Regulation of Guanine Nucleotide Exchange Activity Associated with Wheat Germ Initiation Factor-2 (eIF-2); Effect of Phosphorylation of Wheat Germ and Reticulocyte eIF-2

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DOCTOR OF PHILOSOPHY

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CONTENTS **Page No.**

ABBREVIATIONS

ADP	-	Adenosine 5' diphosphate
AP		Alkaline phosphatase
APS	-	Ammonium per sulphate
ATP	-	Adenosine 5' triphosphate
BCIP	-	5-bromo-4-chloro-3-indolyl phosphate
Binary complex	-	complex of eIF-2 and GDP/GTP
Bis-acrylamide	-	N, N'-methylene-bis-acrylamide
BMV RNA	-	Brome mosaic virus RNA
BSA	-	Bovine serum albumin
CH	-	Cycloheximide
Ci	-	Curie
CHO	-	Chinese hamster ovary cells
CK II	-	Casein Kinase II
CM-Sephadex	-	Carboxy methyl sephadex
CP	-	Creatine phosphate
CPK	-	Creatine phospho kinase
cpm	-	counts per minute
DEAE	-	Diethy aminoethyl
DNA	-	Deoxy ribonucleic acid
dsRNA	-	**double stranded ribonucleic acid**
DTT	-	dithiothreitol
EDTA		Ethylene diamine tetra acetic acid
EF	-	Elongation factor
EGF		Epidermal growth factor
eIF	-	Eukaryotic initiation factor
eIF-2	-	Eukaryotic initiation factor 2
eIF-2a		Alpha subunit of eukaryotic initiation factor 2

eIF-2(αP) - Phosphorylated alpha subunit in eIF-2

Fig. - Figure

GCN - General control non derepressable

GDP - Guanosine 5' diphosphate

GEF/eIF-2B/RF/co eIF-2C - Guanine nucleotide exchange factor or reversing factor

GNE - Guanine nucleotide exchange

GSSG - Oxidized glutathione

GTP - Guanosine 5' triphosphate

HCR/HRI - Heme regulated inhibitor

HEPES - N-[2-hydroxyethyl] piperzine-N'-[2-ethane-sulfonic acid]

HIV - Human immuno deficiency virus

HSP - Heat shock protein

H_2O_2 - hydrogen peroxide

kDa - Kilodaltons

min - minutes

Mr. - Molecular weight

mRNA - messenger RNA

NADH - Nicotinamide adenine dinucleotide, reduced

NADPH - Nicotinamide adenine dinucleotide phosphate, reduced

NBT - Nitro blue tetrazonium

NEM - N-ethylmaleimide

PAGE - Polyacrylamide gel electrophoresis

PKR/dsI - double stranded RNA activated inhibitor

pmol - pico mole

RF - Reversing factor; see also GEF

RNA - ribonucleic acid

rRNA - ribosomal RNA

RNase - Ribonuclease

poly IC/$((rI)_n$-$(rC)_n)$ - Synthetic polymer of Inosine and Cytosine

S	-	Svedberg
SDS	-	Sodium dodecyl sulphate
S. aureus	-	*Staphylococcus aureus*
SV8	-	*Staphylococcus aureus* protease
TCA	-	Trichloro acetic acid
TEMED	-	N',N N,N', N'-tetramethyl ethyl-ethylene diamine
Ternary complex	-	Complex of eIF-2, Met.tRNA$_i$ and GTP
Tris	-	Tris (hydroxymethyl) amino methane
tRNA	-	transfer RNA
WG.eIF-2	-	Wheat germ eIF-2

LIST OF FIGURES

Fig. 15. Polysome profiles of reticulocyte lysates treated with heme, heme and poly IC and heme, poly 1C and wheat germ eIF-2

Fig. 16. Western immunoblot analysis of the 10-50% sucrose gradient fractions of reticulocyte lysates supplemented with or without wheat germ eIF-2; detection of the presence of wheat germ and reticulocyte eIF-2 in the polysome fractions

Fig. 17. Wheat germ lysate protein synthesis in the presence of varying concentrations of poly IC.

Fig. 18. [^{32}P] phosphoprotein profiles of wheat germ lysates in the presence of different concentrations of poly IC and wheat germ eIF-2

Fig. 19. Effect of poly IC-treated wheat germ lysate on wheat germ eIF-2.[^{3}H]GDP complex dissociation

Fig. 20. Phosphorylation of wheat germ eIF-2 subunits by HRI and CK II *in vitro* for phosphopeptide analysis.

Fig. 21. Cleveland partial peptide digestion of phosphorylated wheat germ eIF-2 subunits

Fig. 22. SV8 protease digestion products of p41-42 subunit of wheat germ eIF-2 phosphorylated by HRI, CK II and HRI + CK II added together or at different time points.

Fig. 23. Cleveland mapping of phosphorylated p41-42 subunit of wheat germ eIF-2 and alpha-subunit of reticulocyte eIF-2 in the presence of SV8 protease enzyme; effect of two concentrations of the protease

LIST OF TABLES

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.

2.0 MATERIALS AND METHODS

2.1 Preparation of heme-deficient reticulocyte lysate:

Heme-deficient rabbit reticulocyte lysates which can respond to added hemin *in vitro,* have been prepared from New Zealand white male anemic rabbits as described (Hunt *et al.*, 1972). Rabbits (approximately 2 Kgs in weight) were made anemic by injecting subcutaneously 2.5 ml of 1% acetyl phenyl hydrazine consecutively for four days. On the 9th day, the rabbits were bled through the ear vein. Blood was collected into a beaker rinsed with heparin solution. 300 units of heparin were added for every 50 ml of blood. Red blood cells were harvested by centrifugation at 2000 rpm for 10 min in a refrigerated centrifuge. The buffy coat containing white blood cells was removed and the cells were washed 3 times with buffered saline solution (containing 5 mM Hepes-KOH, pH 7.2, 5 mM glucose, 0.14 M NaCl, 5 mM KC1 and 5 mM $Mg(OAc)_2$). While removing the coat, care was taken to avoid drawing the red blood cells. Cells were then lysed by the addition of an equal volume of ice-cold double distilled water. The stroma was then removed by centrifugation at 10,000 rpm for 15 min. The supernatant was decanted, distributed into 1 ml eppendorf tubes and was stored in liquid nitrogen.

2.2 Measuring reticulocyte lysate protein synthesis:

A standard incubation mixture contained the following ingradients in a total reaction volume of 25 ul: 60% reticulocyte lysate, 4 μM creatine phosphate (CP), 250 ug creatine phosphofructokinase (CPK), 80 mM KC1, 1 mM Mg(0Ac)2, 200 μM GTP, 33 uM amino acid mix without leucine and 33 uM [^{14}C]leucine (specific activity, 340 mCi/mmol) (Ernst *et al.*, 1978). Where indicated, the reaction mixtures were also supplemented with hemin (20 μM) and other agents or components whose effects were investigated. The components of the incubation mixture were mixed together on ice and the protein synthesis reactions were carried out at 30°C. Portions of 5 ul were removed at different time intervals and spotted on a Whatman No. 1 filter paper. The proteins were precipitated by placing the filter discs in ice-cold 10% trichloroacetic acid

(TCA) for 20 min. The filters were then transferred to 5% boiling TCA for 5 min to remove the non-specific radioactivity. Later, the filters were washed with 5% TCA at room temperature. The filters were again washed with ethanol and acetone. The filters were then dried and placed in 1:1 diluted H_2O_2 solution for 10 min to bleach the red color present on the filters, to avoid any quenching effects while reading the filters in a scintillation counter. The filters were again washed in ethanol and acetone before drying. The proteins containing the radiolabelled leucine incorporated into them were read in a liquid scintillation counter to determine the protein synthesis capacity of the extracts. Protein synthesis of a typical heme-deficient reticulocyte lysate, from two different batches, in the presence and absence of added hemin is shown in Fig 1a and 1b.

2.3 Preparation of wheat germ lysate:

Wheat germ lysate was prepared as described by (Roberts and Patterson, 1973; Ramaiah and Davies 1985). All the glassware and water was autoclaved. About 20 g of wheat germ was floated on carbon tetrachloride and cyclohexane mixture in the ratio of 2.5:1. Three grams of the floated wheat germ was removed with the help of a spatula and was vacuum dried (1 hr in a hood) before processing further. The dried floated germ was powdered in liquid nitrogen and made into a paste with extraction buffer (containing, 40 mM Hepes-KOH, pH 7.2, 100 mM KOAc, 1 mM $Mg(OAc)_2$, 2mM $CaCl_2$, and 1 mM DTT) on ice. All the extraction and subsequent procedures were done as quickly as possible at 4°C. The paste was spun at 15000 rpm for 15 min in a high speed refrigerated centrifuge. The top 3/4th supernatant was collected and clarified again at 10,000 rpm for 15 min. Three milliliters of the supernatant was loaded on a 50 x 2.5 cm Sephadex G-25 column which was preequilibrated with column buffer containing 40 mM Hepes-KOH pH 7.6, 120 mM KOAc, 5 mM $Mg(OAc)_2$ and 1 mM DTT. Elution was done with column buffer and 3 ml fractions were collected. Highly turbid fractions were pooled and spun at 15,000 rpm for 20 min.

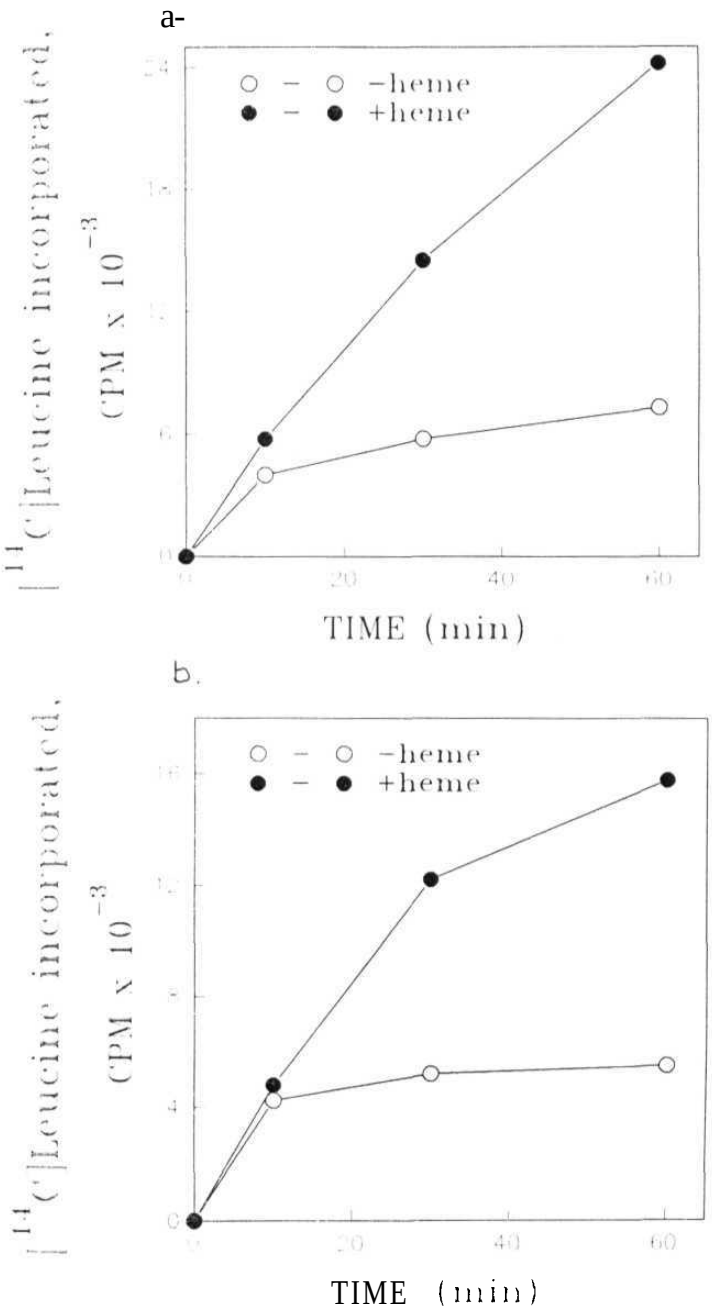


Fig. 1a. **and 1b.** Reticulocvte lysate protein synthesis from two different batches of lysates.

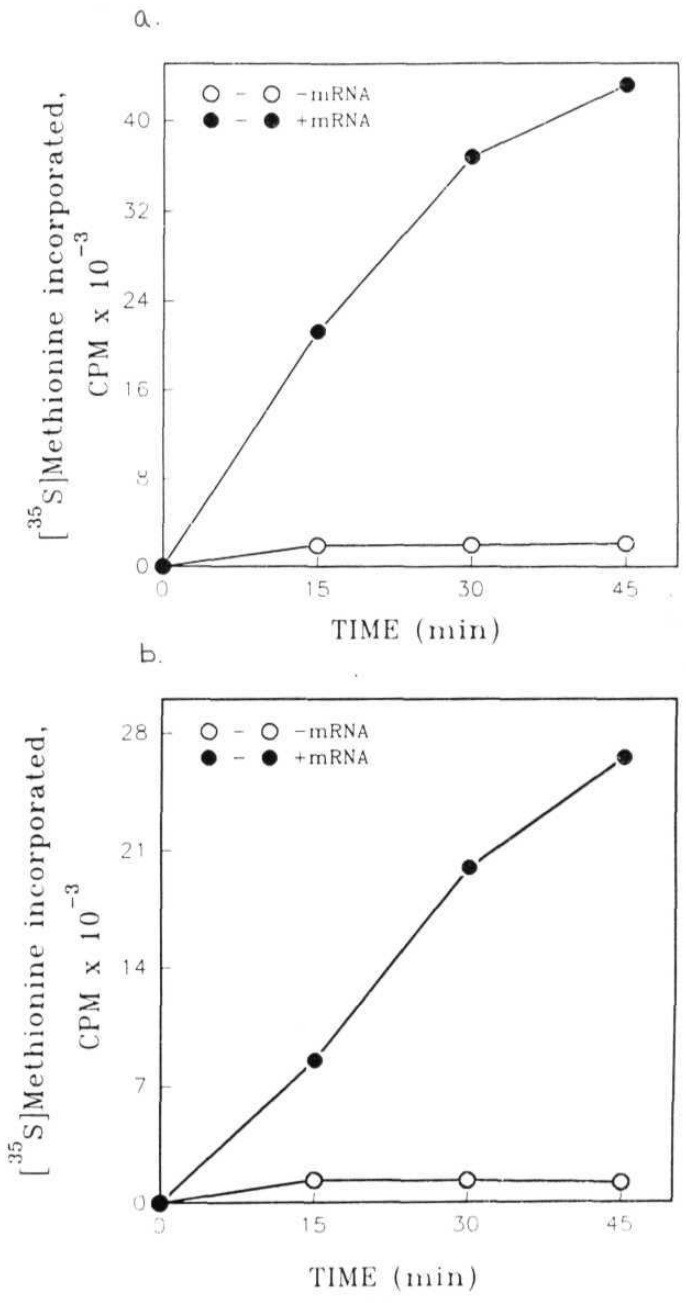


Fig. 2a. **and** 2b. Wheat germ lysate protein synthesis from two different batches of lysates.

The top 3/4th supernatant was collected and stored in 0.5 ml aliquots in liquid nitrogen.

2.4 Wheat germ lysate protein synthesis:

Wheat germ lysate protein synthesis was performed as described (Janaki *et a!.*, 95). Since the endogenous message (mRNA) in wheat germ lysate is almost absent, an exogenous message, Brome Mosaic Virus (BMV) mRNA was used in all the translation experiments. Typically, the reaction mixture in a 25 µl volume contained 20 mM Hepes-KOH, pH 7.6, 1.8 mM ATP, 80 µM GTP, 8 mM CP, 64 µg/ml CPK, 20 µM of all the amino acids except methionine. Optimal concentration of $Mg(OAc)_2$ and KC1 have been determined for each batch of lysate. 20 µM labeled [^{35}S]methionine (specific activity 1100 Ci/mmol) was supplemented to reactions to determine protein synthesis. Samples of 5 µl were removed at different time intervals and spotted on a Whatman filter paper Proteins in the samples were precipitated by keeping the filters in 10% cold TCA for 10 min. Afterwards, the filters were washed with 5% hot TCA for 3-5 min and with 5% TCA at room temperature to remove any non-specific radioactivity. Later the samples were washed with ethanol and acetone and finally air dried. Radioactivity of the filters was counted by a liquid scintillation counter. Protein synthesis of a typical wheat germ lysate preparation (from two different batches of wheat germ) in the presence and absence of added mRNA is shown in Fig. 2a and 2b.

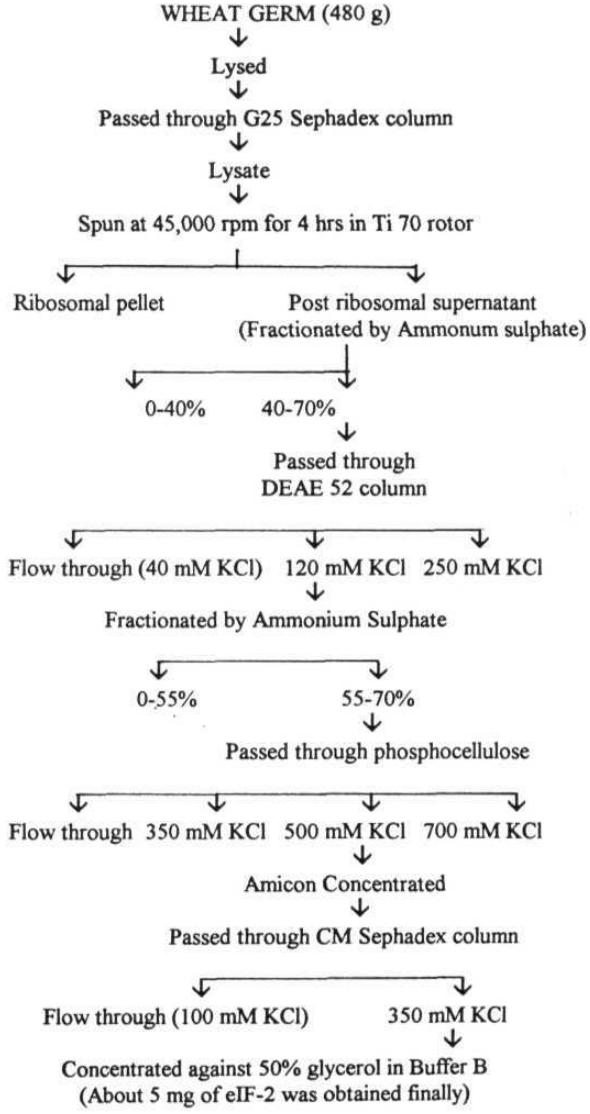
2.5 Preparation of wheat germ eIF-2:

Wheat germ eIF-2 was prepared as described earlier by Lax *et ai,* (1986) with some minor modifications to enhance the purity of the preparation. All the steps in the procedure were carried out at 4°C unless otherwise indicated. All the various steps in the purification scheme are as follows (Fig. 3).

a) *Preparation of post-ribosomal supernatant:* 480 g of wheat germ was divided into four batches, ground into a fine powder in liquid nitrogen and mixed with

Fig. 3. Schema for the **wheat** germ **eIF-2 purification:** Wheat germ eIF-2 is purified from the post ribosomal supernatant (PRS) by ion-exchange chromatography as described in 'Materials and Methods'

SCHEMA FOR WHEAT GERM eIF-2 PURIFICATION



buffer A containing 20 mM Hepes-KOH, pH 7.6, 120 mM KC1, 1mM $Mg(OAc)_2$, 2 mM CaC12, 6 mM β-mercaptoethanol and with protease inhibitors like soybean trypsin inhibitor (0.1 mg/ml) and phenyl methyl sulphonyl fluoride (0.5 mM). For every gram of wheat germ 1.15 ml of buffer A was used. The paste was centrifuged at 12,000 rpm for 20 min. The top yellowish fatty layer was removed with a spatula and the top 3/4th content was drawn out and passed through 650 ml G-25 column. The protein eluate of the void volume was collected and spun at 16,000 rpm. The 16 K supernatant was spun at 45,000 rpm for 3.5 hours in a Ti70 Beckman rotor. The supernatant thus obtained is referred here as the post-ribosomal supernatant (PRS). A total of 35 g of protein was obtained from 480 g of wheat germ at this step.

b) *Ammonium sulphate fractionation of the post-ribosoma! supernatant (PRS).* The entire PRS (1 liter) containing 120 mM KCl obtained as described above was brought to 40% saturation by the gradual addition of 226 g of ammonium sulphate. The contents were stirred for 45 min at 4°C and centrifuged at 10,000 rpm for 60 min. The 10 K supernatant was brought to 70% saturation by the gradual addition of 200 g of ammonium sulphate. The contents were stirred and centrifuged at 10,000 rpm again. The 10 K pellet obtained in this step was resuspended in about 50 ml of buffer B containing 40 mM KCl. (Buffer B contains 20 mM Tris- HCl, pH 7.6, 1mM DTT, 0.1 mM EDTA and 10% glycerol). The suspension was then dialysed against 100 volumes of buffer B in 40 mM KCl and then clarified by centrifugation at 10,000 rpm for 10 min prior to storage.

c) Separation of 40-70% ammonium sulphate fraction of PRS on DEAE-52:

The 40-70% ammonium sulphate fraction of PRS (105 ml containing 11.1 g of total protein was diluted with buffer B containing 40 mM KCl (in the ratio of 1:4 and was applied to a 200 ml DEAE-52 column which is equilibrated with the above buffer B. The column was washed with the same buffer until the absorbency of the washed fraction at 280 nm was less than 0.1. The proteins of

the column were then eluted with 120 and 250 mM KC1. Twenty milliliter fractions were collected and those fractions whose absorbency was above 0.4 were pooled and concentrated by Amicon YM 10 membrane.

d) *Purification of eIF-2 on phosphocellulose and on CM Sephadex C-50 column (CM Sephadex):* The 120 mM KC1 concentrated fractions of DEAE-52 was fractionated and concentrated by 0-55% and 55-80% ammonium sulphate. The fractions were dialysed with buffer B containing 100 mM KC1. The dialysed fraction (25 ml containing 1.1 g of total protein) was applied to a 45 ml phosphocellulose column equilibrated in the above buffer B. The protein in the column was then eluted with buffer B containing 350, 500 and 700 mM KC1. Three milliliters fractions were collected, pooled and concentrated by 0-70% ammonium sulphate fractionation.

The fractions were dialysed against buffer B containing 100 mM KC1. The proteins that were eluted with 500 mM KC1 contained most of the eIF-2 in it as evidenced by gel electrophoresis (Fig. 4a). The concentrated 500 mM KC1 fraction of phosphocellulose column (2.7 ml containing 8.1 g of protein) was further chromatographed on a CM Sephadex column. The column was preequilibrated with 100 mM KCl in buffer B. After washing the column with 100 mM KC1, the bound proteins were eluted with 350 mM KCl. The eIF-2 fractions of the CM Sephadex column were dialysed against buffer B containing 50 mM KC1 and was concentrated against buffer B containing 50% glycerol.

