Translational Regulation in Wheat Germ Lysate Mediated by eIF2α Phosphorylation

Thesis submitted for the Degree of

DOCTOR OF PHILOSOPHY

By

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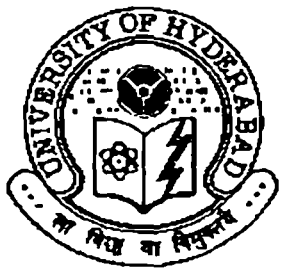

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September, 2002

Dedicated to my parents

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DECLARATION

I hereby declare that the work presented in this thesis entitled ***"Translational Regulation in Wheat Germ Lysate Mediated by eIF2α Phosphorylation"*** has been carried out by me under the supervision of Prof. K. V. A. Ramaiah and this has not been submitted for any degree or diploma of any University.

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This is to certify that Burela Laxminarayana has carried out the work in the present thesis under my supervision for a full period prescribed under the Ph. D. ordinances of the University. I recommend his thesis entitled ***"Translational Regulation in Wheat Germ Lysate Mediated by eIF2α Phosphorylation"*** for submission for the award of the degree of ***Doctor of Philosophy*** of this University.

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Burela Laxminarayana..

ABBREVIATIONS

A, Ala	: Alanine
ADP	: Adenosine 5' diphosphate
AMV	: Alpha mosaic virus
AP	: Alkaline phosphatase
APH	: Acetyl phenyl hydrazine
APS	: Ammonium per sulphate
A-site	: aminoacylated tRNA binding site
Asp	: Aspartic acid
ATP	: Adenosine 5' triphosphate
AUG	: Initiation codon
BCIP	: 5-bromo-4-chloro-3-indolyl phosphate
Bis-acrylamide	: N, N'-methylene-bis-acrylamide
BMV RNA	: Brome mosaic virus RNA
BPs	: binding proteins
BSA	: Bovine serum albumin
DNA	: Complementary DNA
CHO	: Chinese hamster ovary cells
Ci	: Curie
CK II	: Casein kinase II
CM-S	: Carboxy methyl sephadex
CP	: Creatine phosphate
CPK	: Creatine phosphokinase
cpm	: Counts per minute
DAI/dsI/PKR	: Protein kinase regulated by RNA
DEAE	: Diethyl aminoethyl
DMSO	: Dimethyl sulphoxide
DNA	: Deoxy ribonucleic acid
DNA-PK	: Protein kinase regulated by DNA
dNTP	: Deoxy nucleotide triphosphate
dsRNA	: Double stranded RNA
DTT	: Dithiothreitol

IFN	: Interferon
E. coli	: *Escherischia coli*
EDTA	: Ethylene diamine tetra acetic acid
eEF	: Eukaryotic elongation factor
EF	: elongation factor
EGTA	: Ethylene-bis (β-amino ethyl ether) N, N', N"-tetra acetic acid
eIF2	: Eukaryotic initiation factor 2
eIF2(αP)	: Phosphorylated alpha subunit in eIF2
eIF2B/GEF/RF	:Guanine nucleotide exchange factor of eIF2/reversing factor
eIF2α	: Alpha subunit of eukaryotic initiation factor 2
eIFs	: Eukaryotic initiation factors
eRF	: eukaryotic releasing factor or termination factor
E-site	: exit site
Fig.	: Figure
GCN	: General control non-derepressible
GDP	: Guanosine 5' diphosphate
GNE	: Guanine nucleotide exchange
GSH	: Reduced gluthathione
GSK3	: Glycogen synthase kinase 3
GSSG	: Oxidised gluthathione
GTP	: Guanosine 5' triphosphate
H_2O_2	: Hydrogen peroxide
HCV	: Hepatitis C Virus
HEPES	: N-[2-hydroxyethyl] piperizine-N'-[2-ethane-sulfonic acid]
His RS	: histidyl-tRNA synthetases
HIV	: Human immuno deficiency virus
HRI	: Heme regulated inhibitor
hrs	: Hours
Hsp	: heat shock protein

IREs	: Internal ribosome entry site or iron-responsive elements
Kbp	: Kilo base pair
kD	: Kilo daltons
l	: Litre
LB	: Luria-Bartani
$Met.tRNA_i^{Met}$	: Initiation transfer RNA
Mg	: magnesium
mg	: Milligram(s)
min	: Minutes
Mr	: Molecular weight markers
mRNA	: Messenger RNA
mTOR	: mammalian target of rapamycin
NADPH	: Nicotinamide adenine dinucleotide phosphate, reduced
NBT	: Nitro blue tetrazolium
NEM	: N-ethyl maliemide
P11	: Phosphocellulose
PABP	: poly A binding protein
PAGE	: Polyacrylamide Gel Electrophoresis
PERK	: PKR like endoplasmic reticulum kinase
PKR	: Protein kinase regulated by RNA
pmol	: Pico moles
PMSF	: Phenyl methyl sulfonyl fluoride
poly IC	: Synthetic polymer of Inosine and Cytosine
PP	: protein phosphatase
PQQ	: Pyrroline quinoline quinone
P-site	: Peptidyl-tRNA binding site
RNA	: Ribonucleic acid
RNase	: Ribonuclease
RNAsin	: ribonuclease inhibitor
rpm	: Rotations per minute
rRNA	: ribosomal RNA
RT	: Room temperature

S 300	: Sephacryl 300
S	: Svedberg
S. aureus	: Staphylococcus aureus
SDS	: Sodium dodecyl sulfate
Ser	: Serine
-SH	: Protein sulfhydryl groups
SV 8	: Staphylococcus aureus protease
TCA	: trichloro acetic acid
TEMED	: N', N N, N', N' - tetramethyl ethyl-ethylene diamine
Tris	: Tris (hydroxymethyl) amino methane
tRNA	: transfer RNA
uORF	: open reading frames
WG	: Wheat germ
μ	: Micro

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Proteins occupy a position high on the list of molecules important for life processes. They account for a large fraction of biological macromolecules, they catalyze most of the reactions on which life depends and they serve numerous structural, transport, regulatory and other roles in all organisms. Accordingly, a large proportion of the cell's resources are devoted to translation.

The biosynthesis and concentration of a protein in the cell can be controlled at any of the 6 major points indicated viz.,

- synthesis of primary RNA transcript (transcription)
- maturation of RNA or post-transcriptional modification of mRNA
- mRNA transportation from the nucleus to cytosol
- translation
- post-translational modification of the proteins and
- protein degradation

Among these, two of the steps, transcription and translation, are especially critical for the cell. Both are biosynthetic steps in which the cell makes large investments of energy. Indeed, transcription is subject to a multitude of controls. However, based on several examples one has to agree with the famous statement made by Tim Hunt years ago that 'Translational controls are facts and not a fantasy any more' (Hunt, 1985). Translational controls required for survival of the cells are evident from a number of examples and are a part of the cell's arsenal of regulatory mechanisms. In many cases, regulation of gene expression at the translational level may provide a means for fine control in addition to saving the cell's energy. The readily reversible nature of translational control mechanism is economical in energetic terms, a feature that is of particular biological significance in energy deprived cells. In some systems such as reticulocytes, oocytes, and RNA viruses there is little or no opportunity for transcriptional control, and gene expression is controlled mostly at the translational level. Translational controls are widely used to regulate gene expression during development, suggesting that this mode of control preceded transcriptional control in evolution. Further, translational controls are essential where concentration gradients of proteins determine the pattern of early development.

1. An Overview of Protein Synthesis

Translation is the process in which the genetic information stored in the form of a chain of nucleotides (messenger RNA) is converted into a functional form, the protein.

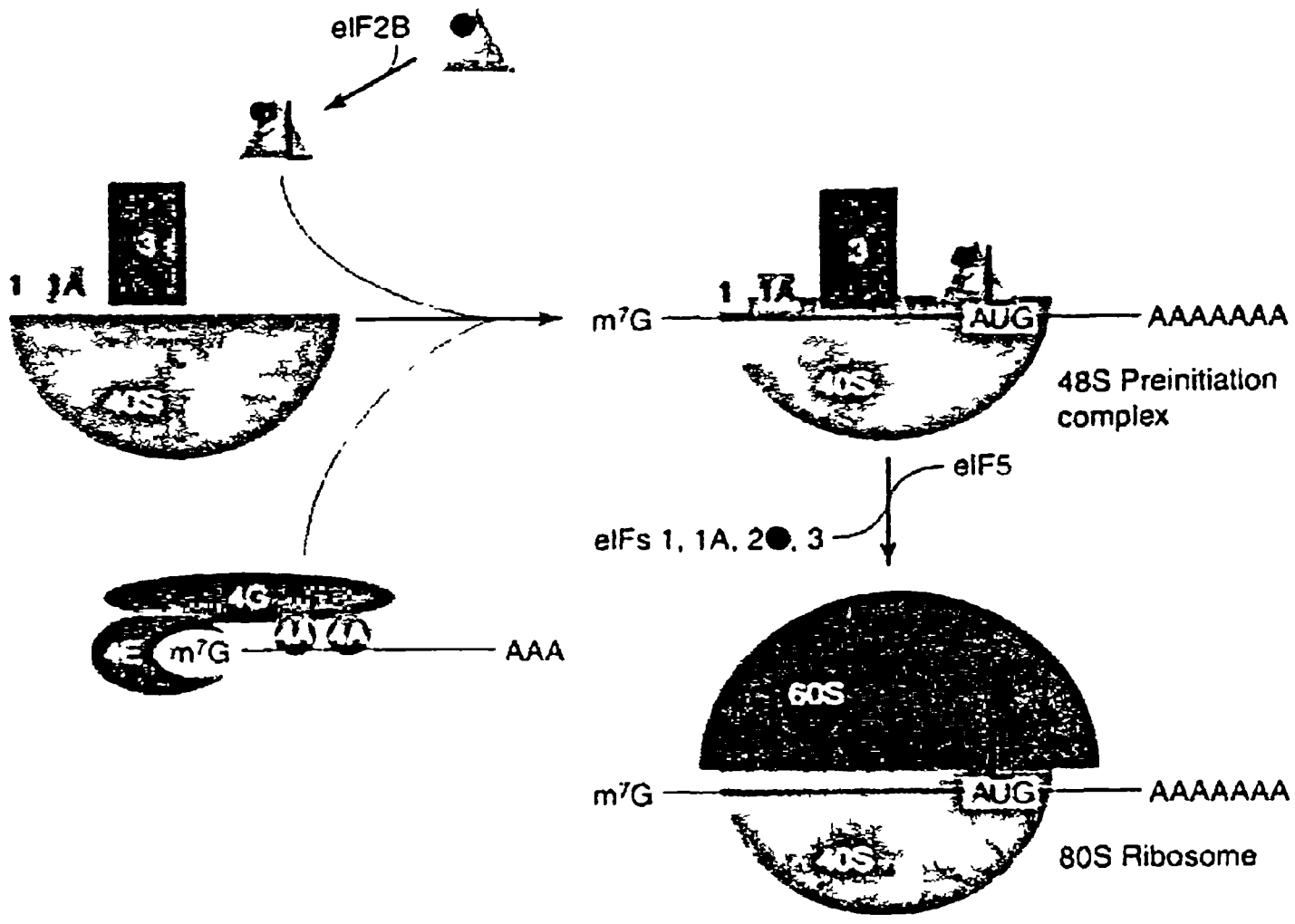
Proteins are long sentences strung using the 20 standard amino acids. With the discovery of selenocysteine (Bock, 1994 and James et al, 2001) and pyrrolysine (Srinivasan et al, 2002), two non-standard amino acids, which are not created by posttranslational modifications and are directly, specified by the genetic code, the protein language has 22 alphabets. In order for the cell to work and multiply, the instructions coded into four letter (A, T, G, C) 64 – word, gene language (codons) needs to be translated into these 22 – letter protein language. Translation by itself is a very elaborate process and requires translation machinery consisting of numerous protein factors, enzymes, ribosomes, nucleotides (ATP and GTP), and RNA (tRNA and mRNA). The process, for convenience, is divided into three steps viz: initiation, elongation and termination. The factors involved in the translation of eukaryotic systems at each step are designated as eukaryotic initiation factors (eIFs), elongation factors (eEFs) and termination factor(s) or the releasing factor(s) (eRFs) (Ochoa, 1983; Hershey, 1991). The factors catalyze or mediate specific steps in the pathway.

