981; Grace *et al.,* 1982; Matts *et al,* 1983) and anti-heme regulated eIF-2α kinase (anti-HRI, Amesz *et al,* 1979), eIF-2B (Konieczny and Safer, 1983) etc.. In addition to exchanging guanine nucleotides of eIF-2, the multi polypeptide eIF-2B factor may also have a role in releasing eIF-2.GDP from the SOS initiation complex (Thomas *et al.,* 1984; Ramaiah *et al,* 1992) and is involved in the recycling of eIF-2. (Please see also the **section 1.3.2.3.** on eIF-2B for details).

1.1.2 Elongation and termination.

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, Merrick, 1992). Four elongation factors (eEF's) have been characterized and each factor is known to catalyze a step in the elongation process. The process of elongation can be divided again into four sub-steps, as mentioned below.

a) eEF-1α catalyzes the binding of the aminoacylated tRNA to the A site of the ribosome.

b) The ejection of eEF-1a is accompanied by the GTP hydrolysis. The eEF-1a.GDP thus released is recycled by the eEF-1β/γ. While eEF-1a is comparable to EF-Tu of prokaryotes, the eEF-1β and eEF-1γ, which are involved in the exchange of GTP for GDP on eEF-1α.GDP, are comparable to EF-Ts of prokaryotes and to the initiation factor eIF-2B of eukaryotes.

c) Formation of the peptide bond between the nascent polypeptide and the incoming amino acid is catalyzed by the peptidyl transferase center presumably located on the 60S ribosomal subunits.

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. Protein synthesis continues till the ribosome reaches the termination codon, at which point the releasing factors (RF's) assist in release of the completed nascent polypeptide chain from the ribosome (Spirin, 1986).

1.2 Regulation of initiation of protein synthesis.

Regulation of translation can occur at various stages of protein synthesis. In such a complex sequence of reactions it is natural for the cells to exert control at the first step of initiation of protein synthesis. Two distinct types of translational control occur.

(i) General control of overall protein synthesis affecting the bulk of mRNA's of the cell, and,

(ii) Selective regulation of specific mRNAs or a subset 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 have been well documented (Hershey, 1991; Merrick, 1992, Proud, 1992, Jeffries and Thomas, 1996).

In the initiation step of protein synthesis, phosphorylation of eIF-2 and eIF-4E proteins play a major role in the regulation. There is a strong correlation to enhanced eIF-4E phosphorylation, that occurs, in response to growth factors, mitogens, and cytokines, to increased protein synthesis (Morley and Traugh, 1989; Kasper *et al,* 1990, Fredrickson *et al.,* 1992; Donaldson *et al.*, 1991). eIF-4E is hypo-phosphorylated during mitosis (Boneau and Sonenberg, 1987), following heat shock (Duncan *el al.,* 1987) or infection with several viruses (Huang and Schneider, 1991; Feigenblum and Schneider, 1993) concomitant with a reduction in the translation rates Interestingly, these conditions which reduce eIF-4E phosphorylation can also enhance eIF-2 phosphorylation. However, the connection (if any) between the phosphorylation states of these two proteins is not understood.

1.3 eIF-2 structure, function and regulation.

1.3.1 Subunit structure and function of eIF-2.

Initiation factor-2 is a key protein involved in the initiation step of protein synthesis. It is a heterotrimer with three subunits a- (38 kDa), β- (50 kDa) and γ- (52 kDa). The factor mediates the binding of Met.tRNAj to the 40S ribosome in a GTP-dependent manner: when bound to GDP, eIF-2 cannot bind to $Met.tRNA_i$ At the end of initiation, eIF-2 is released as eIF-2.GDP binary complex Since the off-rate for GDP is very low, another factor, eIF-2B, is necessary under physiological conditions to allow the replacement of GTP for GDP in eIF-2.GDP binary complex and then to mediate the recycling of eIF-2,

eIF-2 is also a phosphoprotein. Two of its subunits, the a- and p- are known to be phosphorylated Collectively, the activity of eIF-2 can be regulated

by eIF-2 kinase(s), phosphatase(s), and by the eIF-2B factor. The β-subunit in mammalian cells migrates with an apparent molecular mass of 36-55 kDa depending on the gel system used (Lloyd *el al.*, 1980; Meyer *el al.*, 1981; Panniers and Henshaw, 1983; Colthurst and Proud, 1986) The differential migration of the P-subunit may be due to the presence of large blocks of lysine residues, which could alter its electrophoretic mobility (Pathak *et al.*, 1988a). cDNA clones for eIF-2α subunit have been isolated form rat, human (Ernst *et al.*, 1987) and yeast (Cigan *el al.*, 1989) cDNA clones for eIF-2P have been isolated from human (Pathak *et al.*, 1988a; Gasper *et al.*, 1994) and yeast (Donahue *et al.*, 1988).