The purity of eIF-2 was tested by

1) separation on SDS-PAGE and comparing it with purified wheat germ eIF-2 obtained from a laboratory well known for their work on wheat germ eIF-2 purification, 2) its ability to bind labeled GDP to form an eIF-2.GDP binary complex, 3) its ability to form a ternary complex with Met.tRNAi and GTP, 4) cross reacting the polyclonal antibodies raised against purified WG eIF-2 subunits to WG eIF-2 obtained from a reputed laboratory, and also 5) its ability to get

phosphorylated on one of its subunits by reticulocyte heme-regulated eIF-2α kinase (HRI) *in vitro* (please see section A).

2.5.1 Assays for eukaryotic initiation factor -2:

eIF-2 activity was measured by two methods. First, by its ability to bind labeled GDP by forming a stable binary complex [eIF-2.[^{3}H]GDP] in the presence of Mg^{2+}. Second, by its ability to form a ternary complex with GTP and Met.tRNA$_i$ as described (Chakraborty *et al.*, 1987).

2.5.1.1 Preparation of wheat germ binary complex, eIF-2.[^{3}H]GDP, and its dissociation in vitro:

Purified wheat germ eIF-2 (2 ug) was incubated in 20 µl reaction mixtures containing Tris-HCl (20 mM, pH 7.8, KCl (100 mM), creatine phosphofructokinase (CPK, 100 µg/ml) and [^{3}H]GDP (2 µM, ~1900 cpm/pmol) at 25°C for 10 min to form an eIF-2.[^{3}H]GDP binary complex as described (Janaki *et ah,* 1995). The complex was stabilized by the addition of 2.5 mM $Mg(OAc)_2$). Exchange of unlabeled GDP for labeled GDP was studied by the addition of 40 µM unlabeled GDP. Reactions were carried out at 25°C for specified time intervals as described in the figure legends. The reaction mixtures were stopped by the addition of 3 ml of cold wash buffer (containing 20 mM Tris-HCl, pH 7.8, 80 mM KCl and 2.5 mM $Mg(OAc)_2$ and the contents were filtered through HAWP 0025 nitrocellulose (0.45 µM) Millipore filters. Undissociated eIF-2.[^{3}H]GDP binary complex was measured by the retention of the complex on the Millipore filters. The filters were dried and the amount of labeled GDP bound to eIF-2 on the filter paper was measured in a liquid scintillation counter.

After drying the filter papers, the amount of labeled GDP bound to eIF-2 was estimated by counting the filters in a liquid scintillation counter.

2.5.1.2 Preparation of [^{35}S]Met.tRNA$_i$

Charging of [^{35}S]methionine was done as described (Lax et al, 1986; Chakraborti *et al.*, 1987). Briefly, the reaction mixture in a total volume of 4 ml contained 100 mM HEPES-KOH, pH 7.6, 5 mM $Mg(OAc)_2$, 2.5 mM ATP, 2 mg E.coli synthetase, 20 A_{260} units of yeast Met.tRNAi (prepared according to Walker and Rajbhandary, 1972) and 3 uM [^{35}S]methionine (1100 Ci/mmol). The reaction mixture was incubated at 37°C in a water bath for 20 min. To the reaction mixtures was added 0.4 ml of 2 M KOAc, pH 6.0 and 4.4 ml of phenol (saturated with 0.1 M KOAc, pH 6.0). Phenol extraction was repeated twice. The aqueous phases were pooled and dialysed twice for 3 hours, first against 50 volumes of high salt buffer 0.5 M KC1, 50 mM KOAc containing 1 mM DTT and then against a no salt buffer, 5 mM KOAc, pH 5.0.

2.5.1.2.1 Formation of eIF-2.Met.tRNA, complexes:

Reaction mixtures (100 |il) containing 20 mM Hepes-KOH pH 7.6, 100 mM KCl, 2.5 mM DTT, 0.2 mM GTP, 20 ug BSA (as a carrier), 25 pmol of [^{35}S]Met.tRNA$_i$ (1000 Ci/mol) and a source of initiation factor was incubated for 10 min at 25°C. After incubation, 2 ml of cold wash buffer (containing 20 mM Tris-HCl, pH 7.6, 100 mM KC1) was added and filtered through 0.45 μM nitrocellulose filters. The filters were washed thrice with 2 ml of cold wash buffer before drying and counting in a liquid scintillation counter.

2.6 Estimation of reticulocyte eIF-2B activity in lysates:

GNE activity of eIF-2B in translating reticulocyte lysates was measured by its ability to dissociate the preformed labeled binary complex, eIF-2.[^{3}H]GDP, as described (Matts and London, 1984; Ramaiah *et al.*, 1994). In step 1, [^{3}H]GDP (2 μM, 2400 cpm/pmol) was incubated with reticulocyte eIF-2 (0.8 ug in 20 μl assay mixture) at 30°C for 10 min to form the eIF-2.[^{3}H]GDP binary complex as described (Matts and London, 1984; Ramaiah *et al.*, 1994, Babu and Ramaiah., 1996). Details and modifications (if any) are mentioned in the figure legends.

The complex was stabilized with the addition of 1 mM $Mg(OAc)_2$. In step 2, protein synthesis was carried out in reticulocyte lysates (25 µl) as described above, except that, unlabeled leucine was used. In step 3, the preformed reticulocyte eIF-2.[^{3}H]GDP binary complex (20 µl) was added to translating lysates (25 µl) under difierent conditions as mentioned in the figure legend. The reactions were terminated at different time intervals with the addition of 4 ml of cold wash buffer (20 mM, Tris- HC1 pH 7.8; 100 mM KC1, 1 mM $Mg(OAc)_2$) and the mixtures were filtered through 0.45 µM-pore-size HAWP 02500 filters (Millipore). The filters were washed twice with cold wash buffer, dried and the radioactivity bound to the filter paper was counted in a Beckman liquid scintillation counter. Picomoles of eIF-2.[^{3}H]GDP dissociated were determined by the difference of the total eIF-2.[^{3}H]GDP added and the amount remaining at the end of reaction.

2.7 Dissociation of wheat germ.eIF-2.[^{3}H]GDP, binary complex, in reticulocyte lysates:

WG.eIF-2.[^{3}H]GDP binary complex was prepared as described above. The ability of this labeled GDP in WG.eIF-2 to exchange with unlabeled GDP or GTP was assayed here in the presence of heme-deficient, hemin- supplemented or heme and poly IC-treated translating reticulocyte lysates, and the amount of WG.eIF-2.[^{3}H]GDP dissociated was estimated as mentioned above. Details and modifications if any, are mentioned in the figure legends.

2.8 Polyclonal wheat germ eIF-2 antibody :

Eight hundred micrograms of WG.eIF-2 protein was run on 10% SDS- PAGE as described (Laemmli, 1970). The bands corresponding to the p36, p41-42 and p52 subunits identified by commassie staining were cut out of the wet gel with the help of a sharp razor blade. The gel slices containing the subunits were pooled and equilibrated in electroelution buffer (containing 25 mM Tris-HCl, 192 mM Glycine and 0.1% SDS) for 1 hr. Part of the gel pieces containing eIF-2 was electroeluted by Bio-Rad electroeluter apparatus (60 V for 4 hr) and the rest of the

sample was electroeluted by a slab gel electrophoresis chamber into a dialysis bag. The elution in the latter case was done for six hours at 30 V. The eluted protein, identified by its blue colour, from both the elution methods was pooled and dialysed extensively against 2.5 mM Tris-HCl pH 7.8, to remove SDS from the electroeluted protein. Concentration of the dialysed sample was done by lyophilization to about 2.0 ml.

2.8.1 Generation of antibodies against wheat germ eIF-2 in New Zealand White male rabbits:

Priming injection (prepared in complete Fruends adjuvent mixed with protein solution in a 1:1 ratio) was done subcutaneously with 300 μg of electroeluted WG.eIF-2 protein in a total volume of 2.0 ml. After about four weeks, a booster injection containing 150 μg of electroeluted WG.eIF-2 in incomplete Fruends adjuvent (mixed in 1:1 proportion) in a total volume of 2.0 ml was administered subcutaneously on both thighs of the rabbit. Again after a week, a second booster injection, similar to the one given above was adminstered.

2.8.1.1 Isolation of control (preimmune) and anti-wheat germ eIF-2 antiserum:

Rabbits were bled through the ear vein after a gap of four to five days after the second booster injection. The blood was collected and incubated overnight at 4°C and spun at 3000 rpm for 30 min at 4°C to collect the supernatant serum. The serum which contains WG.eIF-2 antibody was stored as aliquot at minus 20°C. The blood from the rabbits was also collected prior to injecting them with the antigen to obtain the control serum (preimmune serum).

2.9 Limited proteolytic digestion of wheat germ eIF-2 subunits by Cleveland method:

2.9.1 Phosphorylation of purified wheat germ eIF-2 in vitro:

Phosphorylation of purified WG.eIF-2 and reticulocyte eIF-2 (2 μg each) was carried out at 3 0°C in 10 μl reaction mixtures in the presence of either HRI (0.5 μg) or CK II (10 ng), or both, for the times indicated, as described below. Reaction mixtures (10 μl) contained Tris-HCl (20 mM, pH 7.4), $Mg(OAc)_2$ (2.5 mM), KC1 (80 mM) and [γ-^{32}P]ATP (10 μCi, specific radioactivity 3000 Ci/ mmol) along with the kinase and eIF-2 preparations. Reaction mixtures were terminated by the addition of an equal volume of 2 x SDS sample buffer (see section on SDS-PAGE) . The samples were heated and then run on a 1 mm mini gel apparatus at 100-125 V.

The gels were stained briefly with 1% commassie blue in water and were destained for 10-15 min in excess water. This will help in detecting the molecular weight markers. The gels were then dried and exposed to X-ray film. The phosphorylated bands corresponding to the eIF-2 subunits were identified by overlapping the developed X-ray film on the dried gel.

2.10 Phosphopeptide mapping in one dimension :

Peptide mapping in one dimension by limited proteolysis in SDS-polyacrylamide gels was done as described by Cleveland *et al.*, 1976. The bands corresponding to the p36 and p41-42 subunits of WG.eIF-2 and the a-subunit of reticulocyte eIF-2, which were phosphorylated either by HRI or CK-II or both, were cut out of the dried gel through the X-ray film with a sharp razor blade After removing the gel drying paper from the back of the dried gel piece, the gel slices were equilibrated for at least 60 min in 1 ml of gel slice equilibration buffer (containing 250 μl of 0.5 M Tris-HCl pH 6.8, 10 μl of 10% SDS, 100 μl of glycerol, 2 μl of 0.5 M EDTA, 3 μl of β-mercaptoethanol, 630 μl of water, and a trace amount of bromophenol blue). Equilibration was repeated with a fresh

equilibration buffer, so that all the residual acetic acid and gel drying filter papers present in the gel slice were removed. Inefficient removal of acetic acid can cause a streaking effect on the gel lanes in the final autoradiogram.

The above samples were incubated in the wells of a 15% gel (1 5mm thick with 3-5 cm long stacking gels) for 15 min in the presence of a protease and gel slice overlaying solution that contains 20% v/v glycerol. X-ray films of length 6 x 0.3 cms were used to insert the gel pieces containing the protein sample into the wells.

Electrophoresis was carried out at 100 V until the bromophenol blue clears the stacking gel. Afterwards, the voltage was increased to 150 V. To achieve greater proteolysis with the same amount of protease, the polarity of current was reversed for 3 min just before the bromophenol blue dye enters the resolving gel and then turned again to the normal mode till the end of the run. This ensures maximum digestion of the protein with the protease enzyme. After completion of the run, the gel was dried and exposed to x-ray film.

2.11 Separation of total ribosomes by sucrose gradient fractionation:

After incubation at 30°C, protein synthesis mixtures (125 µl) were chilled on ice and diluted with an equal volume of 2X ice-cold TMK buffer (20 mM Tris-HC1 pH 7.6, 1 mM $Mg(OAc)_2$ and 100 mM KC1). Samples were layered over a 4.8 ml 10-50% sucrose gradient made in TMK buffer and spun in a SW 50.1 rotor for 1 hr at 40,000 rpm in a Beckman centrifuge. 10% and 50% sucrose solutions were made in TMK buffer as follows. 2.4 ml of 10% sucrose solution was layered carefully on a 2.4 ml of 50% sucrose solution in a 5 ml gradient tube The gradients were kept horizontally for 3 hr at room temperature and then again carefully reverted back to the vertical position and kept at 4°C. After about 20 min at 4°C, the reaction mixtures were layered on the top of the gradients. The gradients were spun as described (Ramaiah *et al.*, 1992). Gradient

fractions were collected by upward displacement with continuous monitoring at 280 nm in an ISCO UA-6 density gradient fractionator.

To detect the presence of eIF-2 on ribosomes, 8 drops (350µl) were collected on ice by upward displacement of gradients in the UA-6 density gradient fractionator. The fractions were adjusted to pH 5 by the addition of 1M HO Ac, and the proteins were precipitated on ice for 1 hour. Precipitates were collected by centrifugation at 10,000 rpm for 20 min and the pellets were resuspended in protein dissociation buffer and electrophoresed in a 10% SDS-PAGE as described (Ramaiah *et al,* 1992). The gels were immunoblotted as mentioned below and probed with human anti-eIF-2 a monoclonal antibody or with anti wheat germ eIF-2 polyclonal antibody.

*2.12 **Immunoblot** analysis of **reticulocyte** eIF-2 in the 15S complex of reticulocyte lysates:*

Reticulocyte lysate protein synthesis reactions (125 µl) were carried out at 30°C for 10 min in the presence ofheme (20 µM) , heme and poly IC (20 µM and 75 ng/ml),or heme, poly IC and WG.eIF-2 (10 µg). The lysates were diluted with an equal volume of chilled dilution buffer (20 mM Tris HCl, pH 7.6, 100 mM KCl, and 1 mM $Mg(OAc)_2$) and layered over a 4.8 ml exponential 10-30% sucrose gradient containing 20 mM Tris HCl pH 7.6, 100 mM KCl and 1 mM $Mg(OAc)_2$. Samples were run at 45,000 rpm for 5 hr. 30 min at 4°C in a SW 50.1 rotor to separate free eIF-2 from [eIF-2(aP).eIF-2B] complex. Fractions (350 µl) were collected by upward displacement of the gradients with the help of an ISCO gradient fractionator. Fractions were concentrated by pH 5.0 precipitation as described (Ramaiah *el al.,* 1992) and separated on 10% SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose membrane and the membranes were incubated with the solution containing anti-human eIF-2α monoclonal antibody (a gift from Dr. Edward Henshaw laboratory,

obtained from Dr. Chen in MIT) followed by a second incubation with rabbit anti-mouse alkaline phosphatase conjugated antibody.

2.13 Protein phosphoryiation in translating reticuiocyte lysates:

Protein synthesis in reticuiocyte lysates was carried out (in 25 µl) as described above, except that unlabeled leucine was utilized. Lysates were supplemented briefly with [γ-^{32}P]ATP (10 µCi, specific activity, 3000 Ci/mmol) during 10-15 min of protein synthesis to facilitate the labeling of phosphoproteins. Ten microliters of the reaction mixture was taken out at the end of the reaction and was concentrated by pH 5.0 precipitation as described (Ernst *et a/.,* 1978). Phosphoproteins were resuspended in protein dissociation buffer and were separated by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis as described (Ramaiah *el al.*, 1992) and analyzed by autoradiography. Details are mentioned in the figure legends.

2.14 Protein phosphoryiation in translating wheat germ lysates:

Wheat germ lysates were treated with varying concentrations of poly IC in the presence of 100 |iM cold ATP, with or without wheat germ eIF-2 and incubated for 7 min at 25°C. Lysates were then pulsed with 10uCi of [γ-^{32}P]ATP (3000 Ci/mmol), in the presence of 20 mM Tris-HCl, pH 7.8. 80 mM KCl and 2.5 mM $Mg(OAc)_2$ for a brief period (5 min). Reactions were terminated by the addition of SDS sample buffer and the denatured proteins were separated on 10% SDS-PAGE.

2.15 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE):

Proteins were separated on SDS-PAGE according to modified Laemmli method (1970). Protein samples were prepared in protein dissociation buffer (also called sample buffer) containing Tris-HCl, pH 6.8, 10%glycerol, 10% SDS, β-mercaptoethanol and bromophenol blue. Samples were heated for 3 min in a boiling water bath before loading. 10% separating gel mix in a total volume of 30

ml contained 7.5 ml of 1.5 mM Tris-HCl pH 8.8, 10 ml of 30:0.8 Acrylamide-bis Acrylamide mixture, 0.3 ml 10% SDS , 0.1 ml of 10% Ammonium per sulphate, 7.5 µl TEMED and 12.1 ml water. The 4.5% stacking gel mix in a total volume of 6 ml contained 0.9 ml of 30:0.8 Acrylamide-Bis Acrylamide mixture, 1.5 ml of 0.5 M Tris-HCl pH 6.8, 0.1 ml of 10% SDS, 0.06 ml of 10% APS, 3.6 ml of water and 6 µl of TEMED.

Vertical slab gel electrophoresis was carried out at 100V in a Tris-Glycine-SDS buffer (6 g of Tris, 30 g of Glycine and 2 g of SDS in 2 liters of water) till the bromophenol blue dye crosses the stacking gel. Afterwards the voltage was increased to 150V. The separation of proteins was allowed till the dye reaches the bottom of the gel.

2.16 Western Blotting:

After separation of proteins on SDS-PAGE, the proteins were transferred electrophoretically on to nitrocellulose. Transfer of proteins was done 3 hr at 40 V at 4°C in transfer buffer (containing 25 mM Tris and 195 mM Glycine in 40% methanol). Nitrocellulose was carefully removed and stained with Ponceau S red solution. Marker proteins were marked with a ball point pen and the stain was removed with excess double distilled water. Regions of nitrocellulose free of proteins were blocked with TBST (Tris-HCl, pH 8.0, 150 mM NaCl and 0.05% Tween-20) containing 1% blot grade BSA for 1 hr. The blocking solution was decanted and the membrane was rinsed once with TBST solution The membrane was then incubated with a monoclonal antibody (diluted 1:10,000) or a polyclonal (diluted 1:20) antiserum for 2 hr at room temperature or overnight at 4°C with gentle shaking. Antibody solutions were decanted and stored for future use. The nitrocellulose membrane was washed with TBST for three times to remove unbound antibodies followed by three times washing with TBS (without Tween-20). The nitrocellulose was then incubated with alkaline phosphatase conjugated anti-mouse IgG (1:7,500) or alkaline phosphatase conjugated anti-rabbit IgG (1:7,500) for monoclonal or polyclonal antibodies

respectively. Incubation was done for 1 hr at room temperature. Secondary antibodies were decanted and the nitrocellulose was washed with TBST 3 times to remove unbound secondary antibody, followed by 3 washes with TBS to remove Tween-20. The membrane was damp dried and treated with the colour developing solution (66 µl of NBT, 33 µl of BCIP in 10 ml of AP buffer containing 100 mM Tris-HCl pH 9.5, 100 mM NaCl and 5 mM $MgCl_2$). The solution was removed soon after the development of the colour. The blot was thoroughly washed with water, dried between two filter papers, wrapped in an aluminum foil and stored at 4°C.

2.17 Protein Estimation

Protein estimation was done according to standard Bio-Rad method and as per the instructions of the manufacturer.

2.18 Autoradiography

The labeled proteins were separated on SDS-PAGE. The gel was then dried in a Bio-Rad gel drier equipment and exposed to a Kodak X-OMAT X-ray film or Indu film manufactured locally and kept at -70°C. The film after exposure for the required time was developed by a set of photographic solutions obtained commercially and as per the manufacturers instructions.

2.19 Materials:

ATP, GTP, CP, CPK, and DTT were purchased from Boehringer and Mannheim. Poly IC was purchased from Calbiochem, USA. *S. aureus* protease V8 enzyme, total tRNA from yeast, and E.coli synthetase were obtained from Sigma. [8-^{3}H]GDP (9 Ci/mmol), [γ-^{32}P]ATP (3000 Ci/mmol) [^{14}C]leucine (340 mCi/mmol) and [^{35}S]methionine (1100 Ci/mmol) were obtained from Dupont, NEN and BRIT, Bombay, India. Ion-exchange resins DE-52 and Phosphocellulose were obtained from Whatman, USA. CM Sephadex C-50 was purchased from Sigma and G-25 was obtained from Pharmacia. Nitrocellulose membranes, filter paper discs (1.75 cm) were bought from Schleicher and Schuell,

USA. BMV RNA and western blot colour developing kits (NBT and BCIP), secondary antibodies (anti-IgG-AP conjugate) were obtained from Promega. X-ray films were bought from Indu, India. Acrylamide, Bis-acrylamide, APS, TEMED, SDS were obtained from Bio-rad. Other chemicals like Acetyl Phenyl hydrazine, RNase-free sucrose and chemicals required for routine work were purchased from Sigma. New Zealand white male rabbits were procured locally from the National Institute of Nutrition, Hyderabad.

eIF-2a monoclonal antibodies developed in Professor E. D. Henshaw's laboratory were obtained from Dr Jane-Jane Chen, MIT, Cambridge, USA Reticulocyte eIF-2 and HRI were prepared by Dr. Ramaiah, University of Hyderabad, India.

3.0 GENERAL OUTLINE TO RESULTS AND DISCUSSION

There is a paucity of information on the regulation of translational initiation through phosphorylation mechanism in plants. Unlike in animal systems, where there is a definite mechanism of regulation of translation by eIF-2a-subunit phosphorylation, phosphorylation of plant eIF-2 does not show a corresponding regulatory effect on plant protein synthesis (Benne et al., 1980; Browning et a!., 1985; Dezoeten et al., 1989; Shaikin et al., 1992; Janaki et al., 1995). In order to understand the mechanism of eIF-2 regulation in plant systems, we have chosen *wheat germ* system, a model translational system from plants.

The results are divided into *three* sections:

Section A: This section deals with the purification of wheat germ eIF-2, and its ability to form binary (eIF-2.GDP) and ternary complexes (eIF-2.Met.tRNA$_i$.GTP). Polyclonal antibodies were raised against the purified eIF-2 to test their ability to cross react with purified wheat germ and reticulocyte eIF-2 The polyclonal antibodies were also used to determine the presence of eIF-2 in the 15S complexes and in polysomes (please see section B). Heterologous mammalian eIF-2a kinases which are known to phosphorylate the alpha-subunit of reticulocyte eIF-2 (Shaikin *et al.,* 1992) and also, the ability of a multipotential heterologous kinase, like casein kinase II (CK II), to phosphorylate wheat germ eIF-2 have been tested here as a part of characterization of the wheat germ eIF-2.

Section B: Phosphorylation of eIF-2a in reticulocyte lysates is known to inhibit protein synthesis by sequestering eIF-2B, which is essential for the exchange of guanine nucleotides on reticulocyte eIF-2. Existence of such a mechanism has not been reported from plant systems. Despite rigorous purifications, earlier workers could not purify an eIF-2B-like protein from plant systems (Osterhout et al., 1983). Therefore, we have chosen reticulocyte lysate, a well studied protein synthesizing system, to understand the functioning of phosphorylated wheat germ eIF-2. The eIF-2B activity of reticulocyte lysates is determined in the presence of phosphorylated reticulocyte eIF-2a and phosphorylated wheat germ eIF-2 (p41-42 subunit). Since purified wheat germ eIF-2 can exchange guanine nucleotides

without requiring an eIF-2B-like activity, studies have also been carried out to determine the effect of phosphorylated wheat germ and reticulocyte eIF-2 on the ability of wheat germ eIF-2 to exchange guanine nucleotides The findings here indicate that phosphorylated wheat germ or reticulocyte eIF-2 do not inhibit the eIF-2B-like activity associated with wheat germ eIF-2 and wheat germ eIF-2 reduced the protein synthesis inhibition and eIF-2B activity of reticulocyte lysates that is mediated by reticulocyte eIF-2a phosphorylation. A possible mechanism has been suggested based on these findings.