The process is exclusively specific and contains several players whose roles and interactions with each other are fast emerging. In order to understand the regulation of protein synthesis, it is necessary to understand the nature and important roles of these various players.

1.1. Initiation of protein synthesis

The initiation of protein synthesis consists of the following steps. Protein factors involved in the eukaryotic initiation pathway are shown in Fig. 1a-d. These figures give a pictorial representation of the important steps (described below) in intiation catalyzed or carried out by different initiation factors (Fig.1a and 1b), the possible interaction among them (Fig.1c) and the requirements for the activation of different eIF2α kinases that are cloned and characterized (Fig.1d) (Dever, 1999; Asano et al, 1999).

Fig. 1a. Simplified model of translation initiation.



The translation factor eIF2 (purple triangle) binds the initiator Met-tRNA (black 'L') in a GTP (green circle)-dependent manner forming a stable ternary complex. This ternary complex associates with a 40S ribosomal subunit along with other initiation factors (eIFl, eIF1A, and eIF3), although the order of binding of eIF2 and these other factors is not fully understood. The mRNA 5' m7G cap-binding protein eIF4E, together with the factors eIF4G and eIF4A, form the cap-binding complex or eIF4F. This complex binds mRNAs and is thought to unwind cap-proximal mRNA secondary structure and, through interactions with the 40S ribosomal subunit-associated initiation factors, facilitate mRNA binding to the ribosome. Once bound, the 40S ribosome scans the mRNA for the initiating AUG codon and forms the 48S preinitiation complex. Interaction of the factor eIF5 with the 48S complex stimulates GTP hydrolysis on eIF2 and results in release of the initiation factors allowing the 60S ribosomal subunit to join and form the 80S ribosome that is now ready for translation elongation. The factor eIF2, bound to GDP (black circle), is released from the 48S complex and, as indicated at the top of the figure, the guanine nucleotide exchange factor eIF2B is required to regenerate active GTP-bound eIF2 (Dever, 1999).

Fig. 1b. eIF4G as an adapter.

Recruitment of mRNA to the ribosome requires multiple protein-protein interactions involving eIF4G. The m7G cap-binding protein eIF4E interacts with a small region near the center of eIF4G, while eIF4A, an RNA helicase, binds to two regions in the C-terminal half of eIF4G (a single molecule of eIF4A bound to eIF4G is shown but it is possible that eIF4G interacts simultaneously with two molecules of eIF4A). These three proteins comprise the cap-binding complex eIF4F. The polyA-binding protein (PABP) binds to a region near the N-terminus of eIF4G and, together with eIF4E, can circularize mRNAs and promote translation of polyadenylated mRNAs. The multisubunit eIF3 serves as a bridge between eIF4G and the ribosome. Regulation of translation is achieved by modifying and exploiting interactions with eIF4G. The MNK1 protein kinase, which is activated by the mitogen-activated protein kinases (MAPKs) ERK and p38, binds near the C-terminus of eIF4G where it can gain access to its substrate eIF4E. Phosphorylation of eIF4E can increase its affinity for RNA. The binding of eIF4E to eIF4G is regulated by 4E-BP. Phosphorylation of 4E-BP, possibly by a MAPK, blocks its interaction with eIF4E and promotes translation. Finally, as indicated by the scissors, several viruses block cap-dependent translation by cleaving eIF4G (poliovirus, coxsackie virus, rhinovirus, foot and mouth disease virus) or PABP (poliovirus) (Dever, 1999).

Fig. 1c. Multiple protein-protein interactions among factors eIF2, eIF2B, eIF3 and eIF5 promote translation initiation.

(a) Adapter role for eIF3 in ribosome recruitment and AUG codon recognition. The five core subunits of eIF3 from yeast (p110 or TIF32, p93 or NIP1, p90 or PRT1, p39 or TIF34, p30 or TIF35) are depicted along with eIF1 (SUI1), eIF5 (TIF5), heterotrimeric eIF2 (eIF2α,β,γ) with bound GTP (green circle) and Met-tRNA (in black), and the small (40S) ribosomal subunit with bound mRNA. The p93 sub- unit of eIF3 directly contacts both eIF5 and eIF1, whereas a C-terminal segment of eIF5 interacts with the poly-lysine sequences near the N-terminus of eIF2β. It is not known which subunit(s) of eIF3 contact the 40S subunit or mediate the interaction with eIF4G. (b) Modular nature of the eIF2-eIF2B interaction. The pentameric eIF2B can be divided into catalytic (2Bε,γ) and regulatory (2Bα,β and δ) subcomplexes. It appears that eIF2Bε possesses the catalytic guanine-nucleotide-exchange activity, whereas the eIF2Bα,β and δ subunits are required for inhibition of eIF2B activity by eIF2 phosphorylated (P) on Ser51 of the α subunit. Three poly-lysine (K) sequences near the N- terminus of eIF2β interact directly with a segment near the C-terminus of eIF2Bε. The points of contact between the regulatory and catalytic subcomplexes in eIF2B as well as the contacts between eIF2B and phosphorylated eIF2α are not known. The black circle on eIF2γ represents the bound GDP nucleotide (Dever, 1999).

Fig. 1b.

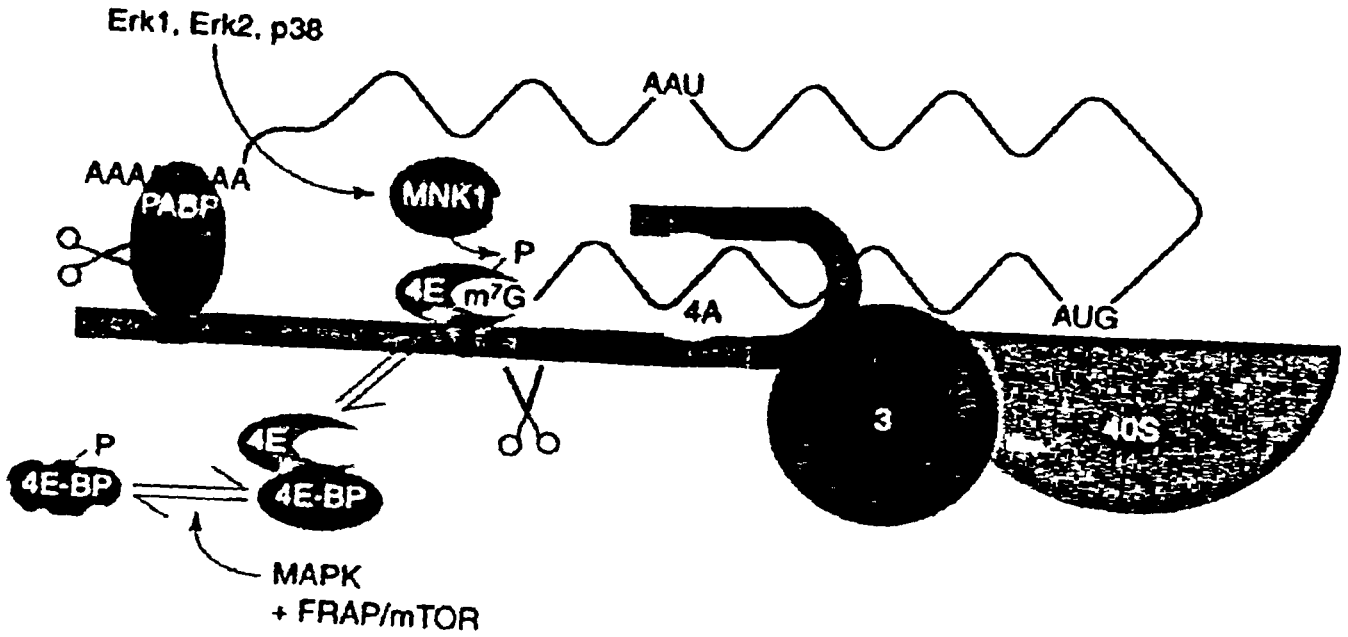
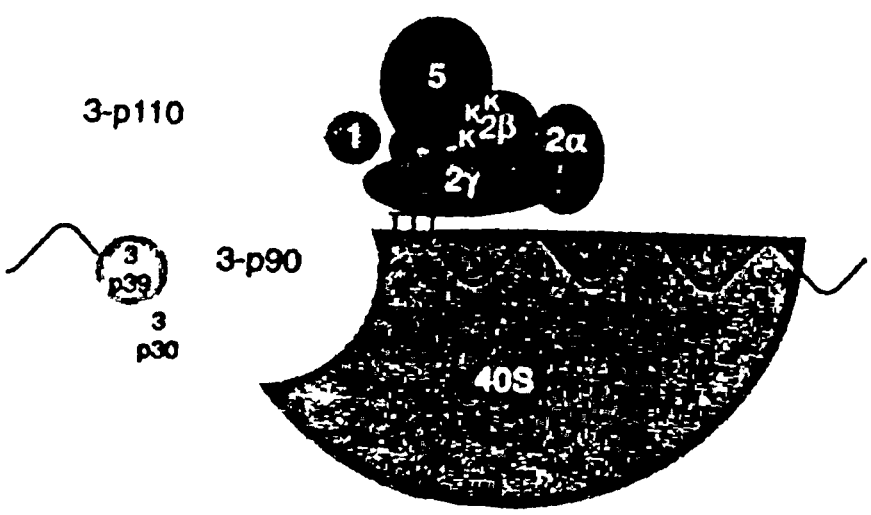


Fig. 1c.