Both eIF-2p and y-subunits appear to be involved in the binding of guanine nucleotides. Studies involving photoaffinity labeling of eIF-2 using appropriate nucleotide analogues and from the cDNA sequence suggest that the γ-phosphate of the guanine nucleotide is in contact with eIF-2y while the guanosine moiety is in contact with eIF-2p (Anthony *el al.*, 1990, Dholakia *et al.*, 1989; Bommer and Kurzchalia 1989). Anthony *et al.*, (1990) have shown that eIF-2 preparations devoid of the alpha-subunit can bind GDP as effectively as the trimeric form of the factor. Pathak *et al*, (1988a) using labeling studies have shown that eIF-2P contains the elements of the consensus guanine nucleotide binding structures found in other GDP/GTP binding proteins They have shown that eIF-2p contains only two of the three elements of guanine nucleotide binding-structures, the third element presumably is present in the y-subunit of eIF-2 (Dever *et al.*, 1987; Pathak *et al.*, 1988b; Suzuki *et al.*, 1990). These findings indicate that the guanine nucleotide binding site in eIF-2 is 'shared' between the β- and γ-subunits.

The studies with cDNA encoding human eIF-2P and y-subunits (Pathak *et al.*, 1988a and Gasper *et al.*, 1994) also suggest that DXXG and NKXD consensus elements for GDP binding are present in both P- and y-subunits, thereby emphasizing the possibility that both subunits might be involved in GTP binding. When the Aspergine residue is altered in the NKXD consensus sequence of y-subunit of eIF-2, protein synthesis is strongly inhibited. In contrast, alteration in

the corresponding Aspergine residue in the p-subunit causes little change in the protein synthesis compared to the wild-type subunit These results support the view that GTP binding requires the NKID in eIF-2γ but does not involve NKAD element in the eIF-2p (Naranda *et al,* 1995). The recent results however, contradict earlier results obtained from affinity labeling of eIF-2 with GDP derivatives in which GDP is shown to bind both β- and γ-subunits of eIF-2 (Bommer *et al,* 1989; Anthony *et al,* 1990). A possible explanation is that eIF-2P lies very close to the GTP binding site in the G domain of eIF-2γ (Bommer et al., 1989; Naranda *et al.* 1995).

Binding of eIF-2 with mRNA supports the concept that eIF-2 interacts specifically with mRNA during protein synthesis and that this interaction is important for translational control. eIF-2 forms a ternary complex with Met.tRNA, and GTP that binds to the 40S ribosomal subunit, yielding a complex that is obligatory for subsequent binding of mRNA (Kaempfer, 1984; Moldave, 1985, Merrick. 1992). Through this property, eIF-2 is already indispensable for binding of mRNA during translation However, eIF-2 also undergoes a direct and specific interaction with mRNA (Kaempfer, 1974; Kaempfer, 1978; Barrieux **and** Rosenfield, 1978; Kaempfer, 1979). Kaempfer *et al,* have shown that in satellite tobacco necrosis virus RNA (Kaempfer *et al,* 1981) or mengo virus RNA (Perez-Bercoff and Kaempfer, 1982), eIF-2 recognizes and protects specific nucleotide sequences that overlap with the ribosome binding sites. This suggested that once bound to the 40S ribosomal subunit, eIF-2 may interact directly with mRNA and thus guide the 43S preinitiation complex to its binding site in mRNA. Indeed, eIF-2, but no other initiation factor, promotes the selection of the 5'proximal translation initiation site by ribosomes (Dasso *et al,* 1990). There is genetic evidence in yeast that eIF-2 recognises the AUG initiation codon: mutations permitting utilization of UUG as initiation codon map into the a- and P-subunits of eIF-2 (Donahue *et al,* 1988; Cigan *et al,* 1989), with those in eIF-2P mapping to a zinc finger motif that may function in mRNA recognition (Donahue *et al,* 1988).

The importance of the interaction between mRNA and eIF-2 for translation control is supported particularly well by the close correlation between the affinity of mRNA species for eIF-2 and its ability to compete in translation (Kaempfer, 1984). Thus, rabbit β-globin mRNA binds to eIF-2 with higher affinity than α-globin mRNA and competes more effectively in translation (Di Segni *et al.*, 1979) Human β-globin mRNA outcompetes the fetal γ-globulin mRNA species, both in translation and binding to eIF-2 (Marsh *et al,* 1990). In each case, translational competition is relieved by an excess eIF-2 (Di Segni *et al,* 1979; Rosen *et al,* 1982). These findings which emphasize the role for interaction between eIF-2 and mRNA for translational control, raises a question as to how the dual binding activities of eIF-2 are regulated. mRNA and Met.tRNAj/GTP are mutually exclusive in their binding to eIF-2, as their recognition involves distinct epitopes in the protein (Harary and Kaempfer, 1990). eIF-2 also binds with high affinity to ATP yet does not hydrolyze it (Gonsky *et al.,* 1990). Interaction of ATP with eIF-2 in ternary complex with Met.tRNA; and GTP results in dual binding activities of eIF-2 during translation, switching from a $Met.tRNA_i$ binding mode into an mRNA binding mode (Gonsky *et al.,* 1990). The eIF-2p but not a- or y- harbors binding sites for mRNA as well as ATP (Gonsky *et al.,* 1992). Hence, during initiation of protein synthesis the mammalian eIF-2p subunit may interact with three ligands, important for translational control: $Met\text{-}tRNA_i$, mRNA, and ATP.