Section C: In order to understand more about the nature of phosphorylation of wheat germ eIF-2, limited proteolytic digestion of wheat germ eIF-2 subunits, phosphorylated by heterologous eIF-2 kinases, has been carried out.

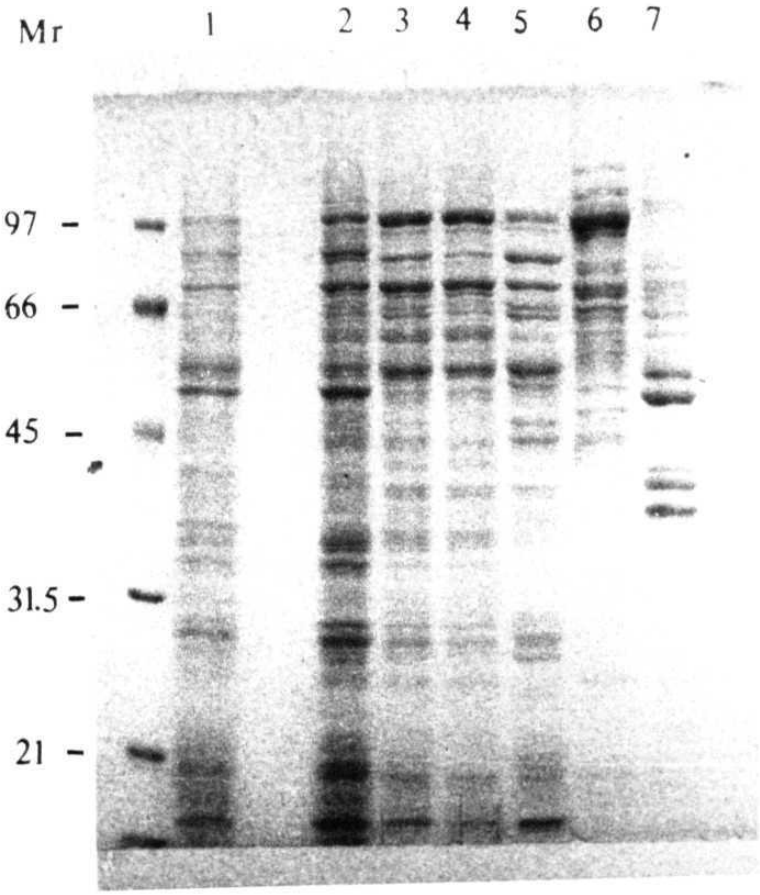
3.1 SECTION A: RESULTS AND DISCUSSION

3.1.1 Purification of wheat germ eIF-2.

Wheat germ eIF-2 was purified by modifying the earlier procedure of Lax et al., (1986). The schema of the wheat germ eIF-2 purification is shown in Fig. 3. Wheat germ (480 g) was taken and passed through various steps as shown in Fig. 3 and as described in 'Materials and Methods'. Modifications made in the procedure were to shorten the time taken for purification, for example, performing a stepwise elution for eluting bound proteins and introduction of a Carboxy Methyl cellulose Sephadex C-50 (CM sephadex) column to enhance the purity of wheat germ eIF-2. An SDS-PAGE gel of the various important fractions that are obtained during the purification of wheat germ eIF-2 has been shown in Fig. 4a Lane 1 of Fig. 4a, shows the protein pattern of post ribosomal supernatant (PRS) from which eIF-2 has been purified and the following lanes (2-7) show the degree of purification of wheat germ eIF-2, as it is being purified from crude fractions Although purification of wheat germ eIF-2 has been done by many workers earlier (Benne et al., 1980; Ranu, 1980; Seal et al., 1983; Lax et al., 1986), except for the preparation of Lax et al., (1986), other preparations contain a number of contaminating bands. With a slight variation in the molecular weights of subunits, the wheat germ eIF-2 is a heterotrimeric protein and the molecular weights of the three subunits are approximately, 36 kDa, 41-42 kDa (a doublet-subunit) and 52 kDa respectively. The p41-42 doublet-subunit is considered to be a single subunit, since the ratio of this doublet form was equal to the p36 and p52 subunits (based on stain intensity) and this subunit is referred here as the p41-42 doublet. However, some authors (Shaikin et al., 1992) referred this doublet as two independent subunits ((3- and γ-). A similar preparation of wheat germ eIF-2 with some high molecular weight contaminants has been obtained here when the 0.12 M KC1 fraction of DEAE Cellulose purified eIF-2 (Fig. 4a, lane 3) is passed through a Phosphocellulose column and eluted with 500 mM KC1 (Fig. 4a, lane 7).

Fig. 4a. Coomassie stained gel of different fractions that were obtained during the purification of wheat germ eIF-2: Different tractions **of** wheat germ eIF-2 as indicated below were separated on 10% SDS-PAGE as described in 'Materials and Methods' The figure is a Coomassie stained gel.

Lane 1, Post ribosomal supernatant (PRS); lane 2, PRS 40-80% ammonium sulphate cut, lane 3, Diethyl aminoethyl cellulose (DEAE), 120 mM KCl eluate (DEAE-120); lane 4, DEAE-120, 0-55% ammonium sulphate cut; lane 5, DEAE-120, 55-80% ammonium sulphate cut; lane 6, Phosphocellulose (P11), 350 mM KCl eluate; lane 7, P11, 500 mM **KC1** eluate


Mr
1
2
3
4
5
6
7
97
66
45
31.5
21

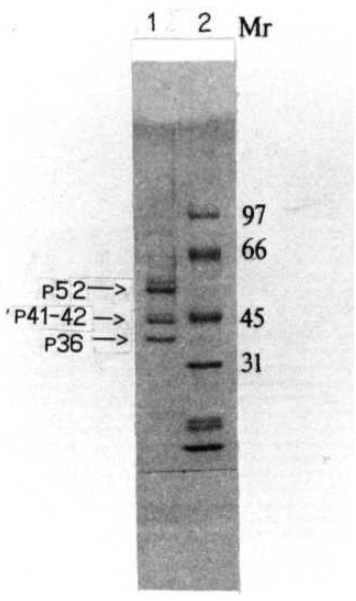


Fig. 4b. Carboxy Methyl Cellulose **Sephadex-C 50 (CM Sephadex) purified** wheat germ **eIF-2:** Wheat germ eIF-2 that has been eluted from the phosphocellulose column with 0.5 M KCl has been **further** purified **using** CM Sephadex column. The eIF-2 fraction bound to the column was eluted and concentrated as described in 'Materials and Methods'. The eIF-2 fraction is separated here by 10% SDS-PAGE and is stained by coomassie. The figure is a coomassie stained gel.

Lane 1, 2 μg of CM Sephadex purified wheat germ **eIF-2.**

Lane 2, Molecular weight markers.

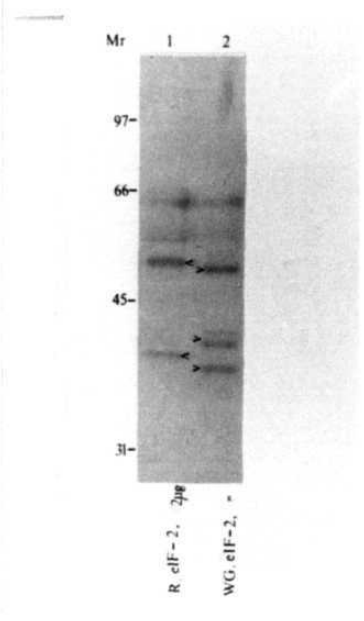


Fig. 4c. Purified wheat germ and reticulocyte eIF-2 fractions separated on 10% SDS-PAGE for subunit comparison: eIF-2 fractions purified from wheat germ and reticulocyte lysates are separated on 10% SDS-PAGE and stained with coomassie blue. The figure is a coomassie stained gel.

Lane 1, 2 μg of purified **reticulocyte eIF-2 (R.eIF-2). The eIF-2** preparation has a 38 kDa subunit (a-subunit) and also two subunits (p and γ) at a position corresponding to 54 kDa.

Lane 2, 2 |ig of purified wheat germ eIF-2 (WG.eIF-2). The p36, p41-42 doublet and the p52 subunits are shown with arrows.

Molecular weight markers (Mr.) are shown on the left hand side of the gel.

Table 1

Fractions (2 (ig)	eIF-2.[^{3}H]GDP Bound, CPM X 10^{-3} (pmol)	
	$+Mg^{+2}$	
- Fraction	444	464
Lysate	245	258
PRS (0-40%)	165	200
PRS (40-80%)	302	222
DE-120 (0-55%)	986	200
DE-120 (55-70%)	166	134
P1 1-350	527	238
P1 1-500	13,034(6.86)	252
CMS-350	19,095 (10.05)	952

Table 1. Formation of wheat germ **eIF-2.GDP binary complex:** To test the purity and the ability of different fractions containing wheat germ eIF-2, fractions with 2 μg of protein are incubated with labeled GDP (2 μM, 1900 cpm / pmol) at 25°C in a 20 μl volume containing 20 mM Tris-HCl, pH 7.8, 1 mM DTT and 10 μg/ml CPK as described in 'Materials and Methods'. The complex is then stabilized by the addition of 2.5 mM $Mg(OAc)_2$. Picomoles of GDP bound to eIF-2 was estimated as described in 'Materials and Methods'.

Table 2

Fraction	Total Protein (mg)	nmol	Specific activity
DEAE Cellulose (120)	8400	206.0	0.024
DEAE Cellulose (120), 0-55% Amm.sulfate cut*	1100	110.0	0.100
DEAE Cellulose (120) 55-80% Amm.sulfate cut*	236	1.180	0.005
Phosphocellulose (500)	8.1	12.96	1.600
CM Sephadex (350)	5.0	9.45	1.890

The figures in the bracket represent the concentration of KCl which was used to elute the bound protein from the respective columns.
* Ammonium sulfate cut

Table 2. Formation of eIF-2.Met.tRNA$_i$.GTP complex. Standard assay conditions as deacribed under 'Materials and Methods' were used. The reaction mixtures contained in a total volume of 100µl: 20 mM HEPES-KOH, pH 7.6, 100 mM KC1, 2.5 mM DTT, 0.2 mM GTP, 20 µg BSA, 25 pmols of [^{35}S]methionine.tRNA$_i$ (1000 Ci/mmol) and 5 µl of a source of initiation factor. The reaction mixtures were incubated at 25oC for 10 min. Aliquots of the reaction mixtures were used for millipore filteration assay as discussed in 'Materials and Methods'. One unit of eIF-2 activity is defined as that amount which binds to 1 nmol of Met.tRNA$_i$ under these conditions.

Further purification of this preparation of eIF-2 has been carried out using a CM sephadex column to minimize the contaminating proteins. As can be seen in Fig. 4b, 2 μg of CM Sephadex purified wheat germ eIF-2, when separated on 10% SDS-PAGE and stained by coomasiee blue, has just one contaminating band of approximately 54 kDa.

There has been a considerable confusion generated regarding the subunit designation of wheat germ eIF-2 by various workers (Benne et al., 1980; Seal et al., 1983; Ray et al., 1986; Lax et al., 1986; Mehta et al., 1987; Shaikin et al., 1992). Based on the substrate specificity of mammalian eIF-2a kinases, like HRI and PKR, the 41-42 kDa subunit of wheat germ eIF-2 can be regarded as the alpha-subunit, since this substrate is phosphorylated by reticulocyte eIF-2a kinases (Fig. 4b). On the other hand, if the migration of individual subunits in SDS-PAGE is considered, the p36 subunit can be regarded as the alpha-subunit, because it is the smallest or the fastest migrating of all the three subunits. The small-subunit of eIF-2 in reticulocyte lysates, brine shrimp and sea urchin is considered to be the alpha-subunit (Safer, 1989; Mateu, 1989; Dholakia et al., 1990). However, to avoid confusion and conflict we have addressed the individual subunits by their respective molecular mobility in SDS-PAGE (Janaki et al., 1995). Therefore, the three subunits are designated here as p36, p41-42 doublet and p52.

3.1.2 Formation of eIF-2.[^{3}H]GDP and eIF-2.GTP.[^{35}S]Met.tRNA$_i$ complexes.

The purified eIF-2 was tested for its ability to bind labeled GDP, ([^{3}H]GDP) and also to form a binary complex, (eIF-2.[^{3}H]GDP), in the presence of Mg^{2+}. As can be seen from the Table 1, 2pg of phosphocellulose purified eIF-2 binds 6.86 pmoles of [^{3}H]GDP where as the same amount of CM Sephadex purified eIF-2 binds 10.008 pmoles of [^{3}H]GDP (Table 1). Other crude or impure preparations have shown little or no binding with labeled GDP. Purified eIF-2 is also tested for its ability to form a ternary complex, eIF-2.GTP.Met.tRNA$_i$ (Table 2). As can be seen from the table (2), wheat germ eIF-2 purified on a CM

Fig. 5a. **Phosphorylation** of **reticulocyte** and wheat **germ eIF-2** *in vitro* by **HRI:**

Phosphorylation reactions were carried out in a standard 20 μl reaction mixtures containing 20 mM Tris-HCl (pH 7.8), 2.5 mM $Mg(OAc)_2$, 30 |iM [γ-^{32}P]ATP (3000 Ci/mmole), pure HRI (~40ng) and with purified wheat germ or reticulocyte eIF-2 (~150 ng). Reaction Mixtures were incubated at 30°C for 10 min. and separated by SDS-PAGE as described in 'Materials and Methods'. The Figure is an autoradiogram.

Lane I, + reticulocyte eIF-2

Lane 2, + wheat germ eIF-2

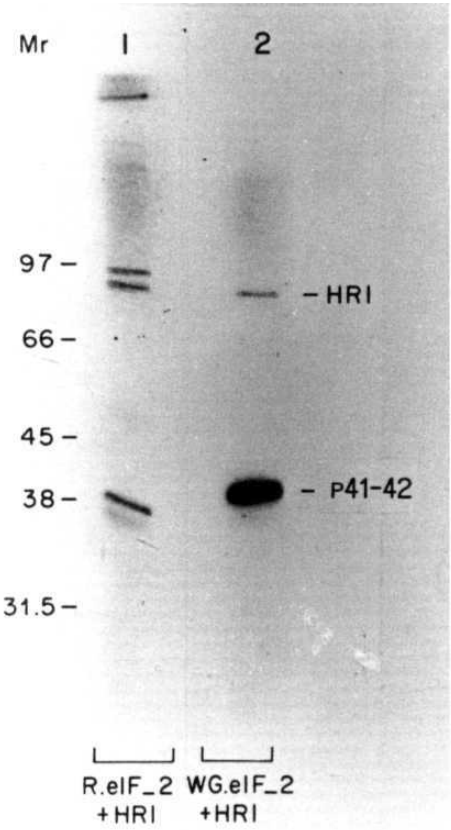

Mr
1
2
97 –
66 –
45 –
38 –
31.5 –
– HRI
– P41-42
R.eIF_2
+HRI
WG.eIF_2
+HRI

Fig. 5b. Phosphorylation of reticulocyte and wheat germ eIF-2 *in vitro* by dsI kinase: Phosphorylation reactions were carried out in a standard 20 µl reaction mixtures containing 20 mM Tris-HCl (pH 7.8), 2.5 mM $Mg(OAc)_2$, 30 µM [γ-^{32}P]ATP (3000 Ci/mmole), dsI kinase (~75ng) and with purified wheat germ or reticulocyte eIF-2 (~150 ng). Reaction Mixtures were incubated at 30°C for 10 min. and separated by SDS-PAGE as described in 'Materials and Methods'. The Figure is an autoradiogram.

Lane 1, + reticulocyte eIF-2

Lane 2, + wheat germ eIF-2

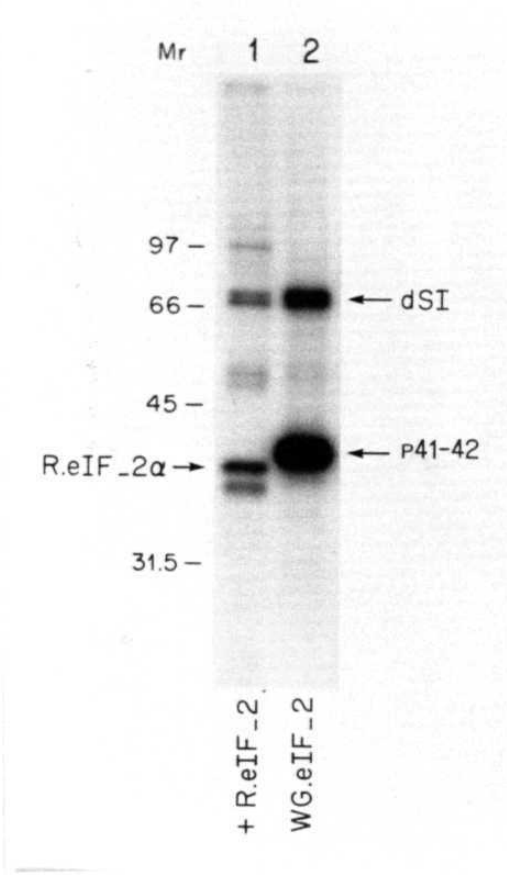

Mr
1
2
97 –
66 –
45 –
31.5 –
R.eIF_2α →
← dSI
← P41-42
+ R.eIF_2
WG.eIF_2

Fig. 5c. Phosphorylation of purified wheat germ eIF-2 *in vitro* by purified CK II and partially purified HRI: Phosphorylation reactions were carried out in a standard 20 µl reaction mixtures containing 20 mM Tris-HCl (pH 7.8), 25 mM $Mg(OAc)_2$, 30 µM [γ-^{32}P]ATP (3000 Ci/mmole), and with purified wheat germ (~150 ng) Reaction Mixtures were incubated at 30°C for 10 min. in the presence of relatively impure HRI or CK II or without the addition of any kinase (control) as indicated. The reaction mixtures were separated by 10% SDS-PAGE as described in 'Materials and Methods'. The Figure is an autoradiogram.

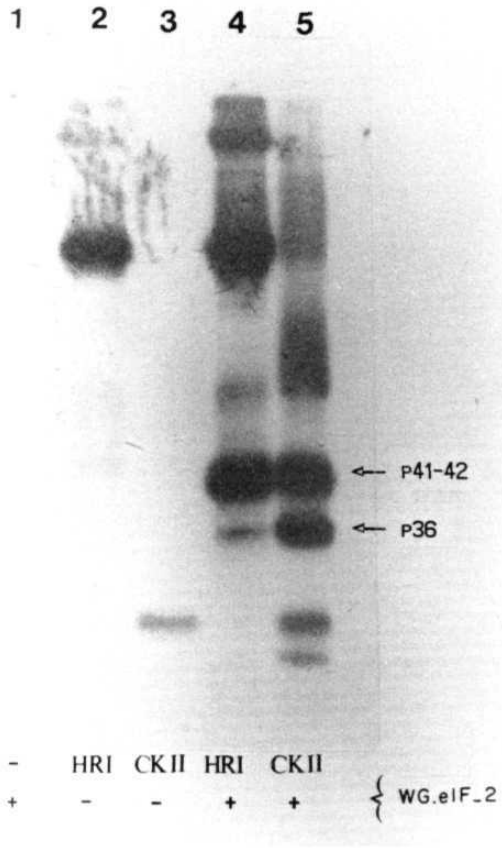
Lane 1, + wheat germ eIF-2, without any kinase (substrate control)

Lane 2, + partially purified HRI alone (kinase control)

Lane 3, + highly purified CK II alone (kinase control)

Lane 4, + wheat germ eIF-2 and partially purified HRI

Lane 5, + wheat germ eIF-2 and CK II


1
2
3
4
5
P41-42
P36
-
HRI
CKII
HRI
CKII
+
-
-
+
+
WG.eIF-2

Sephadex column has higher specific activity compared to that purified on a phosphocellulose column. These results are in agreement with the results obtained in table 1 and also the purification profiles of various fractions on SDS-PAGE (Fig.s 4a and 4b) where it is evident that CM Sephadex purified wheat germ eIF-2 (Fig. 4b) has relatively less contamination of other proteins compared to phosphocellulose purified eIF-2 (Fig. 4a, lane 7).

For a comparison of the migration of subunits of purified wheat germ and reticulocyte eIF-2 preparations, 2 μg of each of the preparations was loaded on a 10% SDS-PAGE. As can be seen from the Fig. 4c, wheat germ eIF-2 has three subunits; p36, p41-42 and p56. In contrast, reticulocyte eIF-2 also has three subunits but with different molecular weights; a- (36 kDa) and β/γ (54 kDa). This Fig. shows that reticulocyte eIF-2α migrates between the p41-42 and p36 subunits of wheat germ eIF-2. This allows the two subunits (reticulocyte eIF-2a and wheat germ eIF-2 p41-42 subunit) to be distinguished clearly in assays where both the subunits are subjected to phosphorylation.

3.1.3 Phosphorylation of purified wheat germ eIF-2 by purified kinases *in vitro.*

It has been shown previously that mammalian eIF-2a kinases, HRI and PKR, can phosphorylate wheat germ eIF-2 in the p41-42 doublet-subunit (Benne et al., 1980; Mehta et al., 1986; Shaikin et al., 1992). Purified reticulocyte kinases, HRI (Fig. 5a) or dsI (Fig. 5b) which could phosphorylate the small-subunit (38 kDa) of reticulocyte eIF-2 (eIF-2a), (Fig. 5a, lane 1 and Fig. 5b, lane 1) phosphorylated the p41-42 doublet-subunit of wheat germ eIF-2 *in vitro* (Fig. 5a, lane 2; Fig. 5b, lane 2). Wheat germ eIF-2 is also phosphorylated in translating heme-deficient and heme and poly IC-treated reticulocyte lysates presumably by the native eIF-2a kinases (see **section B,** Fig. 7). The small-subunit, p36 of wheat germ eIF-2 however, was not phosphorylated under those conditions. In a separate experiment, (Fig. 5c), the phosphorylation of purified wheat germ eIF-2 was tested without the addition of any kinase, in the presence of an HRI preparation which is

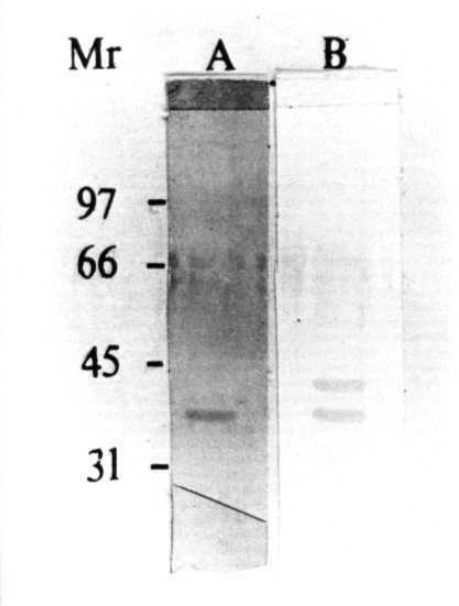


Fig. 6a. Specificity of wheat germ eIF-2 polyclonal antibodies: Polyclona] anti-wheat germ eIF-2 antibodies were raised as described in 'Materials and Methods'. The ability of these antibodies to react with purified wheat germ eIF-2 has been tested here and is compared to the ability of preimmune serum (control) to react to purified wheat germ eIF-2. Two micrograms of CM Sephadex purified wheat germ eIF-2 has been separated on 10% SDS-PAGE, transferred to nitrocellulose membrane and allowed the latter to react with preimmune serum (Lane A) or with the polyclonal antibodies (Lane B) as described in 'Materials and Methods'.