(a)



(b)

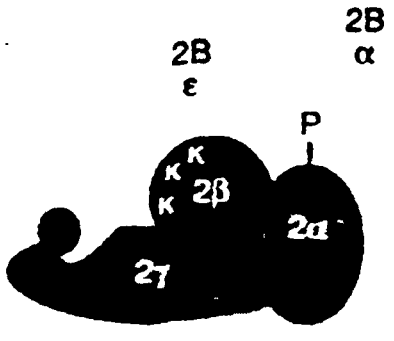


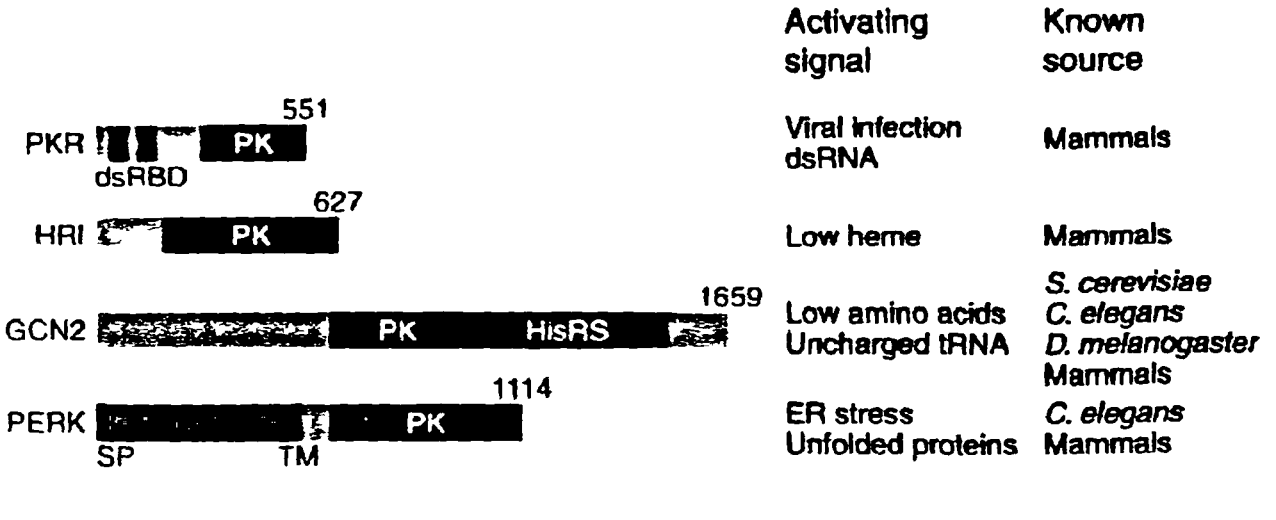
Fig. 1d. Structure of the four identified stress-responsive eIF2α kinases.

The numbers refer to the size (in amino acids) of the primary translation products for each protein. The conserved eIF2α kinase domain (PK) is shown in purple. Also shown are the regulatory domains in the kinases: two double-stranded RNA binding domains (dsRBD) are present in the N-terminus of PKR, a histidyl-tRNA synthetase domain (HisRS) is present immediately C-terminal to the kinase domain in GCN2 and the N-terminus of PERK has a domain that shows similarity to the regulatory domain of IRE1 another kinase involved in the unfolded protein response. PERK also contains a signal peptide (SP) and transmembrane (TM) domain (Dever, 1999).

Fig. 1e. A model for translational control of yeast GCN4 by phosphorylation of eIF2α.

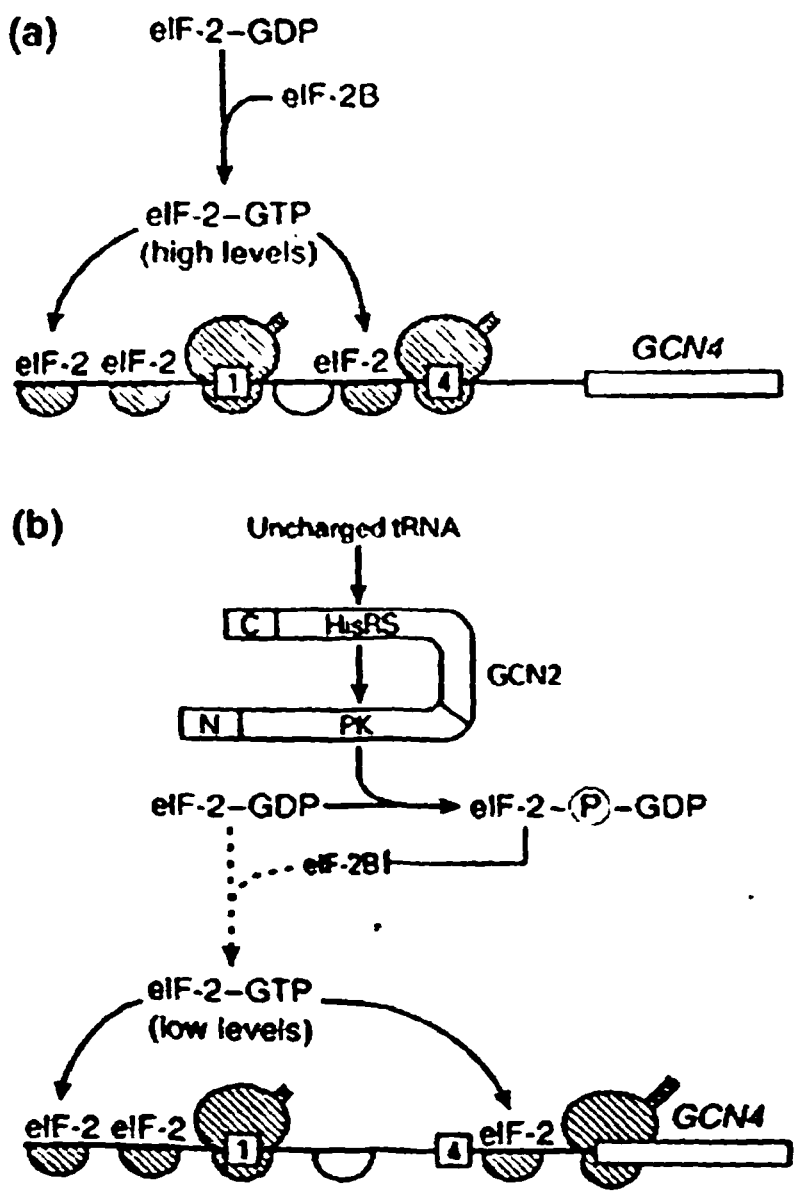
GCN4 RNA is shown with uORFs 1 and 4 and the beginning of the GCN4 coding sequences indicates as boxes. 40 S ribosomal sub-units are shown hatched when associated with eIF2 and thus competent to reinitiate translation (the eIF2 bound to 40S subunits indicates the ternary complex composed of eIF2, GTP and Met-tRNA$_i^{Met}$); unshaded 40S subunits lack the ternary complex and, therefore, cannot reinitiate (a) Under nonstarvation conditions, eIF2 GDP is readily recycled to eIF2 GTP and ternary complex formation. The ternary complexes thus formed reassemble with 40S ribosomes scanning downstream from uORF1, causing reinitiation to occur at uORF4 (b) Under starvation conditions, uncharged tRNA accumulates and stimulates the activity of the protein kinase GCN2. GCN2 phophorylates eIF2α and the phophorylated eIF2 inhibits eIF2B, reducing the recycling of eIF2.GDP to eIF2.GTP. The resulting low level of eIF2.GTP and ternary complex formation diminishes the rate at which initiation complexes are reassembled on 40S subunits following translation of uORF1. Consequently, many 40S subunits scanning downstream from uORF1 are not competent to initiate at uORF4, these subunits acquire ternary complexes while scanning the interval between uORF4 and GCN4 and reinitiate at the GCN4 start site instead (Hinnebusch, 1994).

Fig. 1d.



Activating signal	Known source
Viral infection dsRNA	Mammals
Low heme	Mammals
Low amino acids Uncharged tRNA	*S. cerevisiae* *C. elegans* *D. melanogaster* Mammals
ER stress Unfolded proteins	*C. elegans* Mammals

Fig. 1e.



Step 1. Charging of the initiator tRNA: The initiator tRNA (Met-$tRNA_i^{Met}$) is charged with methionine in the presence of a specific aminoacyl synthetase enzyme. The eukaryotic initiator tRNA has several unique sequences and structural characteristics that distinguish from elongator tRNA and are important to recognize the initiation factors on one side and the ribosome on the other side. The important features of $tRNA_i$ include the base pairing between the A1 and U72 at the end of the acceptor stem and three consecutive G:C base pairs in the anticodon stem (G29: C41, G30:C40, G31:C39). Initiator tRNA also lacks the TψC sequence in loop 4, containing A54 in place of T54 (of the TψC) sequence and contains A60 instead of pyrimidine 60 in this loop. Plant and fungal initiators additionally contain a phosphoribosyl group attached to the ribose 2'-OH at position 64 (RajBhandary and Chow, 1995). The charged methionine in eukaryotes, unlike in prokaryotes, is not formylated due to the lack of transformylase enzyme.

Step 2. Dissociation of 80S subunits to 60S and 40S subunits: At the end of translation the 80S subunits are dissociated into their respective 40S and 60S subunits. The availability of 40S subunits to join the charged initiator tRNA is dependent on anti-association factors such as eIF1A, eIF3 and eIF6.

eIF1A is a small protein of 17 to 22 kD (Wei et al, 1995) and has 21% sequence identity with *E. coli* initiation factor IF1 (Kyrpides and Woese, 1998). This factor is found to interact with another factor called eIF5B (whose role is defined in step 6). The latter is homologous to prokaryotic IF2. On the basis of these functions, it is presumed that eIF1A and eIF5B may occupy the $tRNA_i$ binding A site on the eukaryotic 40S ribosome.

eIF3 by far, is the largest among the initiation factors. It has a molecular mass of 600 kD and has eleven different subunits: p170, p116, p110, p66, p48, p47, p44, p40, p36, p35 and p28. The factor has been purified from mammalian, yeast and plant systems (Benne and Hershey, 1976; Schreier et al, 1997; Naranda et al, 1994; Browning, 1996). This factor is implicated in ribosome dissociation, in Met-$tRNA_i^{Met}$ and binding of mRNA to 40S subunit and in the interaction with several other initiation factors (described later). While eIF1A and eIF3 binds to the 40S ribosomal subunits, eIF6 has been shown to bind 60S subunit. eIF6 is a 25 kD protein

(Valenzuela et al, 1982) and the gene has been cloned from human and yeast (Si et al, 1997; and Si and Maitra, 1999). However, the mechanism by which binding of these factors to ribosomal subunits prevents their association is not yet clearly established. It is likely that the association of these factors to ribosomal subunits creates stearic hindrance for their joining (Goumans et al, 1980).