1.3.2 Regulation of eI F-2 by phosphorylation.

1.3.2.1 Phosphorylation of eIF-2 subunits under various conditions.

The a- and P-subunits of eIF-2 can be phosphorylated *in vitro* as well as *in vivo* (intact cells) (London *et al.,* 1987, Hershey, 1989). Several protein kinases can phosphorylate the P-subunit of eIF-2 *in vitro.* These include protein kinase C (Schatzmann *et al.*, 1983; Clark *et al.*, 1988), casein kinase II (CK II) (Issinger *et al,* 1976; Jagus *et al.,* 1982; Clark *et al,* 1988, 1989) and also by cAMP-dependent protein kinase (Alca'zar *et al.*, 1988). The p-subunit of eIF-2 also undergoes phosphorylation in intact cells (Duncan and Hershey, 1984, 1985).

Several serine residues in the β-subunit are accessible to phosphorylation by different kinases *in vitro* These include serine-13, serine-2 and a serine residue of the C-terminus (Proud, 1992). The functional significance of phosphorylation is however not clear as yet. The activity of mammalian eIF-2 is regulated to a large extent by phosphorylation of the a- or small-subunit (38 kDa), at a specific residue, serine 51, by any of the three highly specific kinases: PKR, a constitutive kinase induced by interferon and activated by dsRNA; HRI, negatively regulated by heme pool mainly during haemopoiesis; or GCN2, a yeast eIF-2α kinase, activated during amino acid starvation (Samuel, 1993). In addition to heme-deprivation, viral infection and amino acid starvation, there are several other conditions such as heat-shock (Clemens, 1982; Duncan and Hershey, 1984; Murtha-Riel *et al.,* 1993), treatment with N-ethylmaleimide (Chen *et al,* 1989), oxidized glutathione, GSSG (Kan *et al,* 1988), heavy metal ions (Matts *et al,* 1991), O-iodosobenzoate (Gross and Rabinowitz, 1972) serum-deprivation (Duncan and Hershey, 1987) and calcium-deprivation (Preston and Berlin, 1992, Prostko *et al,* 1992), which are found to enhance eIF-2a phosphorylation. However, only three eIF-2a kinases that are activated during heme-deficiency (HRI), viral infection (dsI or PKR) and amino acid starvation (GCN2) have been identified and well characterized. The responsible eIF-2a kinases that can get activated under other stress conditions have however not been identified yet.

1.3.2.2 eIF-2 alpha kinases.

To date three eIF-2α kinases have been cloned. These are HRI (Chen *et al,* 1991); PKR from humans (Meurs *et al,* 1990) and mouse (Baier *et al,* 1993); and yeast GCN2 kinase (Wek *et al,* 1989). All the three kinases that have been mentioned above share extensive homology in kinase catalytic domains and all the three phosphorylate the serine 51 residue in the eIF-2α subunit (Colthurst *et al,* 1987; Pathak *et al,* 1988a) However, the regulatory mechanisms of these three eIF-2α kinases are very different. HRI (626 amino acids), is activated under heme-deficiency conditions and the kinase activity is inhibited by heme. PKR (550 amino

acids), is activated by low concentrations of dsRNA (ng/ml) and is inhibited by high concentrations of dsRNA (μg/ml). Yeast GCN 2 (1590 amino acids) is activated by amino acid starvation; the subsequent phosphorylation of eIF-2α is required for the increased translation of GCN4, a transcription activator of genes responsible for amino acid biosynthesis (Samuel, 1993). HRI and PKR can functionally substitute for GCN2 in the yeast GCN4 translational control (Dever *et al.,* 1993).

Each of the eIF-2a kinases mentioned above contains a unique sequence that may be responsible for its regulation. While the amino terminal 160 amino acids of PKR contains two copies of the dsRNA binding motifs rich in basic amino acids, the carboxy-terminus of GCN2 contains an essential 530-residue motif with significant homology to the histidyl-tRNA synthetase, a similarity which has prompted the suggestion that GCN2 senses amino acid starvation by binding uncharged tRNA (Wek *et al,* 1995). The extreme C-terminal 124 amino acids of GCN2 are required for its interaction with the 60S ribosomal subunit and are also required for the GCN2 action *in vivo* (Ramirez *et al,* 1991). While PKR and GCN2 are purified from ribosomal salt wash, HRI is purified from post-ribosomal supernatant. HRI does not have the ribosomal association sequence of GCN2 There is a portion of the sequence between two halves of the conserved kinase domains known as the kinase insertion sequence that is unique to each kinase. In HRI and GCN2, the kinase insertion sequence is large and about 120 amino acids, while in PKR it is only about 40 amino acids long. However, the last 20 amino acids of the kinase insertion sequence of all these three eIF-2a kinases share significant homology among them suggesting a common functional role. The kinase domains 9-10 which are highly conserved among these three eIF-2 kinases are likely to be involved in eIF-2 binding (Chen and London, 1995). Heme inhibits the HRI activity by promoting inter-subunit disulfide bond formation between HRI molecules. HRI also appears to be largely erythroid specific (Chen *et al,* 1991; Chen *et al.,* 1993). However, a recent report by Mellor *et al.,* (1993) indicates that

there is a small amount of HRI mRNA in non-erythroid tissues. The discrepancy between these observations is however, not clear