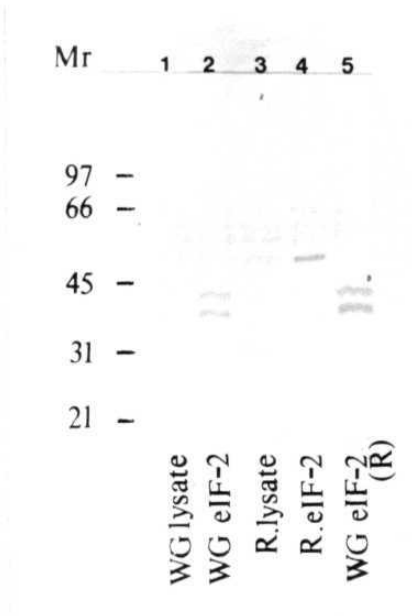


Fig. 6b. Cross-reactivity of wheat germ eIF-2 polyclonal antibodies: Polyclonal anti-wheat germ eIF-2 antibodies were raised as described in 'Materials and Methods'. The ability of these antibodies to identify purified wheat germ and reticulocyte eIF-2 and also the eIF-2 in wheat germ and reticulocyte lysate extracts has been tested here. The eIF-2 containing preparations are separated on 10% SDS-PAGE, transferred to a nitrocellulose membrane and allowed the proteins in the membrane to react with the antibodies as described in 'Materials and Methods'. The various lanes are as follows:

Lane 1, Wheat germ lysate; lane 2, CM Sephadex purified eIF-2; lane 3, Reticulocyte lysate; lane 4, CM Sephadex purified reticulocyte eIF-2; and lane 5, purified wheat germ eIF-2 preparation obtained from Prof. **Joanne M.** Ravel's laboratory, Texas, USA.

relatively less purified, and also by a highly purified multipotential kinase like CK II. Little or no phosphorylation of purified eIF-2 occurred **in** the absence of any kinase (lane 1). Partially purified HRI was autophosphorylated (lane 2) and it phosphorylated both the p41-42 doublet and p36 (weakly) subunits in wheat germ eIF-2 (lane 4). The highly purified CK II was also autophosphorylated (lane 3) and it phosphorylated the p41-42 doublet-subunit as well as the p36 subunit of wheat germ eIF-2 (lane 5).

3.1.4 Cross-reactivity of wheat germ and reticulocytc eIF-2 subunits by polyclonal anti-wheat germ eIF-2 antibodies.

A polyclonal antibody was raised against the purified wheat germ eIF-2 preparation in rabbits as described in 'Material and Methods'. Since the purified CM sephadex fraction of wheat germ eIF-2 contains **a** contaminating band (Fig. 4b), we have electroeluted the three subunits, p36, p41-42 and p52 of wheat germ eIF-2 from the SDS-PAGE gel as described in 'Materials and Methods'. The eluted proteins were mixed and used for raising antibodies in rabbits as described in 'Materials and Methods' To determine whether the serum obtained from the immunized rabbits contained antibodies to the injected wheat germ eIF-2 subunits, a cross-reactivity between the control (pre-immune) and immune serum with purified wheat germ eIF-2 was tested. Fig. 6a lane B, shows the cross reactivity of wheat germ eIF-2 antibodies to purified wheat germ eIF-2 preparation. As can be seen from the figure 6a, the antibodies cross reacted with the p41-42 and p36 subunits. However, no cross reactivity was observed with the p52 subunit. The reason for the non-reactivity with the p52 subunit is not clear. To prove that the reaction is specific to the antisera generated, a control reaction (lane A) with pre-immune serum was also performed. As can be seen from the Fig 6b, lane A, only the p36 subunit of wheat germ eIF-2 shows a cross reactivity with the preimmune serum. However, the cross reaction occurred during an overnight incubation with the color developing solution. In contrast, with the immune serum, the p36 and the p41-42 subunits developed the cross reaction within 10 minutes of

the incubation in the color developing solution. This suggests that the cross reactivity of wheat germ eIF-2 to immune serum is more specific compared to that obtained with the pre-immune serum.

The polyclonal wheat germ eIF-2 antibodies were also tested for their reaction with homologous and heterologous eIF-2 in whole lysates (wheat germ and reticulocyte lysates) and with the purified preparations obtained from wheat germ and reticulocyte lysates. As can be seen from Fig. 6b, lane 1, wheat germ lysate (WG.lysate) faintly cross reacts with the antibodies. The cross reaction occurs probably with all the subunits of endogenous wheat germ eIF-2. Lane 2 shows a cross reaction of purified wheat germ eIF-2 (WG.eIF-2) subunits to the polyclonal antiserum. As can be seen, the p36 and p41-42 subunits are recognized by the antiserum. Fig. 6b, lane 3, shows a cross reaction with reticulocyte lysate, where the p/y subunits of reticulocyte eIF-2 show cross reactivity with wheat germ eIF-2 polyclonal antiserum. This is further confirmed in lane 4, where purified reticulocyte eIF-2 p/y subunit is shown to cross react with antibodies raised against wheat germ eIF-2 subunits. This shows that wheat germ and reticulocyte eIF-2 share antigenic determinants. To check if the immune serum prepared here also cross reacts with wheat germ eIF-2 preparation from other laboratories, we have loaded 2 μg of purified wheat germ eIF-2 preparation obtained from Dr. Joanne M. Ravel's lab, Texas. As can be seen from the Fig. 6b (lane 5), the immune serum cross reacts with p36 and p41-42 doublet subunit of wheat germ eIF-2 and is found similar to the reaction obtained with wheat germ eIF-2 preparation made by us here (Fig. 6a, lane B and Fig. 6b, lane 2).

In summary, these findings indicate that the polyclonal antibody raised against our wheat germ eIF-2 is specific to p41-42 and p36 subunits of purified wheat germ eIF-2 preparation. The purified wheat germ eIF-2 prepared here matches also with the purified preparations obtained from a reputed laboratory based on the cross-reactivity of the polyclonal antibodies that have been raised here. The immune serum also recognizes the β/γ subunits of reticulocyte eIF-2 suggesting that although placed distinctly in the phylogenetic order, mammalian and plant eIF-2 still share antigenic determinants among them.

3.2 SECTION B: RESULTS

3.2.1 Wheat germ eIF-2 phosphorylation in reticulocyte lysates.

Phosphorylation of p41-42 subunit in wheat germ eIF-2 also occurs in inhibited heme-deficient and poly IC-treated reticulocyte lysates due to the active native lysate eIF-2α kinases (Fig. 7). Two eIF-2a kinases are identified and well characterized in mammalian systems. One of them is the heme-regulated eIF-2a kinase (HRI), which becomes active during heme-deficiency (Chen et al., 1995) and the other is an interferon induced eIF-2a kinase (PKR), which is activated during viral infection or in the presence of dsRNA or poly IC (reviewed in Rhoads, 1993; Samuel, 1993). Consistent with these findings, we find here in our standard reactions, that reticulocyte eIF-2a phosphorylation is enhanced during [γ-^{32}P]ATP pulse (between 15-20 minutes of protein synthesis) in heme-deficient (Fig. 7, track 1) or in hemin and poly IC-treated lysates (track 5). Heme inhibits the HRI activity but it cannot inhibit the PKR activity. Hence reduced eIF-2a phosphorylation occurs in heme-supplemented lysates which are not treated with poly IC (Fig. 7, track 2). A standard amount of reticulocyte eIF-2a phosphorylation occurs under those conditions depending on the eIF-2a kinase-phosphatase activities. Addition of wheat germ eIF-2 to heme-deficient (track 3) or hemin and poly IC-treated reticulocyte lysates (track 6) does not however, alter the phosphorylation of reticulocyte eIF-2α, but the conditions also facilitate the phosphorylation of p41-42 doublet-subunit in wheat germ eIF-2 (tracks 3 and 6). The 41-42 kDa subunit of wheat germ eIF-2 migrates above the reticulocyte eIF-2α (38 kDa) and can be seen in the autoradiogram (track 6). The phosphorylation of wheat germ eIF-2 seen in reticulocyte lysates is consistent with the results obtained by the addition of purified reticulocyte eIF-2α kinases to wheat germ eIF-2 *in vitro* (Janaki et al., 1995; Shaikin et al., 1992). Some reports referred this subunit as the α-subunit,

Fig. 7. [^{32}P]-Labeled phosphoprotein profiles of heme-deficient lysates, or heme (20 μM) and poly IC (75 ng/ml) treated lysates with or without wheat germ eIF-2 (2 μg): Protein synthesis in reticulocyte lysates (25 μl) was carried out at 30°C for 10 min. Incubation mixtures were pulsed with [γ-^{32}P]ATP (10 μCi, specific activity 3000 Ci/mmol) at 10-15 min of protein synthesis 10 μl aliquots were taken out and concentrated by pH 5.0 precipitation and separated by sodium dodecyl sulfate-10% polyacrylamide gels as described in 'Materials and Methods'. The figure is an autoradiogram. The various lanes represent the following: lane 1, -heme, lane 2, +heme; lane 3, -heme +WG.eIF-2; lane 4, +heme +WG.eIF-2; lane 5, +heme +poly IC; Lane 6, +heme poly IC +WG.eIF-2.

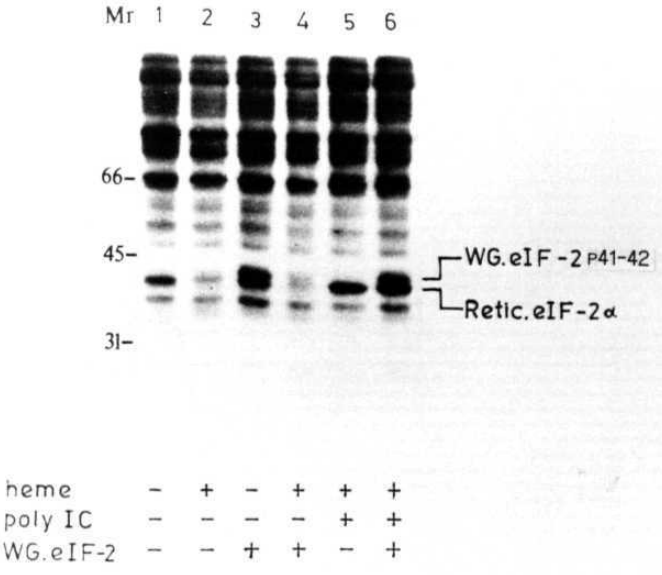
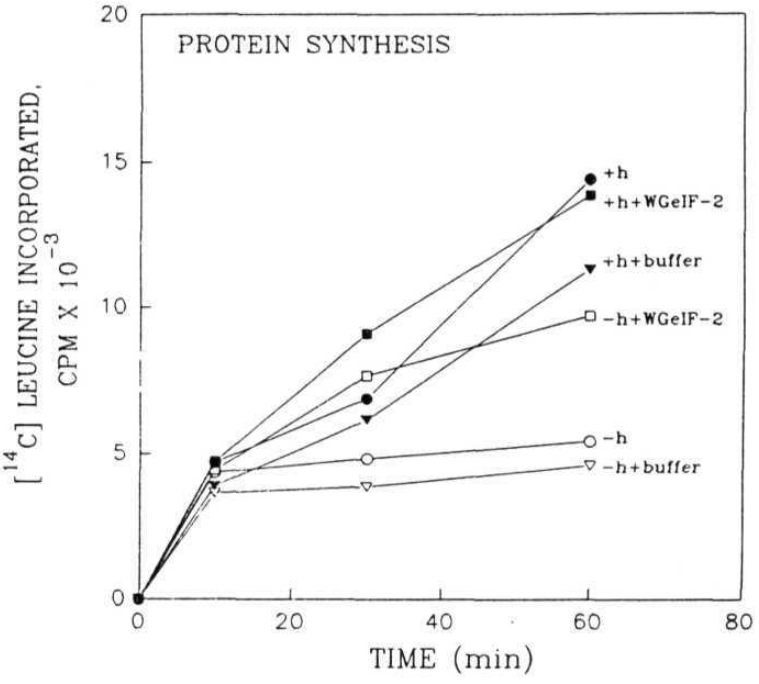

Mr 1 2 3 4 5 6
66–
45–
31–
WG.eIF-2 P41-42
Retic.eIF-2α
heme – + – + + +
poly IC – – – – + +
WG.eIF-2 – – + + – +

Fig. 8. Wheat germ eIF-2 protects protein synthesis in heme-deficient (Fig. 8a), heme and poly IC-treated (Fig. 8b) reticulocyte lysate: Protein synthesis reaction mixtures with 60% reticulocyte lysate (25 μl) were incubated at 30°C for different time intervals without added hemin (-heme). Where indicated, all the additional components like heme (20 μM), poly IC (50 or 100 ng/ml), WG.eIF-2 (2 μg/25 μl) and 2 |il of eIF-2 buffer (20 mM Tris-HCl, pH 7.6; 50 mM KCl, 50% glycerol, 1 raM DTT and 0 1 mM EDTA) were added at the beginning of protein synthesis reactions. The extent of protein synthesis was determined by the incorporation of [^{14}C]leucine in a 5 μl aliquot of the reaction mixtures as described (Ernst *et a/.*, 1978).

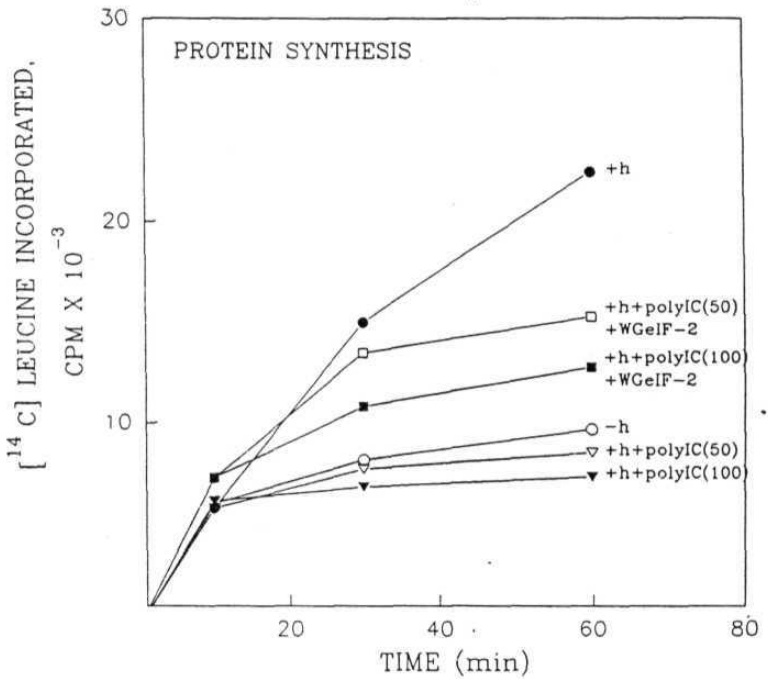
8a. O-O , minus heme (-h); • - • ,plus heme (+h),
V-V , minus heme plus 2 μl WG.eIF-2 buffer (-h + buffer);
D-D , minus heme plus 2 ug WG.eIF-2 (-h + WG.eIF-2);
• -• , plus heme plus 2 ug WG.eIF-2 (+h + WG.eIF-2), and
T-T , plus heme plus 2 μl WG.eIF-2 buffer (+h + buffer).

8b. O·O , minus heme (-h); • - • , plus heme (+h);
V-V , plus heme plus 50 ng/ml poly IC [+h + poly IC (50)];
T-T , plus heme plus 100 ng/ml poly IC [+h + poly IC (100)],
D-D , plus heme plus 50 ng/ml poly IC WG.eIF-2 [+h + poly IC (50) + WG.eIF-2]; and
• - • , plus heme plus 100 ng/ml poly IC and WG.eIF-2 [+h + poly IC (100)+ WG.eIF-2]

8a.


PROTEIN SYNTHESIS
[14C] LEUCINE INCORPORATED, CPM X 10-3
TIME (min)
+h
+h+WGeIF-2
+h+buffer
-h+WGeIF-2
-h
-h+buffer
0
5
10
15
20
0
20
40
60
80

8b.


PROTEIN SYNTHESIS
[14 C] LEUCINE INCORPORATED.
CPM X 10-3
30
20
10
20
40
60
80
TIME (min)
+h
+h+polyIC(50)
+WGeIF-2
+h+polyIC(100)
+WGeIF-2
-h
+h+polyIC(50)
+h+polyIC(100)

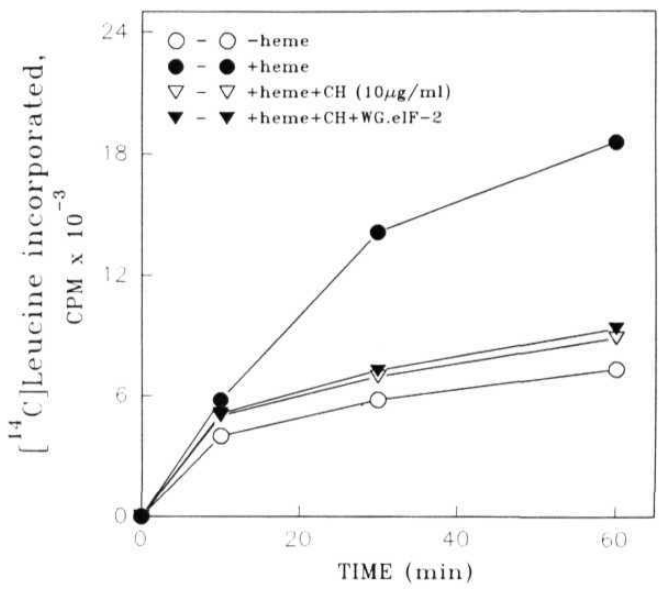


Fig. 9. Wheat germ eIF-2 does not mitigate the protein synthesis inhibition in reticulocyte lysates caused by cycloheximide: Protein' synthesis reaction mixtures (25 μl) with 60% reticulocyte lysates were incubated at 30°C for different time intervals as described in 'Materials and Methods'. Where indicated, the concentration of heme, wheat germ eIF-2 and cycloheximide is 20μM, 2ug and 10μg/ml respectively. The extent of protein synthesis was determined by the incorporation of [^{14}C]leucine in a 5 μl aliquot of the reaction mixture as described in 'Materials and Methods'

probably because, this subunit is phosphorylated by mammalian eIF-2a kinases (Mehta et al., 1986). This subunit is recently reported to have 83% sequence homology around the phosphorylation site with mammalian eIF-2α (Langland et al., 1996).

3.2.2 Protein synthesis.

Typically, heme-deficiency or poly IC-treatment results in the inhibition of protein synthesis in reticulocyte lysates (Fig. 8a & 8b). This inhibition is non-linear and is correlated to the small increment in eIF-2a phosphorylation as has been reported earlier by others (London et al., 1987; Jackson 1991; Merrick, 1992). Addition of wheat germ eIF-2 to heme-deficient or hemin and poly IC-treated reticulocyte lysates reduces the inhibition in protein synthesis (Fig. 8a & 8b). Part of this finding, which indicates that purified wheat germ eIF-2 restores protein synthesis inhibition in heme-deficient lysates, supports some of the very early observations (Benne et al., 1980; Ranu, 1980). No previous reports are known however, which indicate that wheat germ eIF-2 can also overcome the inhibition in protein synthesis caused by poly IC-treatment. This is possible, because both heme-deficiency and poly IC modify reticulocyte eIF-2 through phosphorylation and the added wheat germ eIF-2 can reduce the effect of reticulocyte eIF-2a phosphorylation on protein synthesis (Fig. 8). In contrast, protein synthesis inhibition in heme-supplemented lysates that is mediated by cycloheximide, an inhibitor of translation, is not mitigated by the addition of wheat germ eIF-2 (Fig. 9).

According to available information (reviewed in London et al., 1987; Jackson, 1991; Proud, 1992), 15-25% phosphorylation of reticulocyte eIF-2 can lead to the complete shut down of protein synthesis. This is due to the sequestration of eIF-2B with eIF-2(aP) into a complex, [eIF-2(aP).eIF-2B] in which eIF-2B becomes non-functional. Since eIF-2B is relatively low compared to eIF-2 (15-25% of eIF-2), phosphorylation of a small portion of eIF-2(aP) can complex with all the available eIF-2B having no free elF-2B to promote the

recycling of eIF-2. In contrast, it has been reported that 45-50% eIF-2α phosphorylation is required in Ehrlich ascites cells to inhibit protein synthesis maximally. This is because these cells contain relatively higher level of eIF-2B than in reticulocyte lysates, that is about 50% of the eIF-2 pool (Rowlands et al., 1988b). Also, previous studies have shown that added reticulocyte eIF-2 (depending on its purity) can also restore protein synthesis inhibition in inhibited heme-deficient lysates. Most of the preparations of mammalian eIF-2 co-migrate with eIF-2B protein. If the eIF-2B protein is separated out, the mammalian eIF-2 can restore protein synthesis only stochiometrically, whereas addition of eIF-2B restores protein synthesis catalytically (Jackson, 1991; London et al., 1983). Addition of reticulocyte eIF-2 however, enhances the lysate eIF-2a phosphorylation (London et al., 1983). But in our studies here, added wheat germ eIF-2, which is associated with GNE-like activity, does not enhance reticulocyte eIF-2α phosphorylation any further. But the phosphorylation of one of the wheat germ eIF-2 subunits (p41-42) occurs under those conditions (Fig.7, tracks 3 and 6). These findings suggest that (i) added wheat germ eIF-2 must be competing with reticulocyte eIF-2a for phosphorylation and (ii) the phosphorylated reticulocyte eIF-2a and wheat germ eIF-2 must be competing with each other for interaction with reticulocyte eIF-2B to form a complex. In order to determine if added wheat germ eIF-2 is affecting the ratio of reticulocyte eIF-2 to eIF-2(aP) and to eIF-2B activity, we have measured here the formation of 15S complex and eIF-2B activity in inhibited reticulocyte lysates which are supplemented with or without wheat germ eIF-2.