Step 3. Formation of 43S preinitiation complex: Eukaryotic initiation factor (eIF2) facilitates the joining of the initiator tRNA to 40S subunit. eIF2 is a heterotrimeric protein with three subunits: α (36.5 kD), β (39 kD) and γ (59.1 kD). eIF2 distinguishes the initiator tRNAs from elongator tRNA by recognizing the methionyl residue and the A-U base pair at the end of acceptor stem. The three G:C base pairs present in the anticodon stem of initiator tRNA are required for ribosome binding, but not for eIF2 recognition. In physiological conditions, (in the presence of Mg^{+2}), eIF2 has a higher affinity for GDP than for GTP. However, GTP is required for eIF2 to interact with initiator tRNA. Hence, eIF2.GDP binary complex that is released at the end of initiation cannot join directly with Met-$tRNA_i$, unless GTP exchanges the GDP bound to eIF2. This guanine nucleotide exchange (GNE) is catalyzed by a heteropentameric protein called eIF2B (Web and Proud, 1997). In addition to its ability to bind GDP, the α and β subunits of eIF2 are substrates for several kinases. Phosphorylation of 51 serine residue in the α subunit by a family of eIF2α kinases promotes a tight complex formation between eIF2 and 2B in which the GNE activity of eIF2B becomes nonfunctional (Sudhakar et al, 2000; Babu and Ramaiah, 1996; Pavitt et al, 1988). The eIF2.GTP.Met-$tRNA_i$ ternary complex, thus formed will bind to 40S subunits (bound with eIF3) to form a 43S preinitiation complex.

Step 4: Joining of 43 S preinitiation complex (eIF3-eIF1A-40S-eIF2.GTP.Met-$tRNA_i$) to mRNA to form 48S complex: The 43 S preinitiation complex formed as mentioned above will then join to a mRNA. Most of the eukaryotic mRNAs contain a 5' m^7G cap structure.

With a few exceptions, the 3' end also contains a poly (A) tail. Specific enzymes add these structures during or just after mRNA synthesis. They play important roles in mRNA metabolism such as mRNA splicing, transport, stability, localization and translation. The cap structure is recognized by a cap-binding protein called eIF4E

(~25 kD in mammals) (Altmann et al, 1989; Rom et al, 1998). eIF4E is a part of a heterotrimeric complex called eIF4F. In addition to eIF4E, eIF4F complex consists of a scaffolding protein called eIF4G (~171-176 kD in mammals), also formerly called p220 that brings together other components of the initiation pathway (Yan et al, 1992; Imataka et al, 1998) and eIF4A, a RNA helicase (~ 46 kD in mammals) (Conroy et al, 1990). The discovery and subsequent characterization of the eukaryotic poly A binding protein (Pab1p) supports the idea that poly A tail is involved in translation (Baer and Kornberg, 1983; Adam et al, 1986; Sachs et al, 1986; Sachs and Davis, 1989). The RNA helicase activity of eIF4A is stimulated by a couple of cofactors called eIF4B (69 kD in mammalian systems) that functions as a homodimer (Lawson et al, 1989; Rozen et al, 1990) and eIF4H (25 kD protein in mammals) that functions as a monomer (Richter-Cook et al, 1998). Both eIF4A and 4H have an RNA recognition motif. eIF4A binds ATP and is a helicase capable of bi-directional unwinding of RNA duplexes. It has DEAD sequence motif that is typical of RNA helicases (DEAD box family of proteins). In addition, eIF4B has DRYG (214-327 aa) and ARM (367-423 aa) – rich motifs (Morino et al, 2000).

The binding of eIF4F to 5' m^7G cap commits the translational apparatus to the translation of that mRNA. The formation of eIF4F complex in mammalian systems is regulated by a family of translation repressor proteins (~12 kD) called eIF4E-BPs. Binding of the 4E-BPs to eIF4E occurs through a 'core' sequence contained in eIF4E (YXXXXLΦ) in which X is any amino acid and Φ is a residue possessing an aliphatic portion, most often L, but some times M or F (Mader et al, 1995). Phosphorylation of specific serine and threonine residues of 4EBPs modulates the affinity of the eIF4E-BPs for eIF4E. While hypophosphorylation leads to efficient binding of 4E-BPs to eIF4E, hyperphsophorylation abrogates this interaction (Lin et al, 1994; Pause et al, 1994; Fadden et al, 1997). The cap-binding complex activity of eIF4F is also regulated by eIF4E and 4G (See latter). A decrease in eIF4F activity increases the competition among mRNAs and thereby regulates the class of mRNAs to be translated.

The binding of the 43S preinitiation complex to 5'- terminal region of mRNA requires the melting of the secondary structure in the 5' - proximal region of the mRNA (Rozen et al, 1990). This is accomplished by eIF4A and is stimulated by 4B and 4H cofactors. Once the secondary structure is removed from the 5' – terminal

region, an interaction between eIF4G and eIF3 (bound to the 40S subunit) brings the ribosome to the mRNA (m^7Gcap-eIF4E-eIF4G-eIF3-40S-eIF2.GTP.Met-$tRNA_i$). eIF4G, the scaffolding protein can be divided into three distinct domains. The amino terminal region (1-634 aa) binds the Poly(A)-binding protein and eIF4E, and is required for cap-dependent translation (Lamphear et al, 1993; Imataka et al, 1998). The central domain (635-1039 aa) has an RNA binding site (Pestova et al, 1996) and also binds to eIF3 and eIF4A proteins (Imataka and Sonenberg, 1997). This region is sufficient to bind the 43S preinitiation complex of encephalomyocarditis virus (EMCV) IRES (Internal ribosome entry site) RNA (Pestova et al, 1996) and stimulates the translation of capped mRNAs (De Gregorio et al, 1998). The carboxy terminal region plays a modulatory role and contains a second eIF4A binding site (Imataka and Sonenberg, 1997) and also binds to the protein kinase Mnk 1 (Pyronnet et al, 1999).

ATP binding induces a conformational change in eIF4A which allows RNA binding to the carboxy terminal region of eIF4A (bearing a sequence HRIGRXXR motif), and RNA binding in turn induces ATP hydrolysis followed by more stable RNA binding (Pause et al, 1993; 1994).

Step 5: Recognition of initiation codon AUG: This step involves pairing between the initiation codon (AUG) in mRNA and the CAU anticodon of the initiator tRNA (Met-$tRNA_i^{Met}$). The 5' proximal AUG serves as the initiation codon in more than 90% of mRNAs suggesting that its position in the 5' UTR (untranslated region) of an mRNA plays a dominant role in its selection. Most mRNAs find the start AUG codon by a mechanism called 'scanning' (Kozak, 1999). The hypothesis states that the 40S ribosomal subunit migrates downstream along the mRNA from 5' terminus towards the initiation codon, a process that consumes energy in the form of ATP. However, there is no direct evidence for such 40S subunit migration by stalling it or slowing its movement by omitting specific initiation factors. It is also not known if or when the eIF4F dissociates from the m7G cap and eIF3 during scanning. It is likely, that eIF4G is bound until the initiation codon is recognized. If so, the 40S subunit could be bound simultaneously to the m7G cap and to the AUG, with a single-stranded 5' UTR RNA looping out from the ribosome.

Eukaryotic cellular and viral mRNAs lack a highly conserved motif equivalent to the Shine-Delgarno sequences of prokaryotic mRNA that facilitate the recognition of initiation codons. Efficient initiation codons in eukaryotic mRNAs lie in a context with purines at positions –3 and +4 (where the A of the AUG is +1) but this is not so in yeast. In leaky scanning, the scanning ribosome bypasses the initiation codon that is present in a poor context and starts initiation at another AUG in a more favorable context (Kozak, 1989). This kind of scanning can lead to two protein products. On rare instances, the ribosome instead of migrating along the 5'UTR to encounter the initiation codon, it "hops" from one region to another by a process called 'shunting' or 'repositioning' (La torre et al, 1998).

Initiation at a given site can be enhanced by the presence of an RNA secondary structure inserted some 18 nucleotides downstream from the AUG (Kozak, 1990). In addition to the local sequence in mRNA and secondary structure, the recognition of start AUG in eukaryotic mRNAs requires the presence of several initiation factors. A recent biochemical analysis indicates that eIF1 (12.7 and 12.3 kD in human and yeast respectively) in conjunction with eIF1A (17 – 22 kD) (Wei et al, 1995) promotes the binding of 40S ribosome to the initiation codon in a system that contains eIF2, eIF3, eIF4A, eIF4B, and eIF4F. Earlier, a number of mutations in yeast eIF1 (SUII) that affect initiation codon selection by allowing initiation at UUG have been identified (Yoon and Donahue, 1992; Kasperaitis et al, 1995). Initiation site selection in eukaryotic mRNAs can also occur without any initiation factors like eIF4A, 4B, 4E or 4G or ATP hydrolysis as has been observed in the case of Hepatitis C Virus (HCV) and pestivirus IRES (Internal ribosome entry sites). Also there are variations from the typical scanning-dependent initiation of capped mRNAs that require eIF4E, the amino-terminal and central domains of eIF4G. For example, the translation of Picornovirus IRESs occurs through an internal initiation and requires eIF4G but not eIF4E. The eIF4G requirement is fulfilled by the central domain of the protein. The translation of uncapped Satellite tobacco necrosis virus (STNV) RNA requires binding of eIF4E/eIF4F to an internal site (Timmer et al, 1993). Infact eIF4E binds the 3'UTR of STNV RNA with higher affinity than it binds to conventional cap structures. These findings emphasize the importance of 3'UTR regions in mRNA in the recognition of initiation codon selection.