Heat-shock proteins (HSP70, HSP90 and p54) may also regulate the activation of HRI in reticulocyte lysates and thereby eIF-2α phosphorylation (Rose *et al.*, 1989; Szyszka *et al,* 1989; Matts and Hurst, 1992). However, the mechanism by which these proteins are able to regulate HRI activity is still not clear. A dynamic interaction of HRI with the above heat-shock proteins has been demonstrated by co-immuno adsorption of HRI with these proteins from reticulocyte lysates (Matts and Hurst, 1992). The association of HRI with HSP90 and p54, but not HSP70, is enhanced by hemin. The level of HSP70 in lysates appears to be inversely related to the degree of translational inhibition in hemin-supplemented lysates under conditions of heat shock and oxidative stress (Matts *et al.,* 1992). These results suggest that HSP70 is required to maintain HRI in an active form. Indeed, the addition of denatured protein which sequesters HSP70 activates HRI in heme-supplemented lysates (Matts *et al.,* 1993). More recently, HSP70 has been shown to prevent activation of HRI in heme-deficiency by reducing the optimal concentration of hemin required to suppress HRI activation (Gross *et al.,* 1994). This effect of HSP70 requires dithiothreitol and, to a lesser extent, GTP. These observations fit well with the earlier report that heavy metal ions activate HRI by inhibiting the capacity of heme-supplemented reticulocyte lysate to reduce disulphide bonds (Matts *et al.,* 1991) and with the requirement of a disulphide reducing system to maintain the maximum rate of initiation of protein synthesis. All these studies point to the importance of sulfhydryl groups in the regulation of HRI activity. Although the mechanism by which HSP70 and DTT prevent the activation of HRI in heme deficiency is currently unknown, it is possible that HSP70, a chaperone, would change the conformation of HRI and allow DTT to reduce critical disulphide to free sulfhydry! which can then be oxidized by hemin. Alternatively, the sulfhydryl groups of HRI are required for the

binding of HSP70. Experiential evidence is available supporting both the models (Chen and London, 1995).

Protein kinase regulated by RNA (PKR) is transcriptionally induced by interferon and is activated by RNA-dependent autophosphorylation (Meurs *et al.*, 1990; Clemens *et al.*, 1994). Some naturally occurring ss RNA including reovirus S1 mRNA and HIV TAR RNA, as well as certain forms of synthetic ((rI)n-(rC)n) and naturally (reovirus genome RNA), double stranded RNA (dsRNA) are activators of PKR (Clemens *et al.*, 1994). Depending on their concentration, naturally occurring dsRNA and ssRNA including reovirus genome dsRNA, adenovirus VA_I RNA, Epstein Barr virus EBER RNA, and HIV TAR RNA can inhibit PKR activation Viral RNA binding protein such as σ3 and vaccinia virus E3L also antagonize PKR activation (Jagus and Gray, 1994). PKR is associated with the formation of a stable PKRdsRNA complex that requires atleast 30-50bp of duplex (Manche *et al.*, 1992). PKR is an important component in the anti-viral action of interfercn (Samuel, 1991, Clemens et al ., 1994). Virus that have deleted from their genome genes that antagonize the action of PKR, for example, adenovirus VA_I RNA and Vaccinia virus K3L are reported to have an increased sensitivity to IFN relative to the wild-type virus (Kitajewski *et al.*, 1986). It has been proposed that PKR may also function as a tumor suppresser (Clemens, 1992; Legyel, 1993). Expression of functionally defective PKR-Human in mouse NIH 3T3 cells cause malignant transformation and 3T3 cells overexpressing inactive PKR are highly tumourogenic when injected into nude mice (Koromilas *et al*, 1992, Meurs *et al.*, 1993) Mutations in the dsRNA binding domainl was also shown to result in malignant transformation (Barber *et al.*, 1995). PKR activity is also modulated by compounds which are polyanionic in nature (heparin, dextran sulphate, chondroitin sulphate and poly- L glutamine).

General Control Nonderepressible-2 (GCN-2) kinase is a general control eIF-2a kinase from yeast which is regulated by amino acid availability. The activation of GCN2 kinase during aminoacid starvation conditions is coupled to

the increased translation of GCN4 mRNA leading to synthesis of an important stress response protein GCN4. GCN4 activates atleast 40 different genes encoding aminoacid biosynthetic enzymes thereby enabling the cell to alleviate the nutrient starvation conditions (Hinnebusch, 1994). The increased GCN4 is brought about by overcoming the inhibition effects of multiple short upstream ORFs present in the GCN4 mRNA leader sequence (Dever *et al,* 1992).