3.2.3 Effect of wheat germ eIF-2 on [eIF-2(αP).eIF-2B] complex formation in reticulocyte lysates.

Reticulocyte eIF-2B has atleast 150 fold greater affinity for eIF-2(aP).GDP than for eIF-2.GDP (Rowlands et al., 1988a). This results in an efficient sequestration of eIF-2B with phosphorylated eIF-2a into a 15S complex, [eIF-2(aP).eIF-2B] in which the GNE activity of cIF-2B becomes non-functional

Fig. 10. **Immunoblot** analysis of free **eIF-2** and **eIF-2** in the 15S complex in 10-30% sucrose gradient fractions of normal and inhibited reticulocyte lysates: Protein synthesis reactions (125 μl) were carried out for 15 min at 30°C in heme-supplemented (20 μM) (panel A), heme and poly IC treated (75 ng/ml) (panel B),or, heme plus poly IC and WG.eIF-2 (10 μg) treated reticulocyte lysates as described (Ramaiah *et at.,* 1992). The reactions were then chilled on ice for 5 min, diluted with the equal volume of buffer, layered on a 10-30% sucrose gradient and were spun to separate free eIF-2 from eIF-2(αP) complexed with eIF-2B inorder to determine the formation of 15S complex [eIF-2(αP) eIF-2B] in inhibited lysates treated with or without wheat germ eIF-2.. The fractions (350 μl) were concentrated by pH 5.0 precipitation, separated on 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane to detect reticulocyte eIF-2α with the help of rabbit anti-human eIF-2α antibody as described under 'Materials and Methods' Lanes 1-12 contain eIF-2a detected by an anti-mouse alkaline phosphatase conjugated secondary antibody in various fractions (top to bottom of the gradient) lane 12 in all the panels contains purified reticulocyte eIF-2 (500 ng) as a marker. The migration of molecular weight markers is shown at the left.

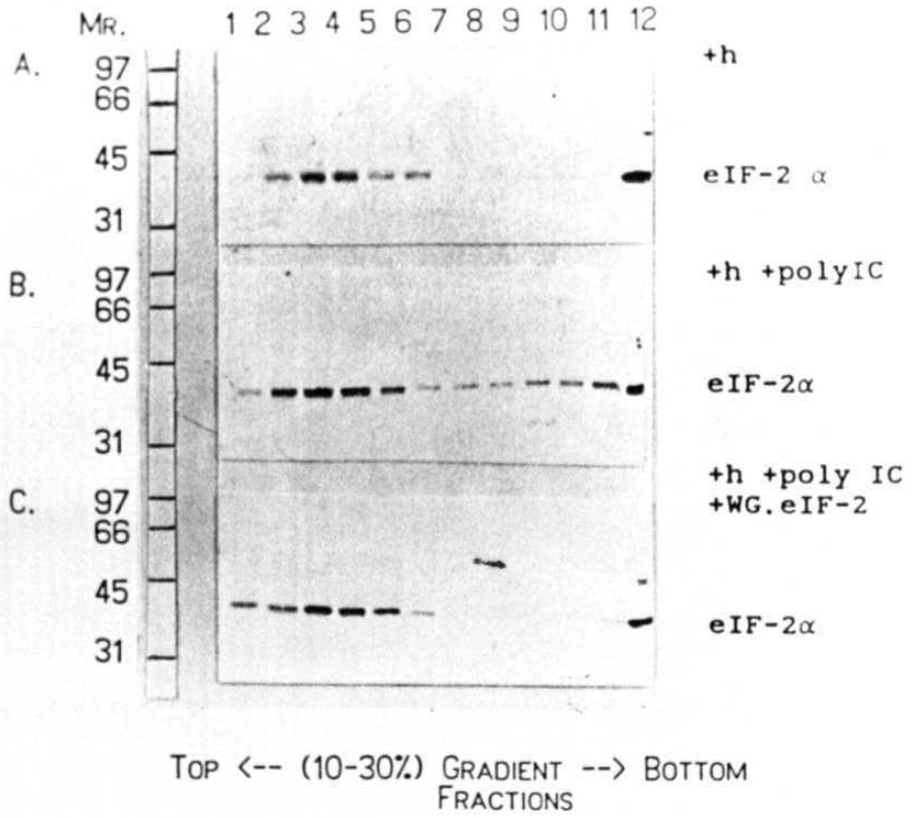
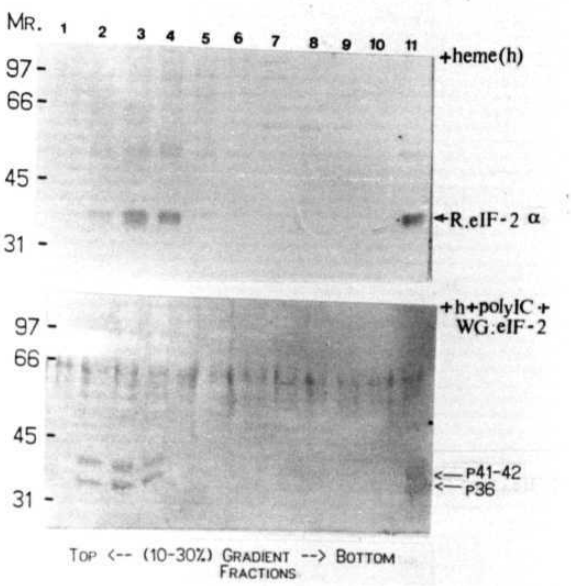

Mr.
1 2 3 4 5 6 7 8 9 10 11 12
A.
97
66
45
31
+h
eIF-2 α
B.
97
66
45
31
+h +polyIC
eIF-2α
C.
97
66
45
31
+h +poly IC
+WG.eIF-2
eIF-2α
Top <-- (10-30%) Gradient --> Bottom
Fractions

Fig. 11. Western immunoblot analysis of **15S** complex **fraction obtained** from **reticulocyte** lysates **supplemented with or without wheat germ eIF-2 to determine the interaction between phosphorylated wheat germ eIF-2 and reticulocyte eIF-2B:** Protein synthesis reactions (125 μl) were carried out at 30°C for 15 min in heme-supplemented (h, 20 μM, Upper panel) and heme, poly IC (75 ng/ml) and wheat germ eIF-2 (12 μg) treated reticulocyte lysates (h+ poly IC+ WG eIF-2, Lower panel) as described in 'Materials and Methods' The reactions were chilled on ice, diluted with equal volume of 2X TMK buffer (20 mM Tris-HCl, pH 7.8, 2 5 mM $Mg(OAc)_2$ and 80 mM KCl), layered on a 10-30% linear sucrose gradient to separate the free eIF-2 from eIF-2(αP) complexed with reticulocyte eIF-2B (15S complex) as described in "Materials and Methods'. The gradients were spun in a Beckman SW 50.1 rotor for 5 hours and 30 min in a Beckman Ultra centrifuge. Fractions (350 μl) were collected by upward displacement of gradients in a ISCO UA-6 density gradient fractionator. The fractions were concentrated by pH 5.0 precipitation; pellets were dissolved in SDS sample buffer and the proteins were separated on 10% SDS-PAGE and transferred to nitrocellulose filter papers.

The nitrocellulose membrane was treated with anti-human eIF-2α monoclonal (Upper Panel) and anti-wheat germ eIF-2 polyclonal antibodies (Lower Panel) to detect the presence of free (fractions corresponding to 1-5) and complexed reticulocyte or wheat germ eIF-2 (fractions corresponding to 5-10).

Lane 11, **in** Upper Panel contains 500 ng of purified reticulocyte eIF-2 and in Lower Panel contains 1 μg of purified wheat germ eIF-2 as markers Left hand side of each panel was loaded with the molecular weight markers. Fraction numbers are indicated on the top of each lane.


MR.
1
2
3
4
5
6
7
8
9
10
11
+heme(h)
97 -
66 -
45 -
31 -
←R.eIF-2 α
+h+polyIC +
WG.eIF-2
97 -
66 -
45 -
31 -
<— P41-42
<— P36
TOP <-- (10-30%) GRADIENT --> BOTTOM
FRACTIONS

(Thomas et al., 1985; Gross et al., 1985). As proposed by Rowlands et al., (1988a), the 15S complex formed by phosphorylated reticulocyte eIF-2 can be in dynamic equilibrium with unphosphorylated eIF-2.GDP. To determine whether wheat germ eIF-2 can exchange phosphorylated reticulocyte eIF-2 from the 15S complex, we have analyzed here the fractions containing free eIF-2 and eIF-2(αP) complexed with eIF-2B in reticulocyte lysates which are treated with hemin alone, or heme and poly IC or, heme, poly IC and wheat germ eIF-2. The translating lysates are fractionated on a 10-30% sucrose gradient and the various fractions are probed with a monoclonal rabbit anti-human eIF-2α antibody, which recognizes the a-subunit of mammalian eIF-2. In heme and poly IC-treated lysates (Fig. 10, panel B) the presence of eIF-2 is evident in all the gradient fractions. The [eIF-2(aP).eIF-2B] complex, which is expected to form in these poly IC-treated lysates, is heavier than free eIF-2 and it migrates towards the bottom of the gradient fractions (fractions 7-11). Top fractions of the gradient (1-6) contain free eIF-2. In hemin- supplemented lysates in which the eIF-2α phosphorylation does not occur, the 15S complex is not formed. This is evident from the absence of eIF-2 signal in the bottom of the gradient fractions (Fig. 10, panel A). Only the top fractions of the gradient contain eIF-2 signal. Addition of wheat germ eIF-2 to heme and poly IC-treated lysates reduces the formation of 15S complex (panel C). These findings suggest that wheat germ eIF-2 exchanges out phosphorylated reticulocyte eIF-2 from the 15S complex (Fig. 10, panel C).

To determine if phosphorylated wheat germ eIF-2 is able to replace phosphorylated reticulocyte eIF-2 and interact with eIF-2B, we have also probed the 15S complex of inhibited poly IC-treated lysates which are treated with wheat germ eIF-2, with the help of a polyclonal anti-wheat germ eIF-2 antibodies. As shown in Fig. 11, only the top gradient fractions obtained from lysates treated with hemin, poly IC and wheat germ eIF-2, reacted with the polyclonal anti wheat germ eIF-2 antibodies. No signal indicating the presence of wheat germ eIF-2 was observed in the lysate fractions corresponding to the 15S. The finding suggests

Fig. 12. Kinetics of eIF-2B activity in hemin and poly IC-treated reticulocyte lysates in the presence and absence of wheat germ eIF-2: In step 1, labeled binary complex, eIF-2.[^{3}H]GDP, was prepared with reticulocyte eIF-2 (1 μg in 20 μl assay) as described in 'Materials and Methods'. In step 2, protein synthesis of reticulocyte lysates without the addition of unlabeled leucine was performed for 10 min at 30°C in the presence of the following agents: 20 u.M hemin (+h, -O-); heme and 75 ng/ml poly IC (+h +poly IC, -•-), heme, poly IC and 2 μg of WG.eIF-2 [+h +poly IC+ WG.eIF-2 (2), -V-] or heme, poly IC and 4 μg of WG.eIF-2 [+h +poly IC +WG.eIF-2 (4), -▼-]. Then, 70 u1 of preformed eIF-2.[^{3}H]GDP complex (21.17 pmol) of step 1 was added to 87.5 μl of protein synthesizing lysates in step 2 and the reaction mixtures were incubated at 30°C. Forty five microliters were taken out at the times indicated and the amount of eIF-2.[^{3}H]GDP bound to nitrocellulose was determined as described in the 'Materials and Methods'. Values indicate the pmols of [^{3}H]GDP dissociated from eIF-2 and are a direct measure of eIF-2B activity present in the reaction mixture.

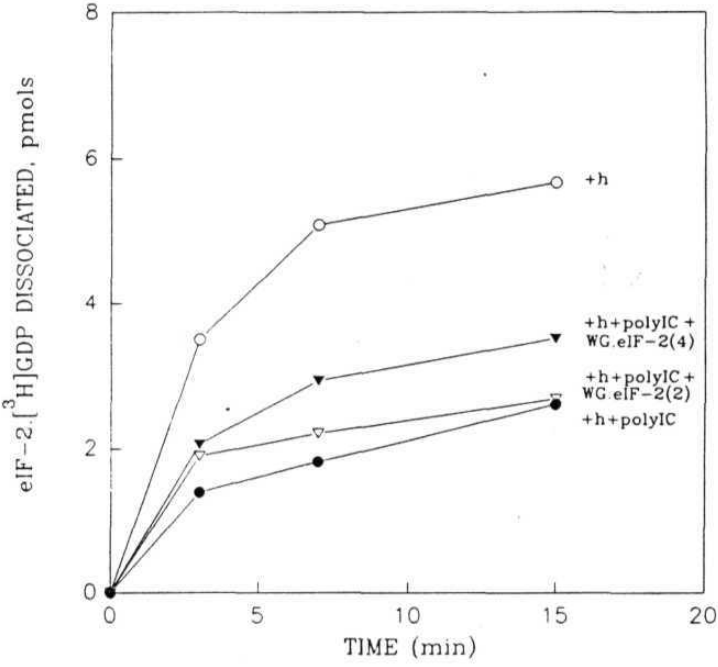

eIF-2.[^{3}H]GDP DISSOCIATED, pmols
TIME (min)
0
2
4
6
8
0
5
10
15
20
+h
+h+polyIC +
WG.eIF-2(4)
+h+polyIC +
WG.eIF-2(2)
+h+polyIC

Table 3

Protein synthesis conditions	eIF-2.[^{3}H]GDP bound, cpm x 10^{-3} (pmol)
-heme	7533(3.13)
+heme	2412(1.00)
-heme + eIF-2 buffer, 4ul	9333 (3.88)
+heme + eIF-2 buffer, 4μl	3973(1.65)
+heme + eIF-2, 4 ug	6818(2.85)

Table 3. cIF-2B activity in heme-deficient lysates treated with or without wheat germ eIF-2: Reticulocyte lysate protein synthesis (25 μl)was carried out at 30°C for 20 min as described under 'Materials and Methods'. Where indicated, incubations (25 μl) were supplemented with hemin (20 uM), WG.eIF-2 (4 ug), or 4 |il of WG.eIF-2 buffer (containing 20 mM Tris-HCl, pH 7.8, 0.1 mM EDTA, 1 mM DTT, 50 mM KCl and 50% glycerol). At 20 min of protein synthesis, 20 μl of preformed eIF-2.[^{3}H]GDP binary complex (5 pmol) was added to protein synthesis mixtures and the reactions were incubated at 30°C for 5 min to determine the amount of labeled GDP bound to eIF-2 as described in 'Materials and Methods'.

that the added wheat germ eIF-2 which gets phosphorylated in heme and poly IC-treated lysates is unable to interact with reticulocyte eIF-2B as efficiently as reticulocyte eIF-2(aP) to form the 15S complex.

3.2.4 eIF-2B activity in inhibited reticulocyte lysate; effect of wheat germ eIF-2.

The exchange of phosphorylated reticulocyte eIF-2 by wheat germ eIF-2 (Fig.s. 10 and 11) in the 15S complex should release functional eIF-2B which can catalyze the dissociation of eIF-2.GDP binary complex. However, the 41-42 kDa subunit in wheat germ eIF-2 is also phosphorylated in reticulocyte lysates (Fig. 7). If the phosphorylated wheat germ eIF-2 is functionally similar to reticulocyte eIF-2(aP), then it should also sequester eIF-2B activity efficiently. Hence, we measured eIF-2B activity of reticulocyte lysates with the help of reticulocyte substrate, eIF-2.[^{3}H]GDP, binary complex. The preformed binary complex, eIF-2.[^{3}H]GDP, is readily dissociated in hemin-supplemented lysates (Fig. 12 and Table 3) in which eIF-2α phosphorylation does not occur significantly and functional eIF-2B activity is available. In heme-deficient lysates or in hemin and poly IC-treated lysates, eIF-2a phosphorylation occurs (Fig. 7 and lanes 1 and 5) and eIF-2B activity is inhibited (Table 3 and Fig. 12). These findings are consistent with the earlier findings (Clemens et al., 1982; Matts and London 1984, Ramaiah et al., 1994; Babu and Ramaiah 1996). In contrast, addition of wheat germ eIF-2 to heme and poly IC-treated lysates or, to heme-deficient lysates restores partially the lysate eIF-2B activity (Fig. 12 and Table 3). Hence, one can see the dissociation of exogenously added reticulocyte binary complex, eIF-2.[^{3}H]GDP, under those conditions in spite of the fact that the added wheat germ eIF-2 does not alter the phosphorylation status of reticulocyte eIF-2α (Fig. 7 tracks 3, 4 and 6). The protection of eIF-2B activity is dependent on the concentration of added wheat germ eIF-2 (Fig. 12). This suggests that wheat germ eIF-2 is probably competing with reticulocyte eIF-2 and eIF-2(aP) to interact with eIF-2B.

These findings, viz,, i) a reduction in the 15S complex formation, ii) absence of wheat germ eIF-2 signal in the 15S complex, and, iii) partial protection of eIF-2B activity in inhibited reticulocyte lysates treated with wheat germ eIF-2 suggest that phosphorylated wheat germ eIF-2 can exchange phosphorylated reticulocyte eIF-2 from the 15S complex in lysates but however, the phosphorylated wheat germ eIF-2 cannot interact with reticulocyte eIF-2B as efficiently as reticulocyte eIF-2(aP).This will facilitate the release of functional reticulocyte eIF-2B activity. However, the released eIF-2B can immediately be sequestered again due to the presence of phosphorylated reticulocyte eIF-2a. The release of eIF-2B under those conditions by wheat germ eIF-2 (briefly before it is complexed again) can catalyze the recycling of a few molecules of reticulocyte eIF-2.GDP. Since the **eIF-2α kinases** are not inhibited, the recycled eIF-2.GDP is also accessible for phosphorylation. Hence one should notice a higher level of reticulocyte **eIF-2α** phosphorylation in inhibited lysates treated with wheat germ eIF-2. Such an enhanced **eIF-2α** phosphorylation is indeed observed previously (London et al., 1983) when inhibited lysates are treated with reticulocyte eIF-2. However, here in our experiments, the level of reticulocyte **eIF-2α** phosphorylation is not enhanced in inhibited lysates treated with wheat germ eIF-2 (Fig. 7). These findings suggest that both these substrates are competing with each other for phosphorylation by reticulocyte eIF-2a kinases. Depending on the competition between wheat germ and reticulocyte eIF-2 for phosphorylation by native kinases and on the ability of these phosphorylated eIF-2 preparation to interact with reticulocyte eIF-2, the translating systems may reach a new steady state level.

3.2.5 Wheat germ eIF-2 dissociation in reticulocyte lysates and in the presence of purified reticulocyte eIF-2(αP).

Our previous studies (Janaki et al., 1995) suggest that dissociation of wheat germ eIF-2.GDP can occur *in vitro* without an eIF-2B-like protein . It is not known if this is due to a contaminant eIF-2B-like protein. To rule out, or support

Fig. 13. Guanine nucleotide exchange on wheat germ eIF-2 in reticulocyte iysates: WG.eIF-2.[^{3}H]GDP, binary complex, was prepared in step 1 as described under 'Materials and Methods' In step 2, protein synthesis in reticulocyte lysates (87.5 µl) was carried out at 30°C for 10 min as described in 'Materials and Mehods'; without heme (-heme, -O-); with 20 µM heme (+heme, -•-) or heme and 100 ng/ml poly IC (+heme +poly IC, -V-). **In** step 3, 70 µl (31.5 pmol) of the preformed binary complex prepared in step 1 was added to protein synthesis mixtures of step 2 and the reaction mixtures were incubated at 25°C. Forty five microliters aliquots were taken at the times indicated and the amount of wheat germ eIF-2.[^{3}H]GDP dissociated was determined as described in 'Materials and Methods'.

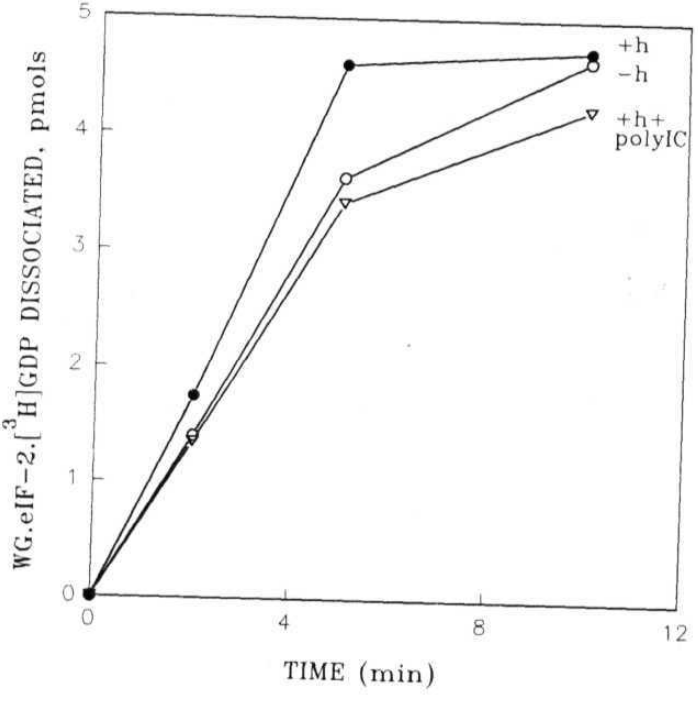

WG.eIF-2.[^{3}H]GDP DISSOCIATED, pmols
TIME (min)
+h
-h
+h+
polyIC
0
1
2
3
4
5
0
4
8
12

Fig. 14. Effect of phosphorylation of wheat germ eIF-2 by HRI *in vitro* on the dissociation of wheat germ eIF-2.[^{3}H]GDP binary complex: In step 1, phosphorylation of WG eIF-2 (1 μg) was carried out at 30°C by HRI and unlabeled ATP as described in Table 4. In step 2, phosphorylated and unphosphorylated reaction mixtures (80 μl) were incubated with [^{3}H]GDP (80 μl binary complex cocktail, without eIF-2) to form eIF-2.[^{3}H]GDP binary complex as described under 'Materials and Methods' Specific activity of [^{3}H]GDP is 2400 cpm/pmol. In step 3, the dissociation of binary complex was monitored in 20 μl aliquots at times indicated, as described in Table 4

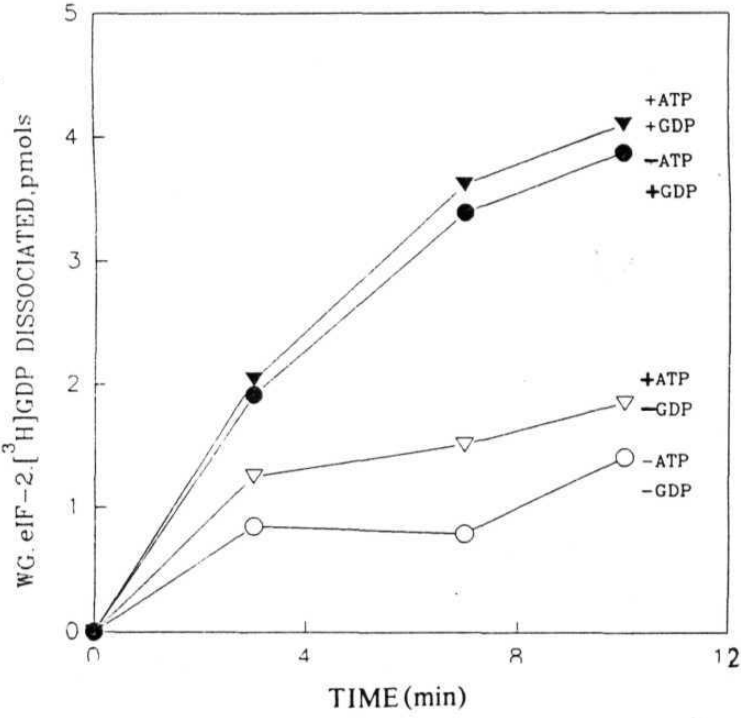

WG. eIF-2.[^{3}H]GDP DISSOCIATED, pmols
TIME (min)
+ATP
+GDP
−ATP
+GDP
+ATP
−GDP
−ATP
−GDP
0
1
2
3
4
5
0
4
8
12

Table **4**

Experimental Conditions		eIF-2.[^{3}H]GDP Bound, CPM X 10^{-3} (pmol)	
		4 min	8 min
-Retic eIF-2 + ATP	- GDP	7648 (3.186)	8394 (3.497)
	+GDP	2278 (0.949)	1142 (0.475)
+Retic eIF-2 - ATP	- GDP	12000 (5.000)	14422 (6.000)
	+GDP	4612 (1.921)	2128 (0.886)
+Retic eIF-2 + ATP	- GDP	10604 (4.418)	9340 (3.891)
	+ GDP	2926 (1219)	1042 (0434)