Step 6. Formation of 80S initiation complex: Joining of 60S subunits to the 48S initiation complex to form 80S initiation complex requires hydrolysis of GTP bound to eIF2, the release of initiation factors bound to the 48 S initiation complex. The process requires two initiation factors eIF5 (~58 – 62 kD in mammals) (Raychaudhuri et al, 1985 a, b; Chakravarti et al, 1993) and eIF5B (~125 – 168 kD, mammals) (Levin et al, 1973; Merrick et al, 1975; Schreier et al, 1977). Infact eIF5B was discovered much earlier to eIF5 and was previously known as eIF5, IF-11 or IF-E5 etc. However, the functional activities of eIF5B are recently established (Pestova et al, 2000).

eIF5 induces hydrolysis of eIF2 bound GTP on 48S complexes (Ghosh et al, 1989; Chakravarti et al, 1993) but differs from eIF5B in that, it is not a ribosome – dependent GTPase. eIF5 protein sequence contains a putative C2-C2 zinc finger in the N-terminal side and is strongly homologous to the zinc finger domain of eIF2B (Das et al, 1997). The C-terminus of eIF5 contains a bipartite sequence motif rich in acidic and aromatic residues (Koonin, 1995). A similar AA motif is also present in the c-terminus of the epsilon subunit of eIF2B that catalyzes the exchange of GTP for GDP bound to eIF2. Recent studies by Asano et al (1999) suggest that these bipartite motifs conserved at the c-terminus of eIF5 and eIF2B ε mediate their binding to the K-boxes in the N-terminal half of eIF2β in yeast. Thus, these bipartite motifs in eIF5 and eIF2Bε facilitate binding to their common substrate eIF2. The exclusivity of these interactions is consistent with the fact that eIF5 and eIF2B promote opposing reactions on the guanine nucleotide bound to eIF2. As eIF2γ subunit binds GDP/GTP, additional (perhaps transient) interactions between eIF5 or eIF2B and the γ - subunit of eIF2 would be important for promoting GTP hydrolysis or guanine nucleotide exchange by inducing conformational changes within the GTP – binding domain of eIF2γ. It is suggested that the signal for eIF5 – dependent hydrolysis of GTP bound to eIF2 will establish base – pairing between an AUG initiation codon and the CAU anticodon of Met-tRNA$_i$ and mutations that uncouple this linkage can lead to recognition of non- AUG triplets as the initiation codon (Huang et al, 1997). Further it has been shown that the bipartite motif in eIF5 is also required for its interaction with eIF3.

The physiological function of eIF5B is understood when eIF5 is found insufficient to promote joining of 60S subunits to 48S complex that had been assembled on a natural mRNA like globin mRNA using a complete set of factors

(Pestova et al, 2000). In a complete assay system lacking eIF5, eIF5B loses the ability to stimulate the hydrolysis of GTP bound to eIF2 and without eIF5B, GTP hydrolysis induced by eIF5 is not sufficient for the joining of 60S subunit to 48S initiation complex. Interestingly, eIF5B has a close homology with the prokaryotic initiation factor, IF2 (Choi et al, 1998). While IF 2 is involved in recruitment of initiation tRNA to the small ribosomal subunit and in the subunit joining reaction in prokaryotes, eIF5B in eukaryotes plays a role in subunit joining only. The initiator tRNA recruitment to 40S subunit in eukaryotes is carried out by eIF2, a factor that has no homologue in prokaryotes. The formation of 80S initiation complex with the Met-$tRNA_i$ properly positioned on the 'start' AUG marks the end of initiation.

1.2. Eukaryotic elongation cycle

Elongation cycle begins at the point when the peptidyl tRNA site (P) in the 60 S subunit of the 80S initiation complex is occupied by the initiator tRNA and the A-site (aminoacylated tRNA site) is vacant. However, it is not known how the Met-$tRNA_i$ reaches the P site. The elongation cycle has three important steps. These are a) joining of amino acylated tRNA to the A site, b) peptide bond formation between adjacent amino acids and c) the translocation of ribosome. During the elongation cycle, the ribosome will add one amino acid at a time to a growing polypeptide chain according to the sequence of codons in the mRNA. The eukaryotic elongation cycle requires three factors namely, eEF1A (50.1 kD, previously known as EF-1 or eEF1α), heterotrimeric eEF1B with three subunits α (24.8 kD), β (31.1 kD) and γ (50 kD) and eEF2 (95.2 kD).

Step1: Eukaryotic elongation is thought to be similar, if not identical, to the prokaryotic elongation. The eukaryotic factors however undergo several posttranslational modifications (discussed later) whereas no such modifications are observed with the prokaryotic elongation factors. Overall structure of eEF1A appears similar to that with bacterial EF1A except that eEF1A has approximately 70 additional amino acids inserted into 16 different sites (Cavallius and Merrick, 1993). The eukaryotic elongation factor, like the initiation factor-2 (eIF2), facilitates the formation of a ternary complex (eEF1A.GTP.aa-tRNA), which is then, bound to the ribosomal A site in a codon-dependent manner. Upon cognate recognition, the eEF1A.GTP is probably brought into the GTPase activating center of the ribosome as

has been shown for the prokaryotic factor (Pape et al, 1998) so that the GTP is hydrolyzed and the eEF1A.GDP leaves the ribosome. The off rate of GDP is slow and the GDP → GTP exchange is catalyzed by eEF1B. The protein is heterotrimeric in most species but, is made up of two subunits in yeast (α, γ). The nucleotide exchange activity is associated with the α and β – subunits but, the γ – subunit has failed to show any activity (Kinzy et al, 1994). The α, β – subunits differ by 130 or so amino acids at the N – terminal region. The α – subunit is shorter but its sequence similarity with the β – subunit suggests that it may have arisen by gene duplication. The eukaryotic eEF1B is found to be unrelated to prokaryotic EF1B and the complexed form with eEF1A is found to be associated with the endoplasmic reticulum (Sanders et al, 1996).

Step II: The peptidyl transferase center of the ribosome catalyzes the formation of a peptide bond between the incoming amino acid and the peptide found in the peptidyl-tRNA binding site (P site).

Step III: The eEF2.GTP then enters the ribosome, probably as in prokaryotic systems, forces the peptidyl tRNA out of the A site and possibly prevents it from binding to the A site. Thus, the peptidyl tRNA is brought fully into the P site and the deacylated tRNA goes to an exit site (E). The GTPase center in ribosome hydrolyzes the GTP bound to eEF2 and eEF2.GDP will then leave the ribosome (Rodnina et al, 1997). The elongation factors function in translation by mimicking the anticodon stem loop of tRNA molecules (Nissen et al, 1995; Nissen et al, 2000). This action of eEF2, i.e. to force the movement of peptidyl tRNA with the P site can account for the precise movement of the mRNA by 3 nucleotides. This model envisages eEF2 dragging the codon of the mRNA from the A site into the P site. Once the translocation is completed, the ribosome will be ready to receive another aa-tRNA with the help of eEF1A and GTP.

A unique elongation factor, eEF3, is found in fungi that appears to be important in the release of nonacylated tRNA from the entry site of the ribosome (Triana-Alonso et al, 1995). The protein uses ATP as a substrate *in vivo*. No homology of this protein has been so far identified in bacteria, archaebacteria or in higher eukaryotes. The protein has a 1044 aa and a sequence homology to ribosomal protein S5 (Gontarek et al, 1998). Although it is an essential protein in yeast, surprisingly it is not found in other organisms.

1.3. Peptide chain termination

Termination of nascent polypeptide chain is aided by termination or release factors (RFs). Two classes of release factors are found. Class I release factors recognize a stop codon and promote the hydrolysis of the ester bond linking the nascent polypeptide with the tRNA present in the P site by the peptidyl transferase center of the ribosome. Class II release factors are GTPases and stimulate class I factor activity (Mikuni et al, 1994; Zhouravleva et al, 1995; Frolova et al, 1996). Prokaryotes have two class I release factors (RF1 and RF2) and one release factor (RF3) that belongs to class II. Eukaryotes have one release factor belonging to each of these classes (eRF1 and eRF2). eRF1 homologs are identified from several species. Sequence of class I release factors had revealed the existence of a GGQ sequence motif that is universal to all species and substitutions in this motif abolished the ability of human eRF1 to trigger peptidyl tRNA hydrolysis (Frolova et al, 1999; Song et al, 2000). There is no sequence similarity between prokaryotic and eukaryotic RF sequences (Frolova et al, 1994). The crystal structure of human eRF1 is recently determined (Song et al, 2000) and the protein has three domains in a structure reminiscent of the letter Y. The overall shape resembles a tRNA molecule.

Events that occur after peptide hydrolysis are not well investigated in eukaryotic systems. The GTPase function of eRF3 is thought to be involved in the dissociation of eRF1 and eRF3 complex from the ribosome (Frolova et al, 1996). Efficiency of translational termination is found to be modulated by transactivating factors and also the sequence context surrounding the termination codon (Poole et al, 1995; Pavlov et al, 1998; Bonetti et al, 1995; Brown et al, 1990, 1993).

2. Regulation of protein synthesis

Translational control is defined as the change in the rate (efficiency) of translation of one or more mRNAs i.e. the number of completed protein products changes per mRNA per unit time. Under steady-state conditions, the number of initiation events per unit time approximates the number of protein products produced during the same time interval (Tsung et al, 1989). Logically, rate of protein synthesis is determined by the rate of initiation. The number of initiation events can be

calculated from the number of ribosomes translating an mRNA (polysome size) and the ribosome transit time as has been described by Palmiter (1975) for the ovalbumin mRNA.

Protein synthesis, as mentioned above, is a multistep and multifactorial pathway. It is economical for the cell to control a pathway of this kind at its onset than to interrupt somewhere in the middle. Consistent with this notion, most of the examples controlling protein synthesis (translational controls) are seen operating at the level of initiation than at the elongation. However, in some well-characterized cases the translational elongation is blocked as a safety measure to halt further peptide chain initiation. One of the best-studied examples of regulation during elongation phase is found in the synthesis of proteins that are destined for secretion (Blobel and Sabatini, 1971, Walter and Blobel 1981). The earliest cases of translational controls essentially came from reticulocytes, developing embryos, virus and phage-infected cells and higher cells responding to stimuli ranging from heat to hormones and starvation & stress.

2.1. Translational regulation at the level of Met-tRNA$_i^{Met}$ binding

Reticulocytes, the immature red cells are enucleated and were found quite suitable to study translational regulation. In 1950s, it was observed that inorganic iron stimulates the synthesis of hemoglobin in reticulocytes (Kruh and Borsook, 1956). Later studies showed that heme, not iron, per se is required for protein synthesis in reticulocyte lysates since the iron-chelating agent, desferrioxamine does not block the stimulatory effect of heme (Bruns and London, 1965; Grayzel et al, 1966). Since iron is required for heme synthesis, iron-deficiency leads to heme-deficiency. Later studies have made it possible to study the effects of heme on the regulation of protein synthesis by developing highly active unfractioned cell-free translational systems from reticulocytes (Lamfrom and Knopf, 1964; Pelham and Jackson, 1976; Hunt et al, 1972). The effects of heme-deprivation on protein synthesis in the reticulocyte or its lysate is also mimicked by

unrelated stimuli such as double-stranded RNA (dsRNA) and oxidized glutathione (Ehrenfeld and Hunt, 1971; Kosower et al, 1971). Heme-deficiency was found to inhibit protein synthesis globally and affects the translation of all mRNAs present or supplemented in reticulocyte lysates (Mathews et al, 1973).