1.3.2.3 Mechanism of protein synthesis inhibition by eIF-2a phosphorylation; importance of eIF-2B activity.

20-30% of eIF-2a phosphorylation can inhibit protein synthesis completely in reticulocyte lysates (Leroux and London, 1983). Also, addition of purified eIF-2 is found to rescue protein synthesis inhibition in reticulocyte lysates caused by heme-deficiency. Further, it has been observed that the rescue by eIF-2 is less effective, the purer the preparation of eIF-2 (Jackson, 1991). These findings suggest that there must be yet another rate-limiting protein which can influence the eIF-2 activity. Eventually this protein factor has been purified from the post-ribosomal supernatant and is named as eIF-2B The eIF-2B factor consists of five polypeptides or subunits These are -a (34 kDa), -β (40 kDa), -*y* (55 kDa), -5 (65 kDa) and -e (82 kDa). Quite frequently, the eIF-2B protein co-migrates with eIF-2 and has been purified from the post ribosomal supernatant PRS (Matts *et al,* 1982; Panniers and Henshaw, 1983; Reichel *et al,* 1985).

Purified eIF-2B protein rescues protein synthesis catalytically (Matts *et al,* 1983). The eIF-2B protein has also been shown to exchange GTP for GDP bound to eIF-2. This is very critical since GDP inhibits the joining of eIF-2 to Met-tRNA$_i$. Hence eIF-2B is important to promote the recycling of eIF-2 Indeed, the standard assay for eIF-2B activity exploits the fact that complexes of eIF-2 with guanine nucleotides bind to nitrocellulose filters, and determine the rate of displacement of tritiated GDP ([^{3}H]GDP) from preformed eIF-2.[^{3}H]GDP binary complexes on incubation with eIF-2B for unlabelled GDP or GTP. The presence of eIF-2B stimulates the displacement process provided that eIF-2 is not phosphorylated.

Phosphorylation of eIF-2α reduces the eIF-2B activity *in vitro* (Clemens 1982). The affinity of eIF-2B for phosphorylated eIF-2α is higher than for unphosphorylated eIF-2. The eIF-2(aP).GDP is regarded as a competitive inhibitor of eIF-2B interaction with eIF-2.GDP, but the magnitude of the difference in affinity is so great that under physiological conditions this type of competitive binding will effectively promote sequestration (Rowlands *et al.*, 1988a). Indeed it has been shown that phosphorylation of a small portion of eIF-2α sequesters all cf the available eIF-2B activity into a complex, 15S complex, (eIF-2(aP).eIF-2B), in which eIF-2B becomes non-functional. The complex can be purified on a sucrose density gradient (Thomas *et al.*, 1984; 1985).

The eIF-2B pool is estimated to be 15% (on a molar basis) of the eIF-2 pool in the case of reticulocyte lysates and about 50% in Ehrlich ascites cells (Rowlands *et al.*, 1988b). Hence, some what higher phosphorylation of eIF-2 is observed (about 50%) in the case of Ehrlich ascites cells for the complete inhibition of protein synthesis.

Yeast eIF-2B also has 5 subunits, these are, GCD1, GCD6, GCD2, GCD7, and GCN3 GCN3 is the smallest subunit of yeast eIF-2B. The induction of GCN4 expression and the inhibition of cell growth that accompanies high level of eIF-2a phosphorylation is dependent on the GCN3 protein.

The observations that mutations in GCN3 makes protein synthesis less sensitive to eIF-2(αP), provides important genetic evidence for the idea that phosphorylation of eIF-2 regulates translation in yeast by reducing eIF-2B function. The GCD7 and GCD2 subunits of eIF-2B share regions of sequence similarity with GCN3 suggesting that all the three proteins are involved in this regulatory function. Probably the GCD1 and GCD6 protein form the active site for nucleotide exchange (Hinnebush, 1994).

Besides eIF-2α phosphorylation, the recent studies suggest that conditions such as phosphorylation of one of the eIF-2B subunits (82 kDa) and changes in

redox levels can also regulate the GNE activity of eIF-2B. The phosphorylation of 82 kDa subunit of reticulocyte eIF-2B by CK II *in vitro* is associated with an increase in the GNE activity of the factor (Dholakia and Wahba, 1988). This finding suggests that the 82 kDa subunit of eIF-2B is apparently associated with the GNE activity. The functions of the other subunits of eIF-2B are however, not clear. Some recent data suggest that eIF-2B may also be involved in the release of eIF-2 GDP from the 60S subunit of 80S initiation complexes (Thomas *et al,* 1984; Ramaiah *et al.,* 1992). Various agents like polyamines, NADPH, NADP+, ATP, heparin, sugars and other ligands are found to modulate the activity of mammalian eIF-2B (Dholakia *et al.,* 1986; Akkaraju *eta/.,* 1991; Oldfield *et al.,* 1992, Kimball and Jefferson, 1995; Singh *et al.,* 1995). While there is no good evidence for changes in intracellular levels of polyamines, the ratios of NADH/NADP+ can be altered in cells under certain conditions. An enhanced NADH/NADP+ ratio can enhance eIF-2B activity (Dholakia *et al.,* 1986; Akkaraju *et al.,* 1991).