Table 4. Wheat germ eIF-2.[^{3}H]GDP binary complex dissociation in the presence of purified phosphorylated reticulocyte eIF-2 *in vitro:* **In** step 1, phosphorylation of reticulocyte eIF-2 was carried out for 7 min at 30°C by HRI (0.1 μg), in a **10** μl reaction mixture in the presence of Tris buffer (20 mM Tris-HCl, pH 7.6, 80 mM KCl and 2.5 mM $Mg(OAc)_2$) with and without the addition of 100 μM ATP. The control unphosphorylated reaction mixtures contained HRI without ATP. In step 2, WG.eIF-2.[^{3}H]GDP complex was prepared as described under 'Materials and Methods'. Specific activity of [^{3}H]GDP is 2400 cpm/pmol. In step 3, dissociation of 50 μl, 10.425 pmols, of preformed binary complex was measured in the presence of 18.75 μl of phosphorylated and unphosphorylated reaction mixtures, with or without the addition of 40 μM unlabeled GDP. At different time intervals (4 and 8 min), 27.5 μl of reaction mixtures were withdrawn at times indicated and the amount of WG.eIF-2.[^{3}H]GDP bound to nitrocellulose was determined as described in 'Materials and Methods'.

such a possibility, it is important to assess the specificity of wheat germ eIF-2.GDP dissociation under conditions in which eIF-2a is phosphorylated. Although our earlier observations suggest that phosphorylation of p36 and also p41-42 doublet - subunit of wheat germ eIF-2 by NEM-treated wheat germ lysates or by purified CK II does not inhibit the GNE on wheat germ eIF-2, it is likely that the above mentioned conditions are unable to phosphorylate the wheat germ eIF-2 at a right site for the inhibition to occur. Hence we tested here the GNE activity of wheat germ eIF-2 in inhibited (minus heme, or heme and poly IC conditions), or in actively translating (plus heme) reticulocyte lysates in which the reticulocyte eIF-2B activity is diminished due to phosphorylation of the serine 51 residue in reticulocyte eIF-2a, or protected due to the inhibition in eIF-2α phosphorylation, respectively (Fig. 13). The dissociation of preformed labeled wheat germ eIF-2.[^{3}H]GDP complex occurs readily and significantly in all these lysates. But it is marginally inhibited in heme-deficient or poly IC-treated reticulocyte lysates. These findings suggest that the GNE on wheat germ eIF-2 can occur independent of an eIF-2B-like protein. However, a functional eIF-2B activity (available in uninhibited heme-supplemented lysates) may enhance the exchange of GDP on eIF-2 (Fig. 13). Also the exchange of unlabeled GDP for labeled GDP in wheat germ eIF-2.[^{3}H]GDP binary complex is estimated *in vitro* in the presence of reticulocyte heme-regulated eIF-2α kinase and ATP (Fig. 14). This kinase preparation phosphorylates the p41-42 doublet-subunit of wheat germ eIF-2 and also the small-subunit of reticulocyte eIF-2 (eIF-2a) (Fig. 5 A). Exchange of GDP on wheat germ eIF-2 is not affected by phosphorylation of wheat germ eIF-2 by reticulocyte eIF-2a kinase (HRI) (Fig. 14).

In another experiment, phosphorylated reticulocyte eIF-2(αP) has been added to wheat germ eIF-2.[^{3}H]GDP binary complex to determine if the exchange of GDP on wheat germ eIF-2 can be inhibited (Table 4). The findings indicate that addition of unphosphorylated reticulocyte eIF-2 or phosphorylated reticulocyte eIF-2a cannot inhibit the GNE on wheat germ eIF-2 *in vitro,* a finding that

matches well with the observations made in reticulocyte lysates (Fig. 13). These findings are also consistent with the previous reports which suggest that eIF-2B analogs are probably not involved in the exchange of guanine nucleotides on wheat germ eIF-2. This may be because the affinity of wheat germ eIF-2 for GDP is only 10 times higher than that for GTP (Shaikin et al., 1992).

3.2.6 Distribution of reticulocyte and wheat germ eIF-2 in polysomes obtained from inhibited reticulocyte lysates treated with or without wheat germ eIF-2.

Earlier observations of Ramaiah et al., (1992) has shown that reticulocyte eIF-2 is translocated from the 48S initiation complex to the 60S subunit of 80S initiation complexs and also of polysomes. The presence of eIF-2 on the 60S subunits of polysomes is incompatible with the conventional model in which eIF-2 is recycled during the joining of the 48S preinitiation complex and the 60S subunit to form the 80S initiation complex. Immunoblot analysis of the eIF-2 distribution in sucrose gradients of actively protein synthesizing lysates indicates that eIF-2 is distributed at low levels throughout the polysome profiles. Further, immobilization of the polysomes by the addition of cycloheximide resulted in higher level of eIF-2a phosphorylation in cycloheximide treated heme-deficient lysates than in heme-deficient lysates. This led to the conclusion that polysomal eIF-2 is a target for HRI and that maintenance of the polysomes is associated with increased phosphorylation of eIF-2a. Based on these findings, and also on other observations (Thomas et al., 1984) which indicated that eIF-2B is required for the release of eIF-2.GDP from the 60S subunits of initiating monosomes, a modified model has been presented for the recycling of eIF-2 and the distribution of eIF-2 on polysomes (Ramaiah et al., 1992). The recycling of eIF-2 by eIF-2B catalyzed exchange of GTP for GDP in the eIF-2.GDP complex may occur only partially from the initiating 80S monosomes due to the limiting amount of eIF-2B, thus permitting the remaining eIF-2.GDP to stay bound to the 60S subunit of ribosomes

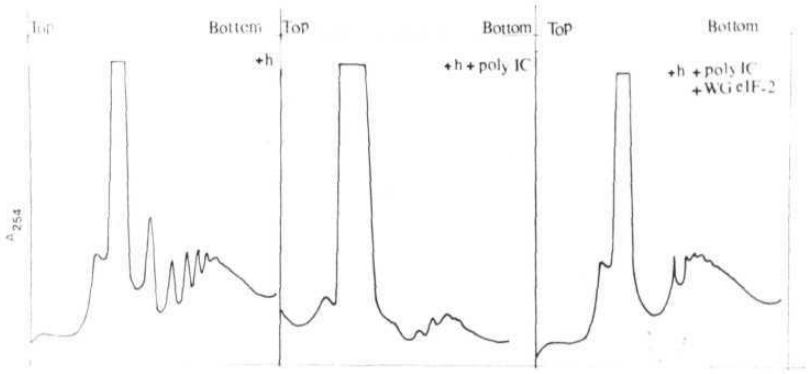
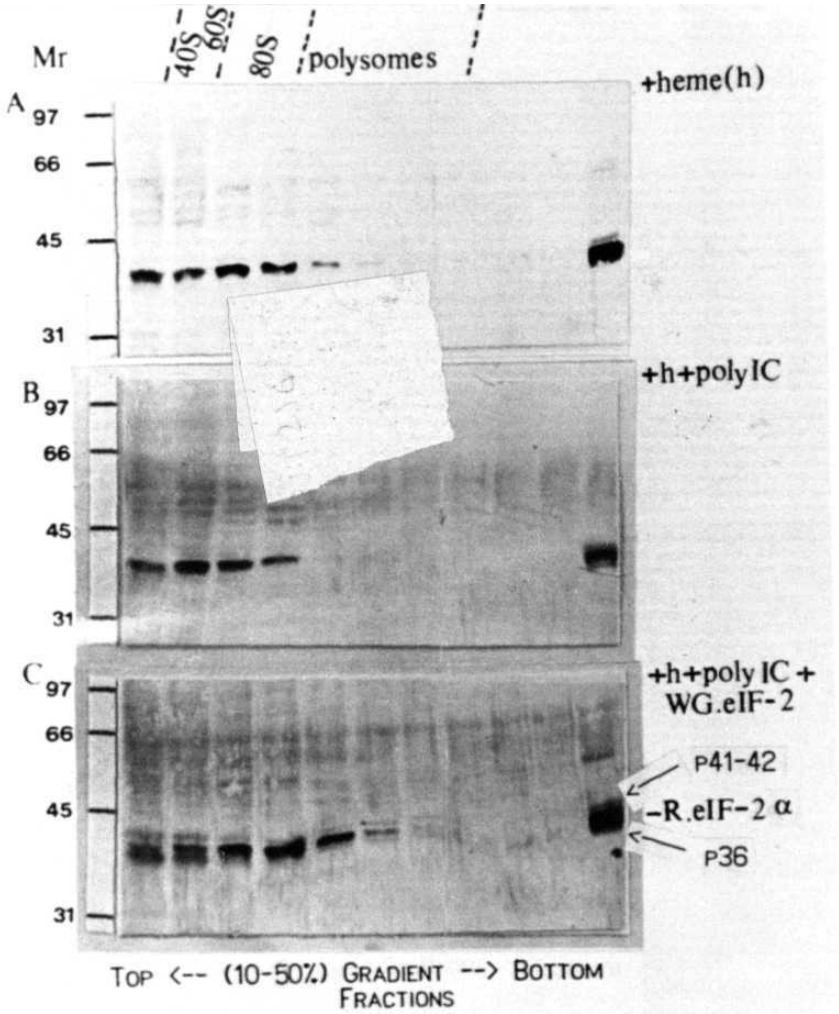


Fig. 15 **Polysome** profiles of reticulocyte lysates treated **with heme,** heme and poly 1C and heme, poly **IC** and wheat germ **eIF-2:** Protein synthesis reactions (125 μl) were carried out at 30°C for 15 min in **heme-supplemented** (+h, 20 μM), heme and poly IC (75 ng/ml) (+h + poly IC) and also heme, poly IC and wheat germ eIF-2 (10 μg) (+h +poly IC +WG.eIF-2) treated reticulocyte lysates as described in 'Materials and Methods' The reaction mixtures were chilled on ice, diluted with equal volume of 2X TMK buffer (containing 20mM Tris-HCl, pH 7.8, 2.5 mM $Mg(OAc)_2$ and 80 mM KC1), and layered on a 10-50% linear sucrose gradient as described in 'Materials and Methods'. The gradients were spun at 40,000 rpm in a Beckman SW 50.1 rotor for 1 hour and then analysed by ISCO UA-6 density gradient fractionator. Top and bottom of the gradients arc shown

Fig. 16. Western immunoblot analysis of the **10-50% sucrose gradient fractions of reticulocyte lysates supplemented with or without wheat germ eIF-2to detect of the presence of wheat germ and reticulocyte eIF-2 in the polysome fractions:** Protein synthesis reactions (125 μl) were carried out at 30°C for 15 min in heme-supplemented (+h, 20 μM; Panel A), heme and poly IC (75 ng/ml) (+h + poly IC; Panel B) and also heme, poly IC and wheat germ eIF-2 (10 ug) (+h +poly IC + WG.eIF-2; Panel C) treated reticulocyte lysates as described in 'Materials and Methods'. The reaction mixtures were chilled on ice, diluted with equal volume of 2X TMK buffer (containing 20mM Tris-HCl, pH 7.8, 2.5 mM $Mg(OAc)_2$ and 80 mM KCl), and layered on a 10-50% linear sucrose gradient as described in 'Materials and Methods'.

The gradients were spun at 40,000 rpm in a Beckman SW 50.1 rotor for 1 hour. Fractions (350 μl) were collected by upward displacement of gradients in a ISCO UA-6 density gradient fractionator. The fractions were concentrated by pH 5.0 precipitation, pellets were dissolved in SDS sample buffer and proteins were separated on 10% SDS-PAGE. The proteins were transferred on to nitrocellulose filter papers and the blots were treated successively with anti-human eIF-2α monoclonal antibody and then with wheat germ eIF-2 polyclonal antibody. The nitrocellulose sheets were then developed to detect the eIF-2 from both the origins as described in 'Materials and Methods'.

The position of 40/60S ribosomes, 80S ribosomes and polysomes is indicated on the top of the figure. The lane towards the extreme right of the figure is a marker lane having purified reticulocyte eIF-2 (500 ng) and wheat germ eIF-2 (1 μg). The position of migration of the **a** subunit of reticulocyte eIF-2 and the p36 and p41-42 subunit of wheat germ eIF-2 is indicated. The left hand side of the figure shows migration of the molecular weight markers.


Mr
40S
60S
80S
polysomes
+heme(h)
A
97
66
45
31
+h+polyIC
B
97
66
45
31
+h+polyIC+
WG.eIF-2
C
97
66
45
31
P41-42
-R.eIF-2α
P36
TOP <-- (10-50%) GRADIENT --> BOTTOM
FRACTIONS

that are involved in the process of elongation. The polysome bound eIF-2 GDP is a target for eIF-2B and also for HRI. The eIF-2(aP).GDP can bind and sequester eIF-2B which is then unavailable for GTP/GDP exchange, but the [eIF-2B.eIF-2(aP)] complex can dissociate from the riboscmes and then appear as 15S complex in the non-ribosomal cytosol (Thomas et al., 1984). Hence, the recycling of eIF-2 and distribution of eIF-2 on 60S subunit of polysomes may require the activity of eIF-2B protein.

Keeping in view of these observations, we have studied here the distribution of eIF-2 in inhibited reticulocyte lysates treated with heme, poly IC and wheat germ eIF-2. Addition of wheat germ eIF-2 mitigates protein synthesis inhibition and inhibition in eIF-2B activity that occurs due to eIF-2a phosphorylation. Under these conditions, the polysomes that are dissociated in inhibited lysates, are partially reformed (Fig. 15). Further, we have collected the various fractions of translating lysates treated with heme or heme and poly IC or heme, poly IC and wheat germ eIF-2 to study the distribution of eIF-2 in these sucrose gradient fractions of the translating lysates. This work has been carried out to determine the distribution of reticulocyte eIF-2 on polysomes in relation to the functional eIF-2B activity that is available under these conditions. The gradient fractions are probed with a monoclonal reticulocyle eIF-2a antibody, to detect the presence the reticulocyte eIF-2 in ribosome and non-ribosomal fractions. Also the fractions have been probed with a polyclonal wheat germ eIF-2 antibody, to determine if wheat germ eIF-2 is able to substitute for reticulocyte eIF-2 in protein synthesis reactions and can be found on polysomes (Fig. 16).

The results indicate that low levels of reticulocyte eIF-2 are found in the 80S and polysome fractions of heme-supplemented actively translating lysates, Most of the eIF-2 is seen in the 15S, 40S/60S fractions (Fig. 16, panel A). In inhibited lysates treated with heme and poly IC, the polysomes are disintegrated due to impaired initiation, hence, reticulocyte eIF-2 is not seen in polysome fractions; the eIF-2 is found associated with 15S / 40S / 60S and 80S fractions

(Fig. 16, panel B). Addition of wheat germ eIF-2 to heme and poly IC treated lysates facilitates the formation of polysomes partially (Fig. 15) and is correlated to partial restoration of protein synthesis and eIF-2B activity (Figs 8 and 12). The gradient fractions obtained from these lysates are probed with both wheat germ and reticulocyte eIF-2 antibodies. As can be seen from the Fig. 16, panel C, the fractions containing the reticulocyte eIF-2 band is flanked by the p41-42 doublet-subunit (above) and p36 subunit (below). The amount of reticulocyte eIF-2a found in the polysomes is higher in panel C (+heme, +poly IC + wheat germ eIF-2) than in panel A (+heme only). These findings suggest that reticulocyte eIF-2 stays bound to the polysomes depending on the availability of eIF-2B. Since, wheat germ eIF-2 protects partially the inhibition in eIF-2B activity of inhibited reticulocyte lysates, one can see higher levels of eIF-2 on the polysomal fractions than in the fractions obtained from actively translating lysates in which the kinase is not activated and the functional eIF-2B activity is available.

Interestingly, the wheat germ eIF-2 is also found to be associated with reticulocyte polysomes, suggesting that it may be able to substitute reticulocyte eIF-2a in protein synthesis.

3.2.7 Addition of poly IC (ng-µg/ml) to wheat germ lysates does not affect wheat germ translation, eIF-2 phosphorylation and the GNE activity of eIF-2.

Previously, this laboratory reported that addition of low concentrations of dsRNA (20-40 ng/ml) to wheat germ lysates does not affect the wheat germ protein synthesis. In contrast, it has been observed here (Fig. 8a) that the same concentration of dsRNA can effectively inhibit reticulocyte lysate protein synthesis (Janaki et al., 1995). Also, as mentioned in earlier results, the PKR kinase activity that gets stimulated in reticulocyte lysates in the presence of poly IC can phosphorylate the small-subunit of reticulocyte eIF-2 (eIF-2a) and also the p41-42 doublet-subunit of wheat germ eIF-2 (Fig. 7). In addition, we have shown that a small amount of purified dsI (PKR) kinase (obtained as a gift) has been shown to

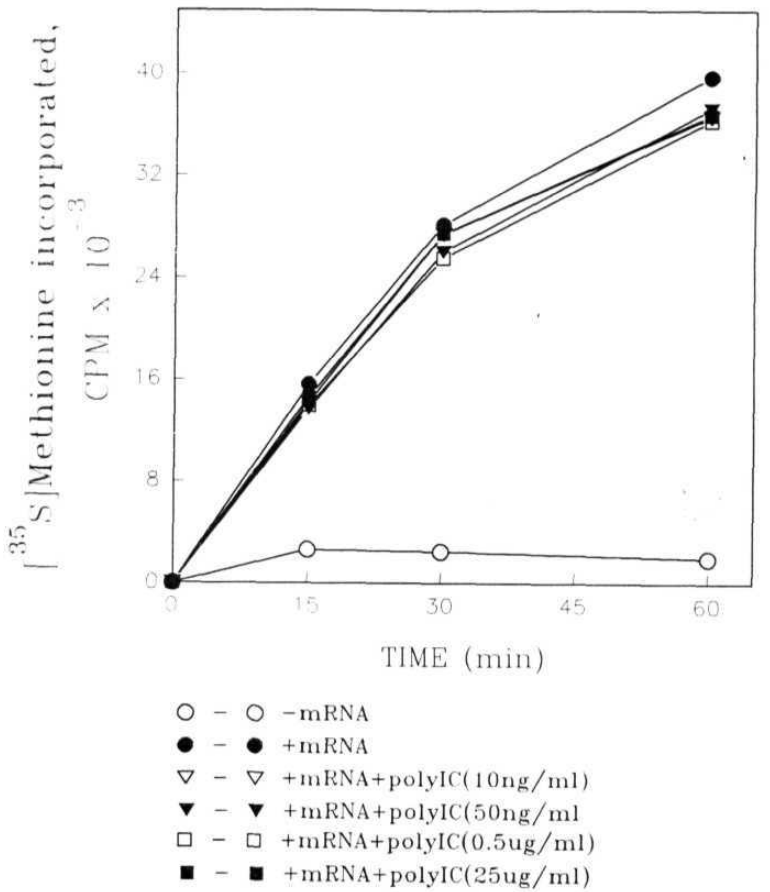


Fig. 17. **Wheat germ** lysate protein synthesis in the presence of varying concentrations of poly **IC:** Protein synthesis in wheat germ lysates was carried out as described in 'Materials and Methods'. Where indicated, varying concentrations of poly IC was added at 0 min. of protein synthesis. The extent of protein synthesis was determined by the incorporation of [^{35}S]methionine in a 5 µl aliquot of the reaction mixture as described in 'Materials and Methods'.

Fig. 18. [^{32}P] phosphoprotein profiles of wheat germ lysates in the presence of different concentrations of poly IC and wheat germ eIF-2: This experiment was carried out in two steps. In step 1, wheat germ lysates (10µl) were treated with varying concentrations of poly IC [0.1 (lanes 3 and 4, 1.0 (lanes 5 and 6), 10 0 (lanes 7 and 8) and 100 (lanes 9 and 10) µg/ml] in the presence of phosphorylation cocktail containing 20 mM Tris-HC1, pH 7.8, 2.5 mM $Mg(OAc)_2$, 80 mM KC1 and 30 u.M ATP. The treated lysates were incubated at 25°C for 10 min. This was done to activate any PKR-like enzyme in our wheat germ lysate preparation. In step 2, 2 |ig of wheat germ eIF-2 was added to reaction mixtures of lanes 2, 4, 6, 8 and 10 and 2 µl of double distilled water to reaction mixtures of lanes 1, 3, 5, 7 and 9, along with 10µCi [γ-^{32}P]ATP (specific activity 3000 Ci/mmol) and incubated for further 10 min at 25°C. Lanes 1 and 2 are control lanes without and with wheat germ eIF-2 respectively. Reactions were terminated with 2X-SDS sample buffer and the phosphoproteins were separated on 10% SDS-PAGE. The gel was dried and exposed to X-ray film. The figure is an autoradiogram.