Later studies have demonstrated that heme-deficiency, addition of low concentrations of dsRNA and oxidized glutathione were found to stimulate the phosphorylation of the small or alpha-subunit of translational initiation factor 2 (eIF2α) in cell-free translational systems prepared from anemic rabbit red blood cells (London et al, 1987; Jackson, 1991). While heme-deficiency activates heme-regulated eIF2α kinase (HRI), dsRNA addition stimulated an RNA-dependent protein kinase, called PKR (Chen and London, 1995; Clemens and Elia, 1997). It is not known if GSSG activates a distinct eIF2α kinase. It may be activating HRI-like kinase in reticulocyte lysates. Both the kinases have been shown to stimulate eIF2α phosphorylation. Incidentally a small fraction of eIF2α phosphorylation (~30%) is sufficient to cause inhibition of protein synthesis (Leroux and London, 1982). This led to the identification of a rate-limiting heteropentameric protein called eIF2B (Webb and Proud, 1997) (Previously known as RF, GEF, Co eIF2C, anti-HRI etc).

In order to generate active eIF2.GTP from eIF2.GDP, eIF2B plays a key role in exchanging the GDP for GTP. While eIF2.GDP is a substrate for eIF2B, eIF2α(P) is a competitive inhibitor of eIF2B (Rowlands et al, 1988a,b). Hence it is presumed that eIF2α(P) forms a complex with eIF2B in which the guanine nucleotide exchange activity of eIF2B becomes non-functional. Studies with site-specific mutants of eIF2α have shown that serine 51 is the only residue in human eIF2α that is phosphorylated (Pathak et al, 1988). Phosphorylation of serine 51 residue promotes a complex formation between eIF2α(P) and eIF2B (Sudhakar et al, 2000, 1999; Krishnamoorthy et al, 2001) and inhibits the GNE activity of eIF2B (Ramaiah et al, 1994) as has been hypothesized earlier (Rowlands et al, 1988b). These events cause an impairment in the recycling of eIF2.GDP, formation of 43S complexes and protein synthesis (Ramaiah et al, 1992; Pavitt et al, 1998). These mutants of eIF2α in which 51 serine residue has been replaced by alanine (S51A) or aspartic acid (S51D) have been used to determine the contribution of eIF2α phosphorylation in the inhibition of protein synthesis during various stresses like heat shock or apoptosis (Murtha-Riel et al, 1993; Srivastava et al, 1998). While S51A in non-phosphorylated form, S51D behaves like phosphorylated form.

In addition to heme-deficiency and virus infection, several other stresses have been shown to stimulate eIF2α phosphorylation. These are amino acid starvation,

unfolded proteins or denatured proteins, exercise, nitric oxide, metal stress, heat shock and serum starvation as listed (Sudhakar et al, 1999). The number of eIF2α kinases that have been discovered during the years also went up from 2 to 4 now. Apart from heme-regulated eIF2α kinase (HRI) and double-stranded RNA dependent protein kinase (PKR), GCN2 is discovered in yeast that is subjected to amino acid starvation and PERK/PEK, endoplasmic reticulum (ER)-resident kinase is discovered that is activated in response to calcium mobilization or accumulation of unfolded proteins (Hinnebusch, 1997; Harding et al, 1999; Shi et al, 1998). All the four kinases are found to phosphorylate the serine 51 residue in eIF2α. Hence they are called eIF2α kinases. They share several structural features with the serine/thrionine kinases but their regulation is different.

Heme availability regulates HRI kinase. Heme binds stably to the amino-terminal region of HRI (Rafie-Kolpin et al, 2000). Heme promotes disulfide bond formation in HRI protein and inhibits the kinase activation (Chen et al, 1989). HRI is strongly activated in heat shocked reticulocyte lysates. The mechanism for activation of HRI by heat shock is complex. Newly synthesized HRI requires the chaperone activity of HSP 90 and HSP 70, the constitutively produced form of HSP 70, for mature folding into a native and active conformation (Uma et al., 1997, 1999). However HSP 70 also blocks HRI kinase activity in the presence of heme (Matts et al., 1993; Uma et al., 1999), accounting for kinase inactivation by heme. Binding of HSP 70 to HRI probably induces a conformational change that prevents kinase activity (Uma et al., 1999). Heme also appears to displace HSP 90 but not HSP 70 from HRI, thereby promoting the formation of a kinase-inactive complex. Heat stress disrupts the negative regulatory HRI-HSP 70 interaction despite the presence of heme, presumably by promoting conformational change in HRI that releases HSP 70, thereby activating HRI kinase activity (Uma et al., 1999). It is not known if these heat shock proteins regulate similarly the activation of other eIF2α kinases mentioned below.

PKR has two double stranded RNA binding regions in the N-terminus (6-79 aa; 96-169 aa). dsRNA binds to PKR in an RNA-sequence independent manner. Viral dsRNA genomes (e.g. reovirus), replication intermediates, mRNA transcripts with extensive secondary structure resembling dsRNA are potent activators of PKR. PKR activation is bimodal: low concentrations of dsRNA activate and high

concentrations of dsRNA inhibit the activity (Hunter et al., 1975). PKR undergoes oligomerization upon binding dsRNA to its amino terminal regulatory domain (Wu and Kaufman, 1997). This triggers transphosphorylation event that enhances PKR activity. In addition to dsRNA, PKR can be activated by heparin *in vitro* (Patel et al, 1994) and by activated caspase-3 present in apoptotic cells *in vitro* (Saelens et al, 2001; Aparna et al, 2002). Recent studies suggest that PACT, a cellular protein that has three double-stranded RNA-binding motifs can interact with PKR and elicits its activation in the absence of dsRNA (Patel and Sen, 1998). These findings suggest that activation of PKR occurs by different mechanisms.

PKR is induced by interferons and activated by dsRNA. This protein poses a serious threat to viral multiplication as judged by the number of mechanisms that viruses have elaborated to counteract its effect on protein synthesis (Kaufman, 1999a). These counter measures are diverse in kind involving viral RNAs and proteins as well as host proteins, which inhibit PKR function (that is its activity to phosphorylate eIF2α) at many different levels. Poliovirus destabilizes PKR (Black et al, 1989) that would dampen antiviral response. Cells expressing *tat* gene or infected with HIV-1 are found to contain reduced PKR levels (Roy et al, 1990). Some viruses produce proteins that bind dsRNA and inhibit PKR activation. These are E3L protein produced by vaccinia virus (Chang et al, 1992; Rivas et al, 1998), σ3, product of reovirus S4 gene (Beattie et al, 1995), NS1 protein of influenza virus and U_S11 of hepatitis simplex virus (Lu et al, 1995; Cassady et al, 1998; Mulvey et al, 1999). Small, highly structured RNA molecules produced by viruses can bind to PKR and antagonize its activation by dsRNA. Adenovirus VA RNA_I (160 nt) prevents PKR activation at late times of infection (Reichel et al, 1985). Similarly dsRNA analogs are produced by Epstein Barr Virus (Bhat and Thimmappaya, 1985; Swaminathan et al, 1992), HIV-1 (Gunnery et al, 1992) and HDV (Robertson et al, 1996). Some of the viral proteins inhibit PKR dimerization. These include $P58^{IPK}$, a cellular protein, a member of the tetratricopeptide family whose activity is masked in uninfected cells by HSP40 but, its activity is triggered by cells infected with influenza virus (Melville et al, 1997). NS5A protein produced by Hepatitis C virus binds PKR and prevents its dimerization and activation (Gale et al, 1998). NS1 produced by influenza virus and vaccinia E3L may also function in part by binding directly to PKR (Romano et al, 1998; Tan and Katze, 1998), Baculovirus PK2 protein resembles sequence homology

to an eIF2α kinase domain and inhibits PKR phosphorylation and thereby eIF2α phosphorylation (Dever et al, 1998; Sudhakar et al, 1999). Some of the proteins produced by the viruses resemble eIF2α. These proteins compete with eIF2α for phosphorylation by PKR. The vaccinia viral proteins E3L (mentioned above) or K3L act as pseudo substrates for PKR and also block the activation of PKR and HRI (Beattie et al, 1995). E2 protein of HCV also contains sequences homologous to eIF2α and inhibits eIF2α phosphorylation catalyzed by PKR (Taylor et al, 1999). HIV-Tat is phosphorylated by PKR leading to inhibition of eIF2α phosphorylation (Brand et al, 1997; Cai et al, 2000). Some of the viral proteins can also dephosphorylate eIF2α(P). The γ 34.5 protein of Herpes simplex virus is a homolog of a phosphatase of eIF2α (He et al, 1998; Leib et al, 2000). SV large T-antigen bypasses the translational block despite continued eIF2α phosphorylation (Swaminathan et al, 1996). The mechanism in this case is however not understood. Alu RNAs are RNA polymerase III transcripts that are induced upon stress conditions, and at high concentrations, can also inhibit PKR *in vitro* and *in vivo* (Chu et al, 1998). p67, a cellular glycosylated protein and L18, 60S ribosomal proteins are found to inhibit PKR activation (Wu et al, 1996; Kumar et al, 1999).

GCN2 kinase is activated in response to amino acid starvation. It was originally observed in yeast (Hinnebusch, 1997). GCN2 like kinases are also cloned from insects (Santyo et al, 1997) and mammalian systems (Sood et al, 2000). In the activation of GCN2, uncharged tRNAs that are accumulated during amino acid starvation appear to be activating the ligands because mutations in amino acyl-tRNA synthetases stimulate GCN2 function without any limitation for the cognate amino acids (Wek et al, 1995; Hinnebusch, 1997). GCN2 (1659 aa) contains about 500 residues of carboxy-terminal to its kinase domain related to histidyl-tRNA synthetases (His RS) (Wek et al, 1989) and a motif preceding to His RS that interacts with the acceptor stem of tRNA (Wek et al, 1995; Zhu et al, 1996). Binding of uncharged tRNAs to His-RS like domain would produce a conformational change in GCN2 that allows the kinase domain to phosphorylate eIF2α.