Matts and London (1984) developed an assay system to study the correlation between eIF-2B activity and protein synthesis in reticulocyte lysates or extracts. In this assay system, the release of labeled GDP or the exchange of unlabeled GDP for labeled GDP in the preformed binary complex is measured. Conditions such as heme-deficiency, addition of dsRNA or oxidized glutathione, which inhibit protein synthesis, are found to inhibit the eIF-2B activity and enhance simultaneously the eIF-2a phosphorylation in reticulocyte lysate (Matts and London, 1984). The specificity of eIF-2α phosphorylation in the regulation of eIF-2B activity in lysates is further demonstrated by using translational inhibitors of protein synthesis namely, pactamycin, puromycin and cyclohexamide. The inhibition of protein synthesis elicited by these agents is not mediated by phosphorylation of eIF-2α and has no effect on the lysate eIF-2B activity or the endogenous eIF-2B activity of reticulocyte lysate (Babu and Ramaiah, 1996). The assay system that measures eIF-2B activity has been used to,

a) correlate the inhibition in protein synthesis with reduction in eIF-2B activity in cells under different physiological stress (Kimball and Jefferson, 1990; Prostoko *et al.*, 1992),

b) measure the rapid activation of eIF-2B in insulin and growth hormone-treated Swiss 3T3 fibroblasts (Welsh and Proud, 1992),

c) determine the inactivation of eIF-2B in insect cells which are expressing the mammalian reconstituted eIF-2a kinase (Chefalo *et al.*, 1994),

d) evaluate the over-expression of wild-type and mutants of eIF-2α subunits in rescuing the eIF-2B activity in CHO cells that is mediated by eIF-2a phosphorylation (Ramaiah *et al.*, 1994), and

e) identify the phosphatase activity that can dephosphorylate eIF-2(aP) and restore eIF-2B activity in translating reticulocyte lysates (Babu and Ramaiah, 1996).

Recently, two models have been proposed for understanding the mechanism of eIF-2B, but the conclusions differ. One group proposes a sequential mechanism (Panniers *et al.*, 1988) and another proposes an enzyme substituted mechanism (Dholakia and Wahba, 1989) However, in spite of these contradictions, there is general agreement that phosphorylation of eIF-2α impairs the eIF-2B catalyzed reaction as has been suggested above.

1.4 Role of mutants of eIF-2α in understanding the translational regulation.

The protein synthesis capacity in eukaryotic cells responds to physiological stimuli through a reversible covalent modification of multiple translation initiation factors as has been mentioned above. Inhibition of protein synthesis correlates with prior phosphorylation of eIF-2a and dephosphorylation of eIF-2B, eIF-4E and eIF-4F (Hershey, 1991). Since multiple changes in eIF-2 modifications can occur in response to different environmental stimuli, it is difficult to attribute any single modification as a causative in the response. To overcome this problem, Kaufman

and Hershey have overexpressed mutant eIF-2as to evaluate the importance of individual sites of modifications on eIF-2a in the regulation of protein synthesis (Kaufman *et al,* 1989). Similar approach has also been used by many others now to understand the importance of eIF-4s and eIF-2a kinases.

The importance of eIF-2a phosphorylation in translational control is elucidated by the expression of wild-type eIF-2a and serine to alanine mutants at residues 48 and 51 (48A and 51A mutants) (Davies *et al.*, 1989 Kaufman *et al,* 1989; Choi *et al,* 1992; Murtha-Riel *et al.*, 1993). Inhibition in the translation of adenovirus mRNA and plasmid derived mRNA's mediated by PKR phosphorylation was rescued by the expression of mutant eIF-2a (Davies *et al,* 1989;. Kaufman *et al.,* 1989). Further, it was demonstrated recently that expression of above mentioned mutants of eIF-2α partially protected cells from the inhibition of protein synthesis in response to heat shock (Murtha-Riel *et al.*, 1993). Although in a 51A mutant, the eIF-2a is not phosphorylated, the serine 51 residue in the 48A mutant is phosphorylated suggesting that these two mutants promote eIF-2α recycling by different mechanisms. Also, a mutant of eIF-2α, in which the amino acid at serine 51 is replaced by aspartic acid, inhibition of translation occurs presumably because aspartic acid mimics phosphorylated serine at position 51. In contrast, the expression of other mutants, 48A or 51A is found to bypass the protein synthesis inhibition mediated by eIF-2a phosphorylation (Choi *et al.*, 1992; Murtha-Riel *et al,* 1993). These studies have shown that the mutant eIF-2α exchanges out eIF-2a in the native trimeric endogenous eIF-2. Since one of these mutants, 51A cannot be phosphorylated, it is predicted that the expression of this mutant eIF-2α can bypass protein synthesis inhibition by protecting the eIF-2B activity, but it is unclear as to how the phosphorylated 48A mutant eIF-2a can overcome the inhibition in protein synthesis. Subsequent studies by Ramaiah *et al,* (1994), have suggested that alterations of serine 48 may effect the interaction of phosphorylated mutant eIF-2a with eIF-2B or the relative affinities of this mutant eIF-2a for GTP and GDP may be altered in such a way as to modify its

requirement for eIF- 2B for its recycling. This is because the expression of either mutant reduces the inhibition in GNE activity of eIF-2B that is mediated by eIF-2α phosphorylation.