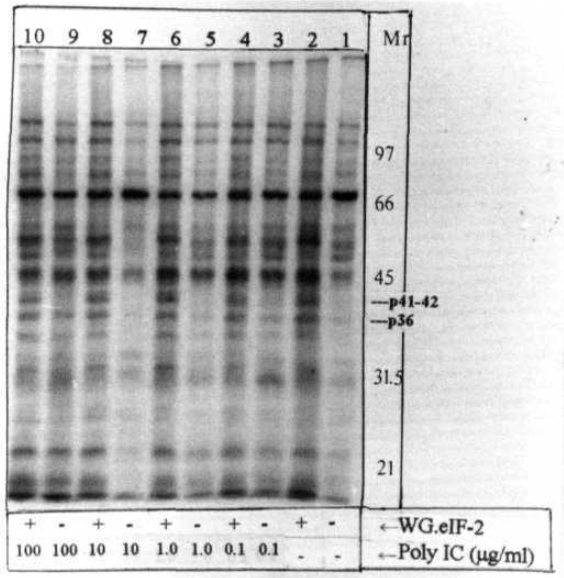
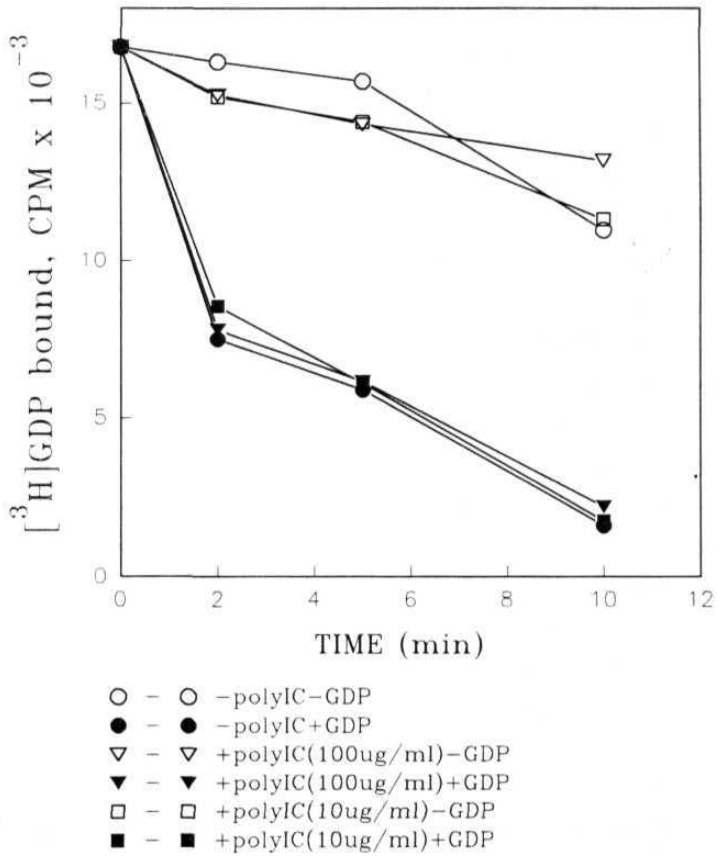

10 9 8 7 6 5 4 3 2 1
Mr
97
66
45
p41-42
p36
31.5
21
+ - + - + - + - + -
100 100 10 10 1.0 1.0 0.1 0.1 - -
WG.eIF-2
Poly IC (µg/ml)

Fig. 19. Effect of **poly IC-treated wheat germ lysate on wheat** germ ***eIF-2.[^{3}H]GDP*** complex **dissociation:** In step 1, wheat germ lysates (10μl) were treated with 2.5 mM $Mg(OAc)_2$ and 100μM ATP and incubated at 25"C for 10 min with or without the addition of poly IC (10 μg/ml and 100 μg/ml) to facilitate the activation of endogenous PKR (if any) and eIF-2 phosphorylation In step 2, wheat germ eIF-2 [^{3}H]GDP binary complex was prepared as described in Material and Methods. In step 3, 35 μl the poly IC-treated and untreated wheat germ lysates obtained from step 1 were incubated for 5 min at 25°C with preformed binary complex (70 μl, 55 pmol) in the presence of 100 μM ATP and 2 5 mM $Mg(OAc)_2$. The dissociation of the preformed binary complex was monitored in 30 μl reaction mixtures at different time intervals with and without the addition of 40 uM unlabeled GDP as described in 'Materials and Methods' One pmol of bound eIF-2. [^{3}H]GDP gives 2,200 cpm. **The** plot indicates the dissociation of labeled GDP from eIF-2.


[3H]GDP bound, CPM x 10-3
0
5
10
15
0
2
4
6
8
10
12
TIME (min)
○ - ○ -polyIC-GDP
● - ● -polyIC+GDP
▽ - ▽ +polyIC(100ug/ml)-GDP
▼ - ▼ +polyIC(100ug/ml)+GDP
□ - □ +polyIC(10ug/ml)-GDP
■ - ■ +polyIC(10ug/ml)+GDP

phosphorylate the same above subunit of purified eIF-2 from both origins *in vitro* (Fig. 5B).

However, the paper by Langland et al., (1996), that appeared at the conclusion of this work suggests that phosphorylation of wheat germ initiation fator-2 by a plant and mammalian PKR inhibits wheat germ translation. These authors have observed that relatively higher concentrations (10-100 μg/ml) of poly IC or dsRNA are required to inhibit wheat germ translation. However, they have not demonstrated that phosphorylation of wheat germ eIF-2a inhibits protein synthesis by reducing the GNE activity of eIF-2B like protein. Also, these authors have not presented any evidence which would indicate that addition of dsRNA can enhance the autophosphorylation of PKR in wheat germ lysates correlating to the enhanced eIF-2 phosphorylation. Hence, higher concentrations of poly IC have been used here, to determine its effects on wheat germ **protein** synthesis, eIF-2 phosphorylation and on the GNE activity of eIF-2.

As has been shown (Fig. 17) that the wheat germ translation is unaffected by the addition of different concentrations of poly IC to wheat germ lysates and is consistent with the data obtained on the activation of wheat germ PKR activity and eIF-2 phosphorylation in lysates (Fig. 18). None of the concentrations of poly IC added to wheat germ lysates could stimulate the phosphorylation of p41-42 doublet-subunit in wheat germ lysates (Fig. 18). Also the addition of poly IC-treated wheat germ lysates does not affect the dissociation of wheat germ eIF-2.[^{3}H]GDP binary complex ***in*** *vitro* (Fig. 19). These findings suggest that poly IC treatments (from a low to a high concentration) fails to activate any eIF-2a kinase like PKR in wheat germ lysates here, which can phosphorylate the p41-42 subunit or other subunits of purified wheat germ eIF-2 and inhibit the GNE activity associated with wheat germ eIF-2. In contrast , it has been demonstrated here, in the earlier results that stimulation of a PKR activity in reticulocyte lysates by the addition of poly IC can phosphorylate the p41-42 subunit of wheat germ eIF-2

(Fig. 7). However, this phosphorylation of wheat germ eIF-2 does not inhibit the GNE activity associated with wheat germ eIF-2 (Fig. 13).

3.3 SECTION B: DISCUSSION

Phosphorylation of serine 51 residue in the eIF-2a subunit inhibits the GNE activity of eIF-2B protein and thereby inhibits protein synthesis in mammalian systems (London et al., 1987; Jackson, 1991, Hershey, 1991; Merrick, 1992; Proud, 1992; Redpath and Proud, 1994), yeast (Dever et al., 1993) and insects (Chefalo et al., 1994). Mammalian eIF-2α kinases can functionally substitute the eIF-2a kinases of yeast and also insects (Dever et al., 1993; Chefalo et al., 1994). Previous results from different laboratories indicate that wheat germ eIF-2 subunits can be phosphorylated by mammalian eIF-2a kinases. However, addition of heme-regulated eIF-2α kinase (HRI), one of the well characterized mammalian eIF-2α kinases (Chen and London, 1995) does not inhibit the wheat germ translation (Janaki et al., 1995; Ranu, 1980). In contrast, a kinase purified from wheat germ had been shown long time ago to phosphorylate one of the wheat germ eIF-2 subunits and inhibit translation (Ranu, 1980). This kinase has not been charecterized any further since then. Recently, a study (Langland et al., 1996) with higher plants indicates that addition of higher concentrations of dsRNA or phosphorylated eIF-2a inhibits the wheat germ translation *in vitro,* presumably due to the activation of a PKR-like activity that stimulates eIF-2a phosphorylation which in turn may inhibit the plant eIF-2B-like activity. However, a direct evidence has not been presented to correlate the protein synthesis inhibition to enhanced PKR activity and eIF-2a phosphorylation in lysates under those conditions. Also, no reports are available which suggest that phosphorylation of a plant eIF-2 subunit can inhibit plant eIF-2B activity. The mechanism by which protein synthesis inhibition occurs in wheat germ lysates treated with high concentration of dsRNA or phosphorylated eIF-2a is not clear.

In a recent study by us (Janaki et al., 1995), it has been shown that both the p41-42 doublet and p36 subunits of wheat germ eIF-2 can be phosphorylated in NEM treated lysates and also by purified CK II. These agents also inhibit wheat germ translation. However, phosphorylated wheat germ eIF-2 is unable to inhibit

the GNE activity associated with purified wheat germ eIF-2. It is likely that these conditions are unable to phosphorylate wheat germ eIF-2 at a right site (like the serine 51 residue in reticulocyte eIF-2a) to inhibit the GNE activity. Also the nature of the GNE activity associated with wheat germ eIF-2 is not understood. It is not known if this is a contaminant eIF-2B-like activity or if it is an inherent property of plant eIF-2.

In this study, mammalian eIF-2α kinases (purified or present in lysates) have been used to phosphorylate wheat germ eIF-2. This is because, we were unable to stimulate the PKR like activity of wheat germ lysates by the addition of low (Janaki et al., 1995) or high concentrations of dsRNA which can stimulate wheat germ PKR-like activity and inhibit protein synthesis through enhanced wheat germ eIF-2 phosphorylation and decreased GNE activity of wheat germ eIF-2 (Fig.s 17, 18 and 19). It is not known if this variation in lysate preparations in terms of dsRNA levels required for the inhibition, with several lysates showing no response to dsRNA, may be due to varying levels of a glycosylated p67 like protein (Datta et al., 1989) as has been suggested by Langland et al., (1996). In our opinion, this may also occur due to other regulators of eIF-2a phosphorylation such as small nuclear RNAs, heat-shock proteins and viral encoded proteins etc (Clemens et al., 1994). The unpublished observations of Langland et al., (1996) suggest that p67 inhibits mammalian and plant PKR autophosphorylation and also eIF-2a phosphorylation. In contrast, our unpublished results from this laboratory suggest that p67 does not influence the autophosphorylation of heme-regulated eIF-2a kinase, but it can inhibit the kinase catalyzed eIF-2a phosphorylation *in vitro* (Babu and Ramaiah 1996). Hence it is difficult for us to believe that p67 inhibits the autophosphorylation of PKR.

Because of conflicting reports on the p67 regulated eIF-2α phosphorylation mechanism and the difficulty to obtain a wheat germ lysate preparation in which endogenous PKR like activity can be stimulated to study the effect of phosphorylation of wheat germ eIF-2 on the GNE activity of an eIF-2B-

like protein present in lysates, we have used here mammalian eIF-2a kinases to phosphorylate wheat germ eIF-2. Our results here indicate that, phosphorylation of wheat germ eIF-2 by HRJ *in vitro* cannot inhibit the GNE activity of wheat germ eIF-2 (Fig. 14). Although significant phosphorylation of wheat germ eIF-2 is observed by added HRI (Fig. 5A) the GNE activity is unaffected under those conditions. Similarly, the phosphorylation of wheat germ eIF-2 occurs in reticulocyte lysates (Fig. 7) in which HRI or PKR is activated but the phosphorylated wheat germ eIF-2 cannot reduce the GNE activity associated with wheat germ eIF-2 (Fig. 12 and Table 4). However, a functional eIF-2B activity that is present in uninhibited hemin-supplemented lysates can enhance marginally the guanine nucleotides on wheat germ eIF-2 (Fig. 13). In contrast, phosphorylation of reticulocyte eIF-2a that occurs in those inhibited lysates decreases reticulocyte eIF-2B activity significantly (Fig. 12) as has been suggested (Clemens et al., 1982; Matts and London, 1984; Ramaiah et al., 1994; Babu and Ramaiah, 1996).

However, the presence of a phosphorylated reticulocyte eIF-2a in inhibited lysates or addition of purified reticulocyte eIF-2(aP) (in which serine 51 residue is phosphorylated) does not inhibit the GNE activity associated with wheat germ eIF-2 (Fig. 13 and Table 4). These findings suggest that the GNE activity of wheat germ eIF-2 is not due to a contaminant eIF-2B-like protein and supports the idea that the nucleotide exchange on wheat germ eIF-2 proceeds under sufficiently high (GTP/GDP) ratios without the mediation of an eIF-2B-like protein as has been suggested by Shaikin et al., (1992). In contrast, the recent paper by Langland et al., (1996) suggests that plants appear to encode a factor like GCN3, yeast equivalent of eIF-2B. These observations raise the physiological relevance of such an eIF-2B-like, protein particularly when the GDP bound to eIF-2 can be exchanged without the mediation of an eIF-2B-like protein. It is likely that the endogenous protein may still be required to perform other functions in addition to exchanging GDP bound to eIF-2. In fact, the previous work in reticulocyte lysates

suggests the involvement of eIF-2B protein to release eIF-2 from the 60S subunit of 80S initiation complexes, in addition to exchanging guanine nucleotides on eIF-2 (Thomas et al., 1985; Ramaiah et al., 1992). eIF-2B is a multi-subunit protein in yeast and mammals and the functions of the different subunits are not yet understood.

We have also shown here that wheat germ eIF-2 mitigates protein synthesis inhibition in reticulocyte lysates that is mediated by reticulocyte eIF-2α phosphorylation (Fig. 8a and 8b). Addition of wheat germ eIF-2 does not decrease the level of reticulocyte eIF-2α phosphorylation (Fig 7, tracks 3 Vs 6) and the inhibited reticulocyte lysates also facilitates the phosphorylation of wheat germ eIF-2 p41-42 subunit (Fig. 7, tracks 3 Vs 6). Consistent with the decrease in the inhibition in protein synthesis, addition of wheat germ eIF-2 to inhibited reticulocyte lysates also protects the lysate eIF-2B activity (Fig. 12 and Table 3). This is possible if wheat germ eIF-2 is exchanging the phosphorylated reticulocyte eIF-2α from the 15S complex, [eIF-2(aP).eIF-2B], and the phosphorylated wheat germ eIF-2 is unable to interact with reticulocyte eIF-2B as efficiently as phosphorylated reticulocyte eIF-2a. This possibility is also tested here by monitoring the 15S complex formation in inhibited reticulocyte lysates treated with wheat germ eIF-2 (Fig. 10). The results indicate that 15S complex formation is reduced in inhibited lysates treated with wheat germ eIF-2 (Fig. 10, panel C) compared to lysates carrying protein synthesis without the addition of wheat germ eIF-2 (Fig. 10, Panel C Vs A) . However, these results do not reveal if phosphorylated wheat germ eIF-2 is able to interact with eIF-2B. Hence, we measured eIF-2B activity under those conditions and also probed the 15S complex of inhibited lysates treated with wheat germ eIF-2 with the help of polyclonal anti-wheat germ eIF-2 antibodies (Fig. 11). Since the eIF-2B activity is partially protected (Fig. 12 and Table 3) and the 15S complex does not show any wheat germ eIF-2 signal (Fig. 11), the findings suggest that phosphorylated wheat germ eIF-2 can exchange phosphorylated reticulocyte eIF-2 in the 15S complex, but it

cannot interact with eIF-2B as efficiently as reticulocyte eIF-2(aP). Hence this should briefly make eIF-2B free before it is complexed again with reticulocyte eIF-2(aP). The free eIF-2B can immediately catalyze the exchange of GDP on reticulocyte eIF-2 so as to enable the latter to enter the initiation cycle.

The above observations, that is, a) the ability of wheat germ eIF-2 to counter the phosphorylation of reticulocyte eIF-2a, b) the inability of phosphorylated wheat germ eIF-2 to form a 15S complex with reticulocyte eIF-2B, c) the inability of phosphorylated wheat germ eIF-2 to inhibit the GNE activity associated with it and d) the ability of plant eIF-2 to carry the guanine nucleotide exchange independent of eIF-2B-like protein suggests that wheat germ eIF-2 is different from mammalian eIF-2. It may be the result of some modifications in the amino acid sequences in and around the phosphorylation site in wheat germ eIF-2. This presumption is supported by earlier evidences which indicate that phosphorylated mutant human eIF-2a (like 48A mutant in which serine 51 residue is phosphorylated) cannot inhibit the eIF-2B activity and helps to overcome the protein synthesis inhibition mediated by eIF-2α phosphorylation (Choi et al., 1992; Murtha-Riel et al., 1993; Ramaiah et al., 1994) and eIF-2B activity (Ramaiah et al., 1994). Also another study with yeast cells identified several mutations within 40 amino acids of the phosphorylation site that can overcome the inhibitory effect of eIF-2a phosphorylation at residue serine 51 mediated by GCN2 kinase (Hinnebusch, 1994). Also a mutation in eIF-2B protein can counter the phosphorylation of eIF-2a. In addition to these supporting observations, our presumption is also consistent with the recent findings of Langland et al., (1996) which indicate that atleast 17% sequences in plant eIF-2α around the phosphorylation domain (45-56 region) are different from human eIF-2a sequence. These modifications in plant eIF-2 may explain in part the properties of plant eIF-2 that are different from mammalian eIF-2, and also the ability of plant eIF-2 to behave like a mutant phosphorylated eIF-2 in rescuing protein synthesis inhibition in reticulocyte lysates that is mediated by reticulocyte eIF-2α phosphorylation.

We have not however, ruled out the possibility that phosphorylated wheat germ eIF-2 cannot interact with its own eIF-2B (if it present). It is possible that homologous factors may interact more efficiently and the phosphorylated wheat germ eIF-2 may still form a complex with wheat germ eIF-2B (if it is present) and can inactivate it. Since wheat germ eIF-2 can exchnage guanine nucleotides with out the requirement of an eIF-2B like protein (based on the observations here) and phosphorylated wheat germ eIF-2 has been shown to reduce translation significantly in wheat germ lysates (Langland et al., 1996), it is likely that the inhibition of protein synthesis reported by Langland et al., 1996, may be due to an impairment of some other activity of eIF-2B protein other than the GNE activity.

3.4 SECTION C: RESULTS AND DISCUSSION

3.4.1 Limited proteolytic digestion of phosphorylated wheat germ eIF-2 subunits.

It is well known that phosphorylation of serine 51 residue in mammalian and yeast eIF-2a decreases the overall protein synthesis by inactivating the GDP/GTP exchange factor called eIF-2B (Price and Proud, 1994; Wek, 1994).

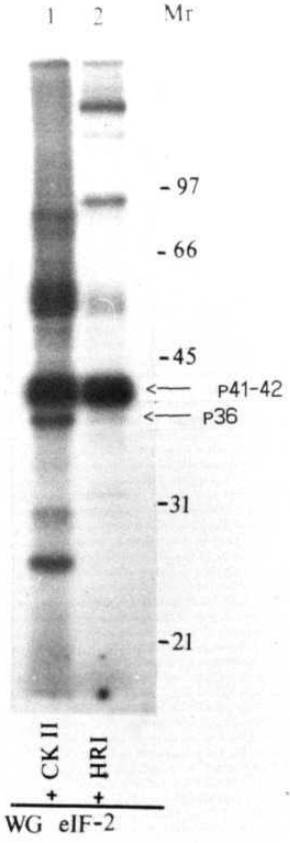
Although some earlier studies suggested that there may be two phosphorylation sites (serine 48 and serine 51) in mammalian eIF-2a, subsequent work by amino acid sequencing (Colthrust et al., 1987), site specific mutagenesis (Pathak et al., 1988a), mutational studies (Vazquez de Aldana et al., 1993) and phosphatase inhibitors (Proud, 1992) has shown that the serine 51 is the only residue in mammalian eIF-2a that can be phosphorylated. Recent studies with yeast indicate that yeast eIF-2α has atleast three potential phosphorylation sites on the C-terminus (291, 293 and 300 serine residues), in addition to serine 51 residue. Phosphorylation of serine 51 residue in yeast eIF-2a by eIF-2a kinases from yeast and mammalian sources stimulate the translation of GCN4 mRNA and decreases the translation of other mRNA's. However, phosphorylation of other residues by CK II does not effect any activities *in vivo* (van den Heuvel et al., 1994).

Here we have found that wheat germ eIF-2 can be phosphorylated by CK II, HRI, dsI (see section A) and also in NEM-treated lysates (Janaki et al., 1995). Studies from this laboratory indicated for the first time that both p36 and p41-42 doublet-subunits of wheat germ eIF-2 can be phosphorylated by purified CK II (section A)and in NEM treated wheat germ lysates (Janaki et al., 1995). In addition to CK II, reticulocyte eIF-2a kinases are also shown to phosphorylate that p41-42 doublet-subunit of wheat germ eIF-2 (section A).

The following work concerning the sites in wheat germ eIF-2 subunits (p36) and p41-42 (which can be phosphorylated by CK II or HRI) has been

Fig. 20. **Phosphorylation** of wheat germ **eIF-2** subunits by **HRI** and CK II *in vitro* for phosphopeptide **analysis:** Phosphorylation reactions were carried in a standard 20 µl reaction mixtures containing 20 mM Tris-HC1, pH 7.8, 2.5 mM $Mg(OAc)_2$, 80 mM KCl, 20 µCi [γ-^{32}P]ATP (3000 Ci/mmol), wheat germ eIF-2 (5 µg) and either CK II (10 ng, lane 1) or HRI (200 ng, lane 2) and incubated at 25°C and 30°C for 10 min respectively. The reaction mixtures were terminated by the addition of SDS sample buffer and separated on 10% SDS-PAGE. Proteins were stained briefly with 1% coomassie blue in water and destained with excess water. This process will help detecting the molecular weight markers without being treated with acidic conditions which are involved while fixing the gel.

The gel was dried and exposed to an X-ray film. The molecular weights of the marker proteins is shown towards the right side of the autoradiogram. The positions of the labeled p36 and p41-42 subunits are shown with arrows. The figure is an autoradiogram. Labeled subunits of wheat germ eIF-2 were cut out from the dried gel in order to carry out, later, a partial peptide digestion by Cleveland method as described in 'Materials and Methods'.


1
2
Mr
-97
-66
-45
<— P41-42
<— P36
-31
-21
CK II
HRI
+
+
WG eIF-2

undertaken as a first step to understand the possible role of phosphorylation of wheat germ eIF-2 subunit in the translational control.

3.4.2 Separation of *S. aureus protease* (SV8) digestion products of phosphorylated p36 and p41-42 subunits of wheat germ eIF-2 in one **dimension.**

3.4.2.1 *In vitro phosphorylation of purified eIF-2 by* ***CK II*** *and* ***HRI*** *and separation of S. aureus protease (SV8) digestion products.*

To determine the possible sites in wheat germ eIF-2 which can be phosphorylated by CK II and HRI, the phosphorylated subunits (p36 and p41-42 subunits as shown in Fig. 20, were separated on 10% SDS-PAGE. The respective bands in wheat germ eIF-2, that is, p36 and p41-42 were excised from the gel and were incubated with SV8 protease as described in 'Materials and Methods'.

SV8 digestion of the p36 subunit of wheat germ eIF-2 subunits was carried out using 200 ng of SV8 protease/lane. The products were then separated on a 15% SDS-PAGE. To obtain optimal digestion of the protein for a given amount of protease, we have used a) a 5 cm stacking gel and b) reversed the polarity of current for 5 min just before the dye enters the separating gel. This was to ensure the proper mixing of protease and the substrate. Since there is a distribution of the incorporated radiolabeled phosphate of the subunit into various peptides obtained after digestion, in order to obtain a clear signal in the final autoradiograph, we have used here a higher concentration of substrate (5 μg of either wheat germ eIF-2 or reticulocyte eIF-2 per 20 μl reaction mixture) in our *in vitro* phosphorylation experiments.