Phosphorylation of eIF2α controls protein synthesis globally as had been observed in reticulocyte lysates (Mathews et al, 1973) or selectively in yeast (Hinnebusch, 1994). Aminoacid starvation in yeast results in GCN2 kinase activation

and increased eIF2α phosphorylation. These events are associated with an increase in the translation of GCN4 mRNA, which encodes a transcriptional activator of many amino acid biosynthetic genes (Hinnebusch, 1994). General translation and cell growth are inhibited in yeast cells only when eIF2α is phosphorylated to higher levels, that occurs when GCN2 is activated by amino acid limitation. Specific induction of GCN4 translation in response to eIF2α phosphorylation is mediated by four short open reading frames (uORFs) in the leader of GCN4 mRNA, of which the first and fourth uORFs are sufficient for nearly wild-type translational control. Under non-starvation conditions, virtually all of the reinitiating ribosomes rebind the ternary complex (eIF2.GTP.Met-tRNA$_i$) and reinitiate at uORF4, after which they dissociate from the mRNA. In starvation condition, eIF2α phosphorylation occurs, eIF2B activity is decreased and the ternary complex formation is lowered. Because of the lack of Met-tRNA$_i$, 40S ribosomes bypass the inhibitory uORF4 start codon and reinitiate at GCN4 instead (Fig. 1e).

The endoplasmic-reticulum (ER) resident kinase called PEK/PERK is activated by ER stress like the accumulation of unfolded proteins. PERK shifts from a complex of relatively low molecular weight (mean size ~240 kD) to one of a very high molecular weight in ER-stressed cells. The high molecular weight is entirely phosphorylated (Bertolotti et al, 2000) suggesting that it is oligomerized to form a high molecular weight complex in which transphosphorylation and kinase activation takes place. PERK kinase has a ER lumenal domain that is similar to the N-terminal region of ire1 gene. This region perceives the ER-stress and transduces to the cytoplasmic effector domain of the PERK. The effector domain contains the eIF2α kinase sequence. It is found that *perk*$^{-}$ $^{-}$ cells are severely impaired in activation of *chop* gene transcription in response to ER-stress agents such as tunicamycin, DTT, thapsigargin etc. This is due to the failure of eIF2α phosphorylation. *Chop* encodes ER-stress inducible transcription factor that may play a role in promoting programmed cell death. This finding suggests that PERK activation and eIF2α phosphorylation causes translational attenuation on one side and probably stimulates transcription of certain genes (Zinszner et al, 1998; Kaufman, 1999b) like the ones involved in apoptosis and protein folding on the other side.

The β-subunit of eIF2 (333 aa) also contains a number of interesting features including the phosphorylation sites for CKII (ser^{2}, ser^{67}), protein kinase C (ser^{13}) and CAMP-dependent protein kinase (PKA) (ser^{218}) (Welsh et al, 1994). Phosphorylation of eIF2β by PKA has been shown to modulate indirectly the guanine nucleotide exchange activity of eIF2B, at least *in vitro* (Kimball et al, 1998a). The N-terminal half of the β-subunit of eIF2 contains three domains, each consisting of 6-8 lysine residues (K-boxes). The binding site for a second initiation factor, eIF5, overlaps the second polylysine domain (Das et al, 1997). The N-terminal half of the protein contains two of the three guanine nucleotide binding domains. However, the distance between the two domains is larger than usual and mutations in the second have no effect on GDP binding (Naranda et al, 1995) suggesting that eIF2β is not involved in GDP binding. The C- terminal portion of eIF2β contains the binding domain for the catalytic subunit (epsilon subunit) of eIF2B, the eIF2 guanine nucleotide exchange factor (Kimball et al, 1998a). The C-terminus of eIF2β also has a zinc finger motif but zinc has no effect on the activity of the protein. A recent study demonstrates that eIF2β is phosphorylated by DNA-dependent protein kinase and a portion of eIF2 localizes to the nucleus. This finding suggests that eIF2 might play a physiological role in DNA repair through its interaction with DNA-PK (Ting et al, 1998).

The γ-subunit of eIF2 (472 aa in humans) is implicated in guanine nucleotide binding and Met-$tRNA_i$ binding based on mutational studies (Naranda et al, 1995) and cross-linking studies (Gaspar et al, 1994).

Two serine/threonine phosphatases PP1, PP2 have been shown to dephosphorylate eIF2α *in vitro* (Redpath and Proud, 1990). Dephosphorylation of eIF2α bound to ribosomes, tRNAs or other proteins is observed to be far slower than the uncomplexed form *in vitro* (personal observations of Ramaiah). These observations prompted Babu and Ramaiah to determine the physiologic phosphatase that is responsible for the dephosphorylation of eIF2α(P). Since eIF2α(P) formation leads to inhibition in the GNE activity of eIF2B and has a higher affinity for eIF2B, Babu and Ramaiah (1996) used inhibited heme-deficient reticulocyte lysates to monitor the delayed effects of added hemin on the restoration of protein synthesis and eIF2B activity in the presence of type 1 and type 2 phosphatase inhibitors. Their findings in quasiphysiological conditions suggest that type 1 phsophatase is

responsible for the dephosphorylation of eIF2α(P) and restoration of eIF2B activity. These findings are supported by recent results obtained from other laboratories wherein it has been shown that a) type 1 phsophatase involvement in the accumulation of eIF2α phosphorylation in response to ischemia in PC 12 cells (Munoz et al, 2000) and the dephsphorylation of eIF2α(P) by γ1 34.5 protein produced by Herpes-Simplex virus1 (He et al, 1998; Leib et al, 2000) which is a homolog of a phosphatase PP1 regulating subunit. Also, genetic studies in yeast support the idea that a type 1 phosphatase (GLC7 gene in yeast) is responsible for the dephosphorylation of eIF2α(P) in physiological conditions that is also involved in the regulation of glycogen metabolism (Wek et al, 1992).

A number of physiological conditions result in an increase in the GNE activity of eIF2B that parallels elevated rates of mRNA translation. For example insulin, glucose, T-cell activation, growth factors like EGF and phorbol esters all rapidly stimulate eIF2B activity (within 15 minutes of treatment), although the level of eIF2α phosphorylation remains unchanged (Welsh and Proud, 1992; Kleijn et al, 1998; Welsh et al, 1996, 1997). These findings suggest that presumably eIF2B activity is also regulated directly independent of eIF2α phsophorylation and by other mechanisms. For example, the catalytic subunit (ε) of eIF2B from mammals or insects is a substrate for glycogen synthase kinase 3 (GSK3) and this inhibits eIF2B (Welsh et al, 1998; Williams et al, 2001). GSK3 is inactivated in response to insulin and, concomitantly with this, the corresponding site in eIF2Bε undergoes dephosphorylation and the GNE activity of the protein is stimulated (Welsh et al, 1998). eIF2Bε is also phosphorylated *in vitro* by casein kinases 1 and 2 (CK1 and CKII) although the reports showing the effect of such a phosphorylation on the eIF2B activity varies (Dholakia and Wabha, 1988; Oldfield and Proud, 1992). Recent studies have identified the ε or catalytic subunit of mammalian eIF2B that is phosphorylated by four protein kinases at different sites (Wang et al, 2001). Two conserved sites ($ser^{712/713}$) are phosphorylated by CKII. They lie at the extreme C-terminus and are required for the interaction of eIF2Bε with its substrate eIF2, *in vivo* and eIF2B activity *in vitro*. GSK3 phosphorylates Ser^{535}. This regulatory phosphorylation event requires both the fourth site (ser^{539}) and a distal region that acts to recruit GSK3 to eIF2Bε *in vivo*. The fifth site that lies outside the catalytic domain is phosphorylated

by CKI. However, it is not known yet how these phosphorylation events would affect eIF2B or its interaction with other proteins.

Several allosteric mechanisms regulating eIF2B have also been identified. NAD^+ and $NADP^+$ have been shown to inhibit the activity of purified mammalian eIF2B, whereas, their reduced forms stimulate it. Indeed eIF2B has been reported to contain bound $NADPH^+$, which is required for its own activity (Price and Proud, 1994). Karnich et al (1993) demonstrated that eIF2B activity was reduced in skeletal muscle but not in cardiac muscle in diabetic rats. NADPH reversed $NADP^+$-induced eIF2B inactivation. Following induction of diabetes the NADPH/$NADP^+$ ratio was increased in heart but was unchanged in skeletal muscle. This is consistent with the idea that NADPH prevents inhibition of eIF2B in the heart and the changes in eIF2 phosphorylation may not be the reason. Consistent with these findings, Ramaiah et al (1997) have shown that a redox compound like pyrroloquinoline quinone (PQQ) stimulates eIF2B activity in Chinese hamster ovary cell extracts. However, a similar increase in eIF2B activity is not observed in hemin-supplemented reticulocyte lysates that are treated with PQQ. This is due to the activation of heme-regulated kinase and increased eIF2α phosphorylation in reticulocyte lysates. On the contrary, PQQ inhibits HRI kinase activity *in vitro*. These findings suggest that PQQ is reduced in lysates to $PQQH_2$ and the reducing equivalents can be transferred to eIF2B or HRI kinase present in the lysates. Action of HRI kinase leads to eIF2α phosphorylation and inhibition of eIF2B activity as has been observed in reticulocyte lysates (Ramaiah et al, 1997). However in CHO cells that presumably lack any such HRI-like kinase, one can see an increase in the eIF2B activity in the presence of PQQ.

The GNE activity of eIF2B is also regulated by amino acid deprivation through mechanisms distinct from eIF2α phosphorylation. In cultures of L6 myoblasts, deprivation of either leucine or histidine results in an inhibiton of protein synthesis that is associated with enhanced eIF2α phsphorylation as well as a reduction in eIF2B activity (Kimball et al, 1998b). However, the proportion of phsophorylation is never more than 5% of the total in cells-deprived of amino acids. Based on the levels of eIF2B relative to eIF2, the L6 myoblasts can tolerate upto 40-50% of eIF2 phosphorylation (Kimball et al, 1998b). Likewise in perfused rat liver an excess of leucine in relation to other amino acids results in an inhibition of protein synthesis with a 50% reduction in the GNE activity of eIF2B (Shah et al, 1999) without a

change in eIF2α phosphorylation. These results are thus different from what has been observed in yeast. In yeast, amino acid deprivation leads to inhibition in eIF2B activity through increased GCN2 kinase activation and eIF2α phosphorylation. But in L6 myoblasts and perfused rat liver the decrease in eIF2B activity that occurs due to amino acid deprivation appears to be independent of eIF2α phosphorylation. Based on several other studies, phosphorylation of the ε-subunit of eIF2 by GSK3, as the cause for inhibition of eIF2B activity during amino acid starvation is also eliminated (Kimball et al, 1998b). Leucine, but not histidine, also stimulates phosphorylation of 4E-BP1, $p70^{S6K}$ and ribosomal S6 protein and also preferentially increases in the synthesis of ornithine decarboxylase (ODC), over and above the observed stimulation of global rates of protein synthesis. The effect of leucine resupplementation is prevented by rapamycin, a specific inhibitor of the protein kinase known as the mammalian target of rapamycin (mTor). Thus, it is likely lecuine effects are mediated through mTor-dependent processes that result in preferential translation of specific mRNAs through stimulation of eIF4F function and activation of $p70^{S6K}$ (Kimball et al, 1998b).