Further, the availability of mutant eIF-2a clones help to characterize the inhibition of protein synthesis that is mediated by eIF-2α phosphorylation. For example, calcium depletion is known to inhibit protein synthesis and increases eIF-2a phosphorylation (Preston and Berlin, 1992, Prostoko *et al,* 1992). With the help of mutants of eIF-2α and also mutants of PKR kinase, it has been recently demonstrated that calcium depletion from the endoplasmic reticulum activates PKR and the inhibition of protein synthesis is confirmed to be mediated by eIF-2a phosphorylation (Srivastava *et al.,* 1985). Since the mutants of eIF-2α (48A or 51A) can bypass protein synthesis inhibition caused by endogenous wild-type eIF-2a phosphorylation, coexpression with mutants of eIF-2a are also found helpful in overexpressing the eIF-2a kinases whose expression is otherwise inhibitory to protein synthesis (Chefalo *et al.,* 1994). Consistent with these findings, the recent studies also indicate that abrogation of translation initiation factor-2 phosphorylation causes malignant transformation in NIH 3T3 cells (Donze *et al.,* 1995).

1.5 Regulation of initiation factor-2 activity in plant systems.

It is now clear that phosphorylation of eIF-2a is a major mechanism in the regulation of protein synthesis in animal systems and yeast as mentioned above. Mammalian eIF-2a kinases like HRI and PKR can functionally substitute for yeast eIF-2a kinase (GCN2) in the GCN2 translational control (Dever *et al.,* 1993) and also the expression of mammalian eIF-2a kinase inhibits insect cell (non mammalian) protein synthesis and eIF-2B activity (Chefalo *et al.,* 1994). However, it is not known if such a mechanism exists in the regulation of protein synthesis in plants. In the case of plant objects, the translational initiation factors from wheat germ have been investigated the most (Benne *et al.,* 1980; Seal *et al.,* 1983; Mehta

et ai, 1986; Shaikin *et ai,* 1992). The wheat germ eIF-2 has been isolated and studied by a number of research workers (Spermulli *et ai,* 1977; Lax *et ai,* 1986; Clark and Ranu, 1987; Shaikin *et ai,* 1992; Krishna *et ai,* 1994) but the mechanism for the regulation of eIF-2 activity in plant cells has not yet been demonstrated. It is generally believed that such mechanisms are similar to those of mammalian cells, but up to now it is not clear whether there are functional analogs for eIF-2B factors in wheat germ (Lax *et ai,* 1982; Seal *et ai,* 1983; Osterhoust *et ai,* 1983) and whether eIF-2B factor in plants is regulated by eIF-2 phosphorylation (Benne *et ai,* 1980; Browning *et ai,* 1985) Also there is a considerable confusion in the literature regarding the phosphorylation of plant eIF-2, in large part, due to the discrepancy in subunit identification: purified wheat germ eIF-2 is composed of three subunits, Mr 36,000, Mr 41-42,000 (doublet subunit) and Mr 52,000 (Benne *et ai,* 1980; Spermulli *et ai,* 1979, Seal *et ai,* 1983; Lax *et ai,* 1986; Mehta *et ai,* 1986; Shaikin *et ai,* 1992). Several studies, including those from this laboratory, have indicated that mammalian eIF-2α kinases can also phosphorylate the 41-42kDa doublet subunit of plant eIF-2 (Janaki *et al.*, 1995).

Recent studies from this laboratory (Janaki *et ai,* 1995) with purified initiation factor-2 (wheat germ eIF-2) indicate that two of the wheat germ eIF-2 subunits can be phosphorylated by CK II and also in translating wheat germ lysates which are treated with sulfhydryl reactive agents such as N-ethylmaleimide (NEM) and dithiothreitol (DTT). These conditions while inhibit protein synthesis however, cannot impair the GNE activity associated with wheat germ eIF-2 Also some of the results in the above study are in agreement with the earlier studies (Benne *et ai,* 1980; Shaikin *et ai,* 1992; Mehta *et ai,* 1986) which suggest that mammalian eIF-2a kinases phosphorylate the p41-42 doublet subunit of wheat germ eIF-2 but cannot however, impair the wheat germ translation. These findings suggest that probably none of the conditions phosphorylate wheat germ eIF-2 at the right site, so that its GNE activity can be inhibited.

While oxidized glutathione does not stimulate eIF-2 phosphorylation, addition of NEM or DTT can stimulate the phosphorylation of several proteins of wheat germ lysate, including the p41-42 and p36 subunits of wheat germ eIF-2 (Janaki *et al,* 1995). Phosphorylation of either of the subunits in wheat germ eIF-2 cannot be correlated to protein synthesis inhibition through a reduction in GNE activity associated with wheat germ eIF- 2 (Janaki *et al,* 1995).

Earlier studies to purify eIF-2B equivalents from wheat germ did not meet with any success (Lax *et al,* 1982; Osterhout *et al,* 1983) A study by Shaikin *et al,* (1992) suggests that unlike in mammals wheat germ eIF-2 may not require an eIF-2B-like protein and the affinity of wheat germ eIF-2 for GDP and GTP is not markedly different.

In contrast to these observations, another study dealing with higher plants describes that tobacco mosaic virus infection or addition of dsRNA to uninfected host-cell extracts enhances the phosphorylation of a host encoded protein (p68) which appears to share many properties with PKR of mammalian cells (Crum *et al,* 1988) Whether p68 has an eIF-2α kinase-like activity is not known till recently. Just at the conclusion of this thesis work, a paper by Roth's group (Langland *et al,* 1996) suggested that higher concentrations of dsRNA (10-100 μg/ml) stimulates plant PKR activity and phosphorylation of p41-42 doublet subunit of wheat germ eIF-2 Such higher concentrations of dsRNA are infact known to inhibit mammalian PKR activity. This observation is contradicting earlier observations which have suggested that the presence of dsRNA in plant systems in a wide range of concentrations results in the suppression of protein synthesis neither *in vitro* (Reijnders *et al,* 1975; Grill *et al,* 1976, Pratt *et al,* 1978) nor apparently *in vivo* (Dezoeten *et al,* 1989). Also the concentration of dsRNA required as reported by Langland *et al,* (1996), to stimulate plant eIF-2α phosphorylation are relatively very high compared to the concentration of dsRNA required to stimulate mammalian eIF-2a phosphorylation in the lysates In fact

such high concentrations of dsRNA are shown to inhibit the eIF-2a kinase activity in mammalian systems triggered by low concentrations of dsRNA.

To determine the presence of translational inhibitors (if any), in wheat germ cell free translational systems, Rychlik *et al.,* (1980) isolated a 20 kDa protein kinase which phosphorylates two polypeptides present in the preparations of unwashed wheat germ ribosomes. Since the preparation cannot phosphorylate salt washed ribosomes, the above findings suggest that the kinase may be phosphorylating non ribosomal proteins. Interestingly, this kinase inhibits the translation of BMV RNA 1 and 2 but not BMV RNA 4. Since it does not affect the translation of poly U directed polyphenylalanine synthesis, the kinase appears to inhibit translation at the initiation level. The mechanism of this inhibition is not studied as yet.

A second wheat germ kinase, partially purified by Ranu (1980) appears to phosphorylate eIF-2 from both wheat germ and rabbit reticulocyte lysates This kinase is also shown to inhibit wheat germ translation. The physical properties of this kinase and the mechanism of inhibition of translation by this kinase have not been studied so far

A third kinase (32 kDa) isolated from wheat germ by Davies and Polya (1983) appears to be physically similar to a kinase purified by Yan and Tao (1982). This kinase phosphorylates preferentially a 48 kDa polypeptide in wheat germ. It is also shown to phosphorylate the 41-42 kDa subunit of wheat germ eIF-2 and the 107 kDa subunit of eIF-3. In addition, this kinase is shown to phosphorylate three proteins (38, 14.8 and 12.6 kDa) of the 60S ribosomal subunit. Phosphorylation of eIF-2, eIF-3 or 60S ribosomal subunits by this kinase does not affect their activities *in vitro* (Browning *el al.,* 1985).

The recent studies by Langland *et al,* (1996) point out that phosphorylation of plant eIF-2a can inhibit protein synthesis, it is not clear however, if the inhibition in protein synthesis is mediated by a decrease in the GNE activity of eIF-2B-like protein in plants. We are however, unable to stimulate eIF-

2a phosphorylation of wheat germ eIF-2 by the addition of low to high concentrations (100 ng-100 μg/ml) of poly IC or dsRNA in wheat germ lysates Also our recent findings in (Janaki *et al,* 1995) suggest that many of the redox agents or kinases like CK II that inhibit wheat germ translation and promote wheat germ eIF-2 phosphorylation do not affect the GNE activity associated with wheat germ eIF-2. Also the findings of Shaikin *et al,* (1992) suggest that probably wheat germ eIF-2 does not require eIF-2B-like protein to exchange guanine nucleotides, since the affinity of wheat germ eIF-2 for GDP and GTP is not markedly different and appears to be different from mammalian eIF-2.

Keeping in view these observations, the present work has been carried out with the objectives mentioned below.

1.6 OBJECTIVES

Recently Dr Ramaiah's laboratory has undertaken to study the regulation of initiation factor-2 activity both in plants and animals. While one of my colleagues (Janaki, 1996) here in the laboratory has been working on the regulation of eIF-2 activity in translating wheat germ lysates, the present studies here have been addressed to determine

A) the nature of guanine nucleotide exchange activity associated with purified wheat germ eIF-2 and its sensitivity towards phosphorylated wheat germ and reticulocyte eIF-2 subunits.

B) similarities or differences (if any) in the functional aspects and in the phosphopeptides (generated by limited proteolytic digestion) between phosphorylated wheat germ and reticulocyte eIF-2 subunits.

In order to carry out this work, we have

prepared translating lysates and eIF-2 from both wheat germ and reticulocyte lysates,

identified kinases that can phosphorylates both the above substrates *in vitro,*

- raised polyclonal antibodies against wheat germ eIF-2,
- studied the effect of phosphorylation of both the eIF-2 preparations on the GNE or eIF-2B-Iike activity associated with wheat germ eIF-2 and of reticulocyte lysates,
- assessed the ability of phosphorylated wheat germ eIF-2 to interact with reticulocyte eIF-2B to form 15S complex and on reticulocyte eIF-2B activity, and
- assessed the ability of wheat germ eIF-2 to enter into the initiation cycle of protein synthesis in reticulocyte lysates, and finally,
- analyzed the phosphopeptides of wheat germ and reticulocyte eIF-2 by limited proteolytic digestion.