3.4.2.2 S. aureus protease (SV8) digestion products ***of p36*** *subur.it phosphorylated by CK* ***II.***

SV8 digestion of the p36 subunit of wheat germ eIF-2 which is phosphorylated by CK II yielded two major phosphopeptides of 21 kDa and 19 kDa. Several weakly phosphorylated bands corresponding to 28, 26 and 16 kDa

Fig. 21. Cleveland partial peptide digestion of phosphorylated wheat germ eIF-2 subunits: This procedure was carried out as described in 'Materials and Methods'. Briefly, the phosphorylated bands of wheat germ eIF-2 subunits were obtained from **Fig.** 20. The bands were identified by superimposing the developed X-ray film on the dried gel and were then cut out through the X-ray film and the gel with the help of a sharp razor blade. The dried gel pieces containing the phosphorylated subunits were equilibrated and made ready for SV8 protease digestion as described in 'Materials and Methods'. The concentration of SV8 protease used per gel lane was 200 ng and the size of the stacking gel was kept at 5 cm. Other modifications to achieve greater proteolysis are described in 'Materials and Methods'. The protease treated labeled subunit was then separated on a 15% SDS-PAGE.

All the lanes represent SV8 protease digested radiolabeled products; lane I, digestion products of p41-42 subunit of wheat germ eIF-2 phosphorylated by CK II; lane 2, SV8 digest of p36 subunit of wheat germ eIF-2 phosphorylated by CK II and lane 3, SV8 digest of p41-42 subunit of wheat germ eIF-2 phosphorylated by HRI.

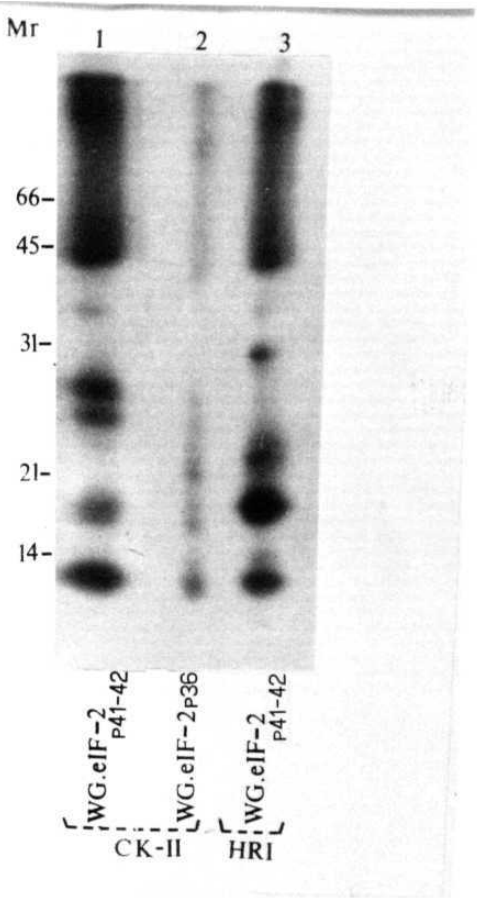

Mr
1
2
3
66-
45-
31-
21-
14-
WG.eIF-2 P41-42
WG.eIF-2 P36
WG.eIF-2 P41-42
CK-II
HRI

Fig. 22. SV8 protease digestion products of p41-42 subunit of wheat germ eIF-2 phosphorylated by HRI, CK II and HRI + CK II added together or at different time points: To determine if there are any overlapping sites of phosphorylation in the p41-42 subunit of wheat germ eIF-2, phosphoryiation of the substrates (5 μg of eIF-2) was carried out with [γ^{32}-P]ATP in the presence of CK II, HRI or a combination of both enzymes as described in 'Materials and Methods'. Where both the enzymes (CK II and HRI) are added, these have been added together at 0 min or added at different time intervals (0 and 7 min) as describe below. Also, to determine the presence of any endogenous kinase like activity associated with purified wheat germ eIF-2, phosphorylation of the latter has been carried out in the absence of any added kinase.

Phosphorylated products were separated on 12.5% SDS-PAGE and the p41-42 subunit was cut out from the gel for SV8 digestion. The various lanes represent the SV8 digestion products of labeled phosphopeptides of p41-42 subunit.

Lane 1, no kinase added.

Lane 2, + CK II

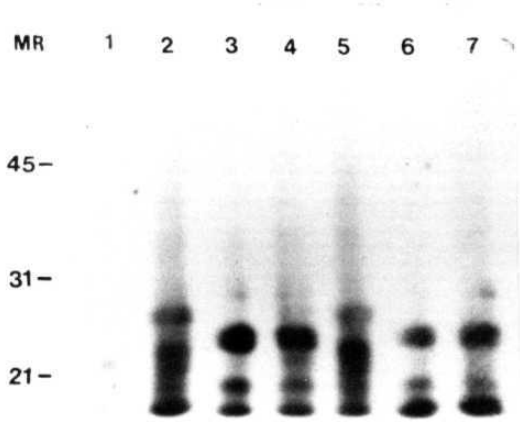
Lane 3, + HRI

Lane 4, + CK II + HRI (added together at 0 min)

Lane 5, no kinase + unlabeled ATP (0-7 min)
CK II + labeled ATP (7-14 min)

Lane 6, no kinase + unlabeled ATP (0-7 min)
HRI + labeled ATP (7-14 min)

Lane 7, + CK II + unlabeled ATP (0-7 min)
+ HRI + labeled ATP (7-14 min)


MR
1
2
3
4
5
6
7
45–
31–
21–

(Fig. 21, lane 2) were also seen. Pure HRI cannot phosphorylate the small-subunit of wheat germ eIF-2 (the p36 subunit), hence, the phosphopeptide analysis of p36 by HRI was not undertaken.

*3.4.2.3 S. **aureus protease** (SV8) digestion products of p41-42 subunit phosphorylated by CK II or HRI.*

SV8 digestion of the p41-42 doublet-subunit phosphorylated by CK II, yielded three strongly labeled bands corresponding to 28, 26 and 19 kDa (Fig. 21, lane 1). Since reticulocyte eIF-2a kinases can also phosphorylate the p41-42 doublet-subunit, we have used here reticulocyte HRI to phosphorylate the p41-42 doublet-subunit and analyzed the phosphopeptide pattern. SV8 digestion of p41-42 subunit of wheat germ eIF-2 which is phosphorylated by HRI also yields three strongly labeled species, but of different molecular weights compared to that obtained by phosphorylation with CK II. The peptides obtained here correspond to 24, 22 and 18-19 kDa (Fig. 21, lane 3). Two weakly labeled phosphopeptides also appeared corresponding to 30 and 13.5 kDa in the above digestion (Fig. 21, lane 3). These findings suggest that

p41-42 subunit of wheat germ eIF-2 appears to have more than one phosphorylation site for both HRI and CK II.

the phosphorylation sites of eIF-2 by HRI and CK II appear to be different.

*3.4.2.4 Limited proteolytic digestion **of the p41-42** subunit phosphorylated by CK II or HRI and a combination of both the enzymes(Fig. 22).*

To further understand the phosphorylation sites of p41-42 subunit for CK II and HRI, phosphorylation of this doublet subunit was carried out in the presence of the respective enzymes alone (lane 2 and 3) and also by a combination of both the enzymes added together (lane 4) or at different time points (lane 7). In the latter case wheat germ eIF-2 was phosphorylated in the presence of CK II and unlabeled ATP for 7 min before the addition of HRI and [γ^{32}-P]ATP. An analysis of the results indicate that the phosphorylation of p41-42 subunit by CK II or HRI

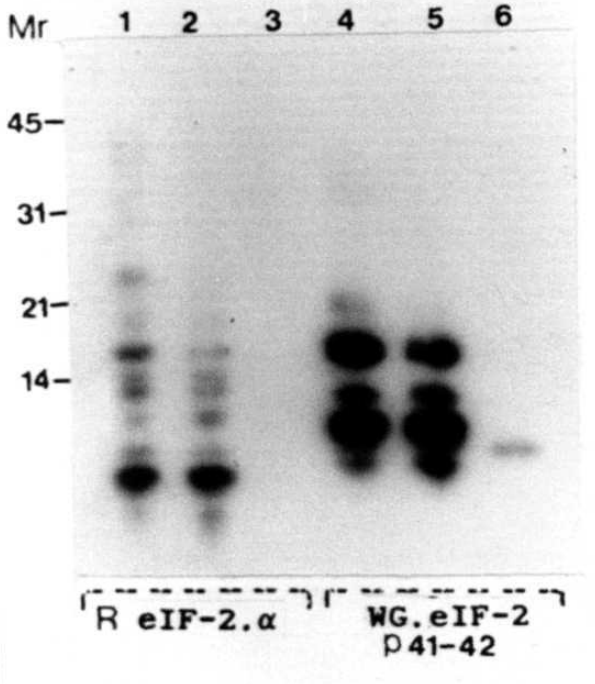
occurs at different sites. This is because, the phosphopeptides generated by SV8 digestion of p41-42 subunit phosphorylated in the presence of these respective enzymes are different (lanes 2 Vs 3). A similar pattern of phosphopeptides were observed here when the substrate was phosphorylated by unlabeled ATP and then with the presence of labeled ATP and respective kinase alone (lanes 5, 6). Consistent with these findings, phosphorylation of the p41 -42 subunit by unlabeled ATP and CK II and, then by labeled ATP and HRI yields a phosphopeptide map similar to the one obtained by HRI alone (lane 7 Vs. lane 2 & 6). This finding suggests that none of the phosphopeptides generated by CK II has any sites of phosphorylation for HRI. However, the phosphopeptides generated by SV8 digestion of p41-42 subunit phosphorylated in the presence of both HRI and CK II (added together at 0 min or at different time points) resemble to the pattern in the presence of HRI alone (lane 4 and 6 Vs. lane 3). This may be possible if wheat germ eIF-2 has greater affinity for HRI than for CK II, a possibility that requires to be further investigated.

As shown above, partial digestion of p41-42 subunit of wheat germ eIF-2 generates more than one phosphopeptide. This could be due to more than one site of phosphorylation or due to generation of fragments of various lengths. Therefore we used higher concentrations of SV8 protease to obtain maximum digestion of the phosphopeptides (Cleveland et al., 1976). A comparison was also made with the reticulocyte eIF-2α which is known to generate one strong phosphopeptide upon digestion with a protease reflecting the presence of one site of phosphorylation (Fig. 23).

3.4.2.5 Comparison of the phosphopeptides of reticulocyte eIF-2α and the p41-42 subunit of wheat germ generated by higher concentrations ofS. aureus (SV8) protease.

Analysis of phosphopeptides of reticulocyte eIF-2α (phosphorylated by HRI) generated by increasing concentration of SV8 protease has also been undertaken and compared to the phosphopeptides pattern generated from wheat

Fig. 23. Cleveland mapping of phosphorylated p41-42 subunit of wheat germ **eIF-2 and alpha-subunit of reticulocyte eIF-2 in the** presence **of** S\8 protease enzyme; **effect of two concentrations of the protease:** The phosphorylated subunits were isolated and digested as **described** in 'Materials and Methods' and Figure. Two concentrations of SV8 protease have been used to determine the effect of digestion on the resolution and pattern of the labeled **phosphopeptides**. Lane 1 and 2 contain reticulocyte **eIF-2**α phosphorylated by **HRI** and digested by 400 ng and 2000 ng of SV8 protease respectively. Lane 3 contains reticulocyte eIF-2a subunit and phosphorylation mixture but without the kinasc and digested by 2000 ng of SV8 protease. Lanes 4 and 5 contain wheat germ **eIF-2 p41-42** subunit phosphorylated by **HRI** and digested by 400 ng and 2000 ng of SV8 protease respectively. Lane 6 contains wheat germ eIF-2 **p41-42** subunit and phosphorylation mixture but without the kinase and digested by 2000 ng of SV8 protease. The labeled phosphopeptides are separated on 15% SDS-PAGE. The migration of molecular weight markers is shown on the left hand side. The figure is an autoradiogram.


Mr
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45–
31–
21–
14–
R eIF-2.α
WG.eIF-2
p41-42

germ eIF-2 p41-42 doublet under thc same conditions (Fig. 23). At low concentrations of SV8 (400ng/lane), the phosphorylated p41-42 subunit of wheat germ eIF-2 yields 4 major peptides corresponding to 16, 12, 10 and 8 kDa (Fig. 23, lane 4). At higher concentration of the protease (2000ng/lane), the 16 kDa phoshopeptide intensity is decreased with a concomitant increase in the intensity of the 8 kDa phosphopeptide (Fig. 23, lane 5). This suggests that 16 kDa phosphopeptide contain further digestion sites in it. In contrast, when reticulocyte eIF-2α was phosphorylated by HRI and digested with two concentrations of SV8 protease, it was found that all the phosphopeptides generated by a concentration of 400ng/lane (Fig. 23, lane 1) were further digested and resulted in a single major phosphopeptide of 6 kDa in the presence of 2000ng of protease concentration per lane (Fig. 23, lane 2). Minor bands which are present even at higher concentrations of SV8 digestion (Fig. 23, lane 2) may have disappeared if a slightly higher concentration of protease has been used. Without the addition of HRI, the reticulocyte eIF-2a subunit is not phosphorylated (Fig. 23, lane 3) and hence the digestion of the a subunit does not generate any labeled phosphopeptides. In contrast, the p41-42 subunit of purified wheat germ eIF-2 is phosphorylated in the absence of HRI and it may be due to a contaminant CK II like activity. Contamination of eIF-2 by endogenous kinases as for the preparation shown here (Fig. 23, lane 6) was often observed for the preparation of eIF-2 from rat and sometimes with reticulocyte eIF-2 (Clarke et.al., 1988). Since the phosphorylation of p41-42 subunit of purified wheat germ eIF-2 by contaminating kinases associated with it is very low, the phosphopeptides generated by SV8 digestion of the peptide is very faint and undetectable (Fig. 23, lane 6).

In summary, these results suggests that CK II phosphorylates both the p36 and p41-42 doublet-subunit of wheat germ eIF-2 . Purified reticulocyte eIF-2α kinase like HRI phosphorylates the p41-42 doublet-subunit of wheat germ eIF-2 Phosphorylation of the p41-42 doublet by CK II and HRI appears to occur at

different sites. Unlike reticulocyte eIF-2α, p41-42 subunit of wheat germ eIF-2 has more than one site of phosphorylation.

4.0 SUMMARY

PHOSPHORYLATION OF WHEAT GERM eIF-2 BY PURIFIED KINASES *in vitro*

Purified wheat germ eIF-2 was resolved into three subunits on 10% SDS-PAGE. Molecular weights of these three polypeptides are 36 kDa, 41-42 kDa and 52 kDa. Purified reticulocyte kinases, HRI or PKR which can phosphorylate the small subunit (38 kDa) of reticulocyte eIF-2 (eIF-2a), phosphorylated the p41-42 doublet subunit of wheat germ eIF-2 *in vitro*. The small subunit, p36 subunit, of wheat germ eIF-2 however was not phosphorylated under those conditions. Impure HRI preparations or highly purified multipotential kinase like CK II were found to phosphorylate both the p36 and p41-42 doublet subunits of wheat germ eIF-2. These findings suggest that the phosphorylation of small subunit of wheat germ eIF-2 can be carried out by CK II or by a contaminant CK II like activity in impure HRI.

We have also analyzed the ability of native reticulocyte eIF-2α kinases (which get activated during heme-deficiency or poly IC or dsRNA treatment in reticulocyte lysates) to phosphorylate wheat germ eIF-2. The results are consistent with the ones obtained by purified kinases *in vitro*.

GUANINE NUCLEOTIDE EXCHANGE ACTIVITY OF WHEAT GERM eIF-2 *in vitro* AND THE EFFECT OF PHOSPHORYLATION

Purified wheat germ eIF-2 was able to bind added labeled GDP (2 μM) in presence of 2.5 mM magnesium. In the absence of magnesium, eIF-2 could not bind to GDP efficiently. However, the binary complex was found to exchange its labeled GDP for unlabeled GDP in the reaction mixture very efficiently in the absence of any eIF-2B-like factor being added. The labeled complex was fairly stable in the absence of unlabeled GDP. It is not known however, if the guanine nucleotide exchange activity associated with wheat germ eIF-2 is due to a contaminant eIF-2B-like activity in eIF-2 preparation. Alternatively, it is possible that the ability of wheat germ eIF-2 to bind GDP or GTP is not markedly different and the guanine nucleotide exchange may be occurring independent of an eIF-2B-

like protein. A recent publication that appeared after we have taken up these studies suggests the latter possibility based on the estimation of dissociation constants of eIF-2.GDP and eIF-2.GTP.

Purified eIF-2 from mammalian sources co-migrates with eIF-2 during several steps of purification. Hence the possibility of such an eIF-2B-like contamination with wheat germ eIF-2 preparation has been tested here. In mammalian systems such a contaminant eIF-2B activity is known to be inhibited upon phosphorylation of serine 51 residue in the wild type eIF-2a. The p41-42 doublet subunit of wheat germ eIF-2 can also be considered to be equivalent of mammalian eIF-2α since it is phosphorylated by reticulocyte eIF-2α kinases. So, the guanine nucleotide exchange activity of wheat germ eIF-2 was tested when the latter is phosphorylated by purified or native reticulocyte eIF-2a kinases. As it is not known if the heterologous eIF-2α kinase phosphorylates the wheat germ eIF-2 at the right site we have also determined the guanine nucleotide exchange activity of wheat germ eIF 2 in presence of phosphorylated reticulocyte eIF-2α in which the serine 51 is known to be phosphorylated. The findings indicate that

- phosphorylation of wheat germ eIF-2 by HRI could not inhibit the guanine nucleotide exchange activity associated with wheat germ eIF-2,
- reticulocyte lysates in which HRI or PKR was activated and could inhibit the exchange of guanine nucleotides on reticulocyte eIF-2 however were unable to inhibit the exchange of GDP on wheat germ eIF-2,
- addition of phosphorylated reticulocyte eIF-2a *in vitro* could not inhibit the guanine nucleotide exchange activity associated with wheat germ eIF-2.

These findings suggest that the guanine nucleotide exchange activity of wheat germ eIF-2 is not because of a contaminant eIF-2B-like activity and support the idea that guanine nucleotide exchange activity proceeds under sufficiently high GTP/GDP ratio.

WHEAT GERM eIF-2 MITIGATES PROTEIN SYNTHESIS INHIBITION IN RETICULOCYTE LYSATES MEDIATED BY RETICULOCYTE eIF-2a PHOSPHORYLATION

Addition of wheat germ eIF-2 to inhibited reticulocyte lysates was found to mitigate the protein synthesis inhibition mediated by eIF-2a phosphorylation in the present studies. Hence we have studied further the mechanism by which wheat germ eIF-2 is able to overcome the protein synthesis inhibition in reticulocyte lysates under those conditions. Our findings indicate that wheat germ eIF-2 did not decrease the level of reticulocyte eIF-2a phosphorylation that was required for the protein synthesis inhibition to occur and the conditions in inhibited reticulocyte lysates also facilitated the phosphorylation of p41-42 doublet subunit of wheat germ eIF-2. Consistent with the decrease in the inhibition of protein synthesis, addition of wheat germ eIF-2 to inhibited reticulocyte lysates also protected partially the lysate eIF-2B activity. This is possible if wheat germ eIF-2 is exchanging the phosphorylated reticulocyte eIF-2a from the 15S complex [eIF-2(aP).eIF-2B] and the phosphorylated wheat germ eIF-2 is unable to interact with reticulocyte eIF-2B as efficiently as reticulocyte eIF-2(aP). This possibility is also tested here by monitoring the 15S complex's in inhibited reticulocytes treated with or without wheat germ eIF-2. These results indicate that 15S complex formation is reduced in inhibited lysates treated with wheat germ eIF-2 compared to lysates carrying protein synthesis without the addition of wheat germ eIF-2.

Since the eIF-2 in the 15S complexes is probed by reticulocyte eIF-2a monoclonal antibodies, the results do not reveal if phosphorylated wheat germ eIF-2 is able to interact with reticulocyte eIF-2B. Hence we have measured the eIF-2B activity of reticulocyte lysates under those conditions and also probed the 15S complex with polyclonal anti-wheat germ eIF-2 antibodies.

Reticulocyte eIF-2B activity was partially protected and the 15S complexes did not show any wheat germ eIF-2 signal when probed with a wheat germ eIF-2 polyclonal antibody, suggesting that phosphorylated wheat germ eIF-2 can

exchange reticulocyte eIF-2(aP) from the 15S complexes but however it cannot interact as efficiently as reticulocyte eIF-2(aP) with eIF-2B. Hence this should make briefly the eIF-2B available before it is complexed again with reticulocyte eIF-2(aP). The free eIF-2B can immediately catalyze the exchange of GDP on reticulocyte eIF-2 so as to enable the latter to enter into the initiation cycle. The ability of wheat germ eIF-2 to escape the effect of phosphorylation of reticulocyte eIF-2a draws similarities with mutant mammalian eIFB 2a in which serine 48 residue, adjacent to phosphorylated serine 51 residue, is replaced by alanine. This mutant eIF-2 can still be phosphorylated in its serine 51 residue. The expression of such a mutant eIF-2 is shown to overcome the inhibition in protein synthesis and eIF-2B activity in Chinese hamster ovary (CHO) cells that is mediated by wild type eIF-2a phosphorylation. Based on this information presented, it is likely that wheat germ eIF-2 is different from mammalian eIF-2 and the differences may have occurred in evolution due to a modification of some amino acid(s) in and around the phosphorylation site of eIF-2.

A polyclonal anti-wheat germ eIF-2 antibody raised in these studies was found to recognize the p36 and p41-42 subunit of wheat germ eIF-2. The polyclonal antibody was also found to cross react with the reticulocyte eIF-2 (3-, *y*- subunits. This suggests that although these systems are phylogenitically placed distinctly wheat germ and rabbit reticulocyte eIF-2s still carry conserved sequences among them. This antibody is also found useful to determine the phosphorylated wheat germ eIF-2 interaction with reticulocyte eIF-2B as has been described earlier.

Further the antibody has been used here to determine if wheat germ eIF-2 can enter into the protein synthesis cycle of inhibited reticulocyte lysates. Our findings indicated that polysomes were completely disiaggregated in inhibited reticulocyte lysates and were partially disaggregated in inhibited lysates treated with wheat germ eIF-2. These findings correlate with the reduced inhibition of protein synthesis under those conditions. When these polysomes were analyzed for

the presence of wheat germ eIF-2, it was observed that the 80S subunits and to some extent the disomes and trisomes were found to carry wheat **germ ell-Z'** These observations suggested that wheat germ eIF-2 can probably enter into the reticulocyte initiation cycle.

ANALYSIS OF PHOSPHOPEPTIDES OF RETICULOCYTE eIF-2α (p38) AND p36 AND p41-42 SUBUNITS OF WHEAT GERM eIF-2:

Since the p41-42 doublet subunit of wheat germ eIF-2 can be phosphorylated by reticulocyte eIF-2a kinases, we have tested here the phosphopeptides that are generated by limited proteolytic digestion from phosphorylated wheat germ eIF-2 and reticulocyte eIF-2. The findings suggest that there are more than one phosphorylation site in the p41B42 doublet subunit of wheat germ eIF-2. Also the phosphorylation of small subunit of wheat germ eIF-2, that is p36 by CK II is compared to the phosphorylation of small or a subunit of reticulocyte eIF-2 by HRI (please note that CK II cannot phosphorylate the small subunit of reticulocyte eIF-2 and hence we have used HRI to compare the phosphopeptides of the small subunits in both the preparations). All these studies reveal that wheat germ eIF-2 subunits have multiple phosphorylation sites whether that be in p36 or p41-42 subunits, whereas reticulocyte eIF-2a appears to have a single site of phosphorylation. The latter is consistent with the earlier reports

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