The metabolism of branched chain amino acids (like leucine and valine) is significantly different in yeast compared with other eukaryotes, where amino acids are eventually broken down to components of the tricarboxylic acid (TCA) cycle. For example, yeast cannot use branched amino acid as a sole carbon source. They can be however used as a nitrogen source under nitrogen limiting conditions with the consequent production fusel alcohols (Webb and Ingraham, 1963; Cooper 1982). They are shown to induce in yeast, a range of strain-specific morphological effects like filamentation (Dickinson, 1996; Lorenz et al, 2000). These effects are also seen if yeast is grown on leucine as the sole source of nitrogen. Consistent with the above interpretations, a recent study has shown that fusel alcohols like butanol and isoamyl alcohol inhibit translation probably through the inhibition of the GNE activity of eIF2B directly without changing the status of eIF2α phosphorylation (Ashe et al, 2001).

2.2. Regulation at the level of mRNA binding to ribosomes

Translation of a given mRNA is regulated by *cis*-acting elements such as 5' cap, the length and secondary structure of the 5' UTR, nature and placement of the

initiation codon and the 3' poly A tail and elements in the 3' UTR. The effects of the various *cis*-acting elements in the mRNA 5' UTR and 3' UTR are modulated through the activity of initiation factors and other *trans*-acting factors. Chiefly, phosphorylation of initiation factors controls the rate of mRNA binding to 43 S preinitiation complex.

eIF4E, eIF4G, eIF4B and eIF3 that promote mRNA binding to ribosomes are phosphorylated and the phosphorylation status of these protein correlates positively with both translation and growth rates of the cell (Rau et al, 1996). The best-studied and characterized factor among these is eIF4E. Phosphorylated eIF4E is reported to have a higher binding affinity to the mRNA 5' cap relative to its unphosphorylated counterpart (Minich et al, 1994) and is supported by crystallographic data (Marcotrigiano et al, 1997). In spite of lack of *in vitro* translation data for the importance of eIF4E phosphorylation, many reports have documented that a correlation exists between the state of eIF4E phosphorylation and translational rate in the cells. For example, phosphorylation is low in G_0 phase of the mitosis, increases throughout G_1 and S phases, and is reduced in M phase (Bonneau and Sonenberg, 1987). The pattern correlates well with translational rates in cells. Complementing these observations, it has been shown that cap-independent translation of poliovirus RNA that occurs independent of 4E, proceeds efficiently in M-phase arrested cells (Bonneau and Sonenberg, 1987). eIF4E phosphorylation correlates with the increase in translational rates upon increased cardiac load (Wada et al, 1996). These results are consistent with the findings that treatment of vascular smooth muscle cells with angiotensin II, which induces cellular hypertrophy, also leads to an increase in eIF4E phosphorylation (Rao et al, 1994).

eIF4E has a single phosphorylation site Ser 209 (Joshi et al, 1995). Protein kinase C (PKC) and the MAP kinase signal integrating kinase (MNK1) can specifically phosphorylate eIF4E *in vitro*. MNK1 is a substrate for the mitogen-activated protein kinases (MAPK) – ERK and p38 MAPKs but not to the related C-Jun amino-terminal kinases/stress activated protein kinases (JNK/SAPKs) (Fukunaga and Hunter, 1997). Mnk1 however appears to be physiologically relevant kinase in the phosphorylation of eIF4E (Flynn and Proud, 1996; Waskiewicz et al, 1997). MnK1 does not interact directly with eIF4E; rather Mnk1 binds to the eIF4G family of protein (Pyronnet et al, 1999; Waskiewicz et al, 1999). The interaction between eIF4E

and 4G is required for eIF4E phosphorylation *in vivo* because a mutant eIF4E protein that cannot interact with eIF4G is not efficiently phosphorylated (Pyronnet et al, 1999). Since the eIF4E is phosphorylated by an eIF4G-bound kinase, it is likely that phosphorylated eIF4E "clamps" the bound mRNA in place.

The association of eIF4E with poly A-binding protein, Pab1p is found to be dependent on eIF4G (Tarun and Sachs, 1996). An expected consequence of the simultaneous association of eIF4E and Pab1p with eIF4G is that mRNA would become circularized. Circularization may stabilize mRNA by maintaining the association of translation initiation factors with its two ends (Schwartz and Parker, 1999). Another feature of circularization of mRNA ends, is that, it may stimulate reinitiation on that mRNA.

eIF4E association with N-terminal region in 4G depends on its interaction with eIF4E-BPs (4E-Binding proteins). In other words, the 4E-BP protein competes with 4G for a common binding site on the cap-binding protein, eIF4E. Various types of stimuli activate intracellular signaling pathways leading to hyperphosphorylation of the eIF4E-BPs. Hyperphosphorylation decreases the affinity of the 4E-BPs for eIF4E, leading to eIF4E release. Free eIF4E can then interact with 4G protein forming functional eIF4F complex. In contrast, hypophosphorylation increases the affinity of the 4E-BPs for eIF4E, which inhibits eIF4F formation (Sonenberg and Gingras, 1998).

The general mechanism of ribosome binding to mRNA is conserved in plants with a few notable exceptions. Plants carry two different eIF4F-like complexes, termed eIF4F and eIF iso4F. (The complex consists of 4E, 4G and 4A). In wheat, eIF4E (p26) and iso 4E (p28) are found to be functionally equivalent (Browning et al, 1987; Allen et al, 1992). However, eIF4E and eIF iso4E from *Arabidopsis thaliana* are not functionally equivalent and they are expressed differentially throughout development (Rodriguez et al, 1998). Unphosphorylated eIF4E and eIF iso4E are detected in the maize embryo, whereas, the only phosphorylated isoforms are present in leaves (Gallie et al, 1997). In young shoots and roots, even more acidic forms (suggesting further increase in phosphorylation of eIF4E and iso 4E) are present which disappear in older leaves (Gallie et al, 1997). Oxygen deprivation increases maize eIF4E phosphorylation (Manjunath et al, 1999). This is inhibited by ruthenium red. The hypoxic effect is mimicked by elevation in cytosolic calcium concentrations.

These findings are consistent with a role for calcium signaling in the phosphorylation of plant eIF4E. eIF4BP-like protein has not been isolated from plant cDNA libraries.

Phosphorylation of the mammalian and yeast eIF4A proteins has not been observed. However, during wheat seed development, phosphorylated eIF4A is observed at early stages (Le et al, 1998). In subsequent phases of seed development, and upon induction of germination, only dephosphorylated eIF4A is detected (Le et al, 1998). No correlation is observed between eIF4A phosphorylation and translational rates. During stress response eIF4A phosphorylation is inversely related to translation rates. This is in contrast to tobacco tube germination, in which phosphorylation of some eIF4A isoforms correlates with an increase in protein synthesis (op den Camp and Kuhlemeier, 1998). Since the phosphorylation sites in plant eIF4A are not yet analyzed, these observations are not necessarily contradictory. It is possible that some phosphorylation site(s) may elicit a positive effect and some can cause a negative effect.

eIF4E regulates translation of many mRNAs, which have a long 5' UTR that is G/C-rich and presumably highly structured. For example, ornithine decarboxylase (ODC) mRNA is translated inefficiently in normal cells, but when the highly structured 5' UTR is removed, ODC synthesis is greatly stimulated (Manzella and Blackshear, 1990). ODC mRNA is translated up to 30 times more efficiently in cells overproducing eIF4E (Rousseau et al, 1996). Other examples include cyclin D1 (Rosenwald et al, 1993a), C-Myc, a transcription factor (De Benedetti et al, 1994), human fibroblast growth factor 2 and vascular endothelial growth factor (Kevil et al, 1996).

Cis-acting elements if any, present in the 5' UTR or 3' UTR, like the iron-responsive elements (IREs) can affect the translation. When iron is limiting, cytoplasmic iron-regulatory proteins (IREPs) interact with the IREs present in the 5' UTR of ferritin or 3' end of transferrin receptor mRNA. IREs are usually located within 40 nucleotides of the cap structure, a feature that is functionally important. Ferritin mRNA interaction with the iron regulatory protein (IRPs) to the IRE blocks the cap-mediated recruitment of the 43 S preinitiation complex to mRNA (Gray and Hentze, 1994; Muckenthaler et al, 1998). In contrast, IRP binding to IREs in the 3'UTR of transferrin receptor is proposed to prolong the half-life of transferrin

receptor mRNA by sterically interfering with endonuclease access to a vulnerable cleavage site (Binder et al, 1994).

The "masking" hypothesis initially proposed by Spirin more than thirty years ago (Spirin, 1966) suggests that specific mRNAs are repressed through the action of proteins that hide them from translational apparatus. In response to a stimulus, such as fertilization, the masking proteins are removed, the mRNA is relieved and the translation begins. Masking hypothesis was proposed to explain the dramatic increase in protein synthesis observed in sea urchin eggs at fertilization. Clam ribonucleotide reductase mRNA provides a well-studied paradigm for masking (Standart et al, 1990). Masked ribonucleotide reductase mRNA can be derepressed in oocyte extracts by severing the 3' UTR from the body of the mRNA, using targeted RNAse H-cleavage (Standart et al, 1990). The activation appears to be independent of poly adenylation, even though the mRNA receives poly A tail as it is activated *in vivo*. These results imply that 3'UTR-bound factors are important and that derepression *in vitro* can be uncoupled from poly A addition. 3' UTR contains sequences that control cytoplasmic polyadenylation (CPES). These CPEs may also mediate repression as in cyclin B1 mRNA before being involved in activation (de Moor and Richter, 1999). CPEB, an RNA-binding protein is required for cytoplasmic polyadenylation and translational activation of dormant mRNAs (Hake and Richter, 1994). Consistent with this, mutants lacking a *Drosophila* CPEB homolog, *orb* fail to activate *oscar* mRNA (Chang et al, 1999), and *xenopus* oocytes injected with anti-CPEB antibodies fail to activate or polyadenylate *c-mos* mRNA (Stebbins-Boaz et al, 1996). CPEB homologs can also cause repression (Minshall et al, 1999). Phosphorylation of serine 174 residue in CPEB by Eg2 kinase is found important for polyadenylation (Mendez et al, 2000). Cytoplasmic polyadenylation also regