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.

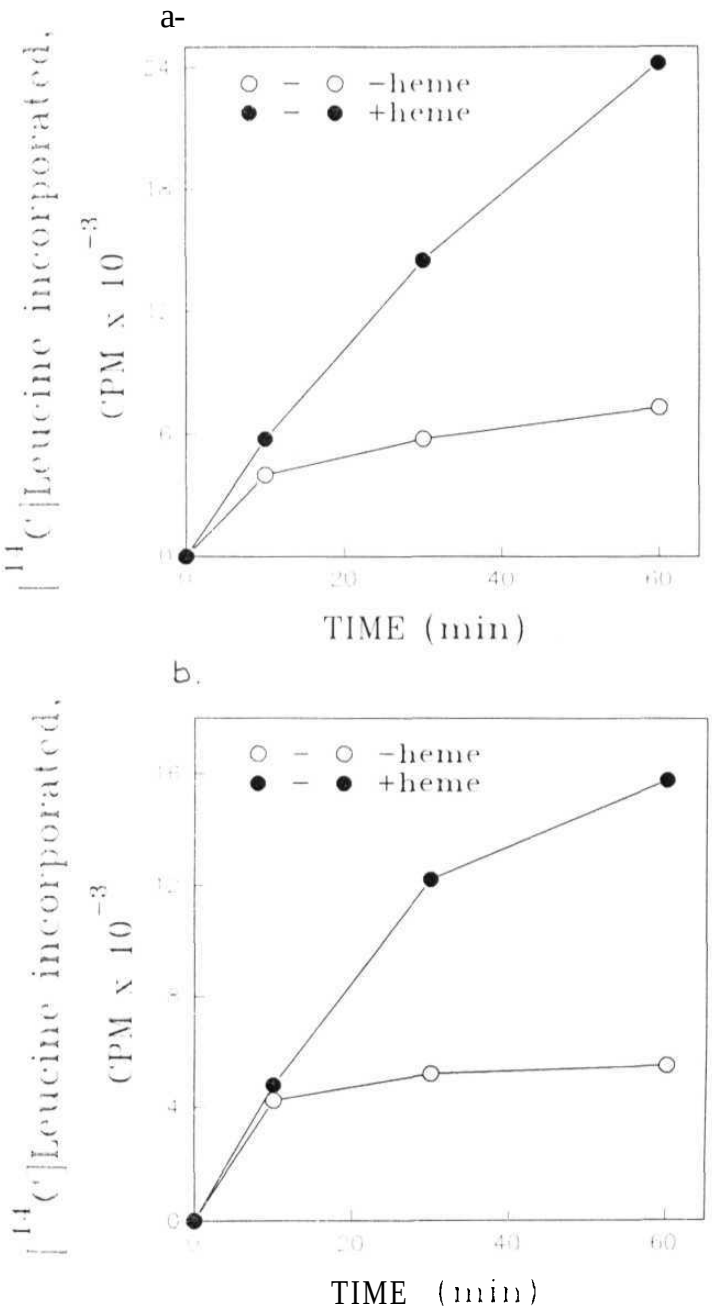


Fig. 1a. **and 1b.** Reticulocvte lysate protein synthesis from two different batches of lysates.

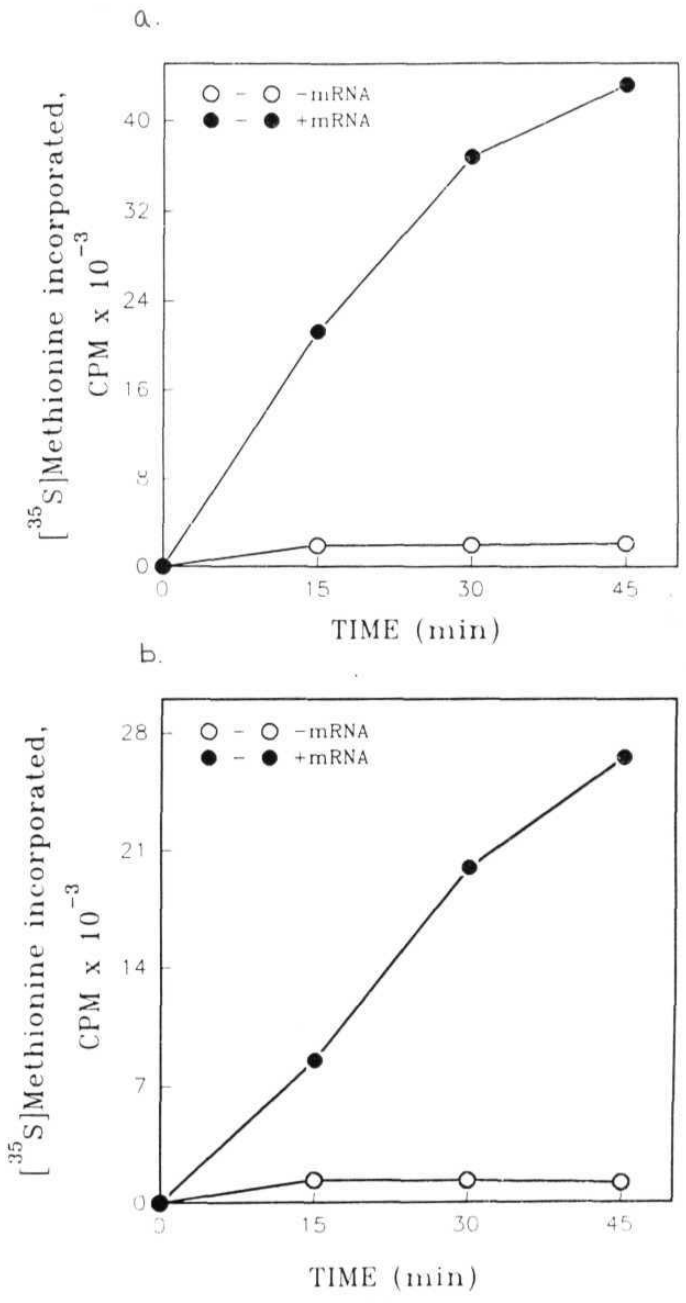


Fig. 2a. **and** 2b. Wheat germ lysate protein synthesis from two different batches of lysates.

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.

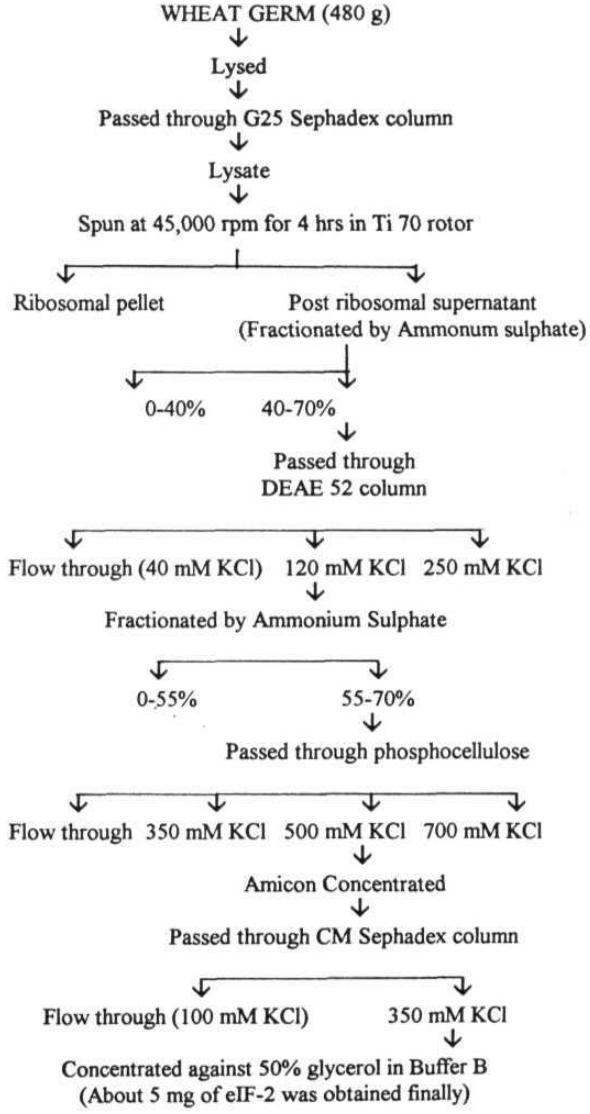
2.5 Preparation of wheat germ eIF-2:

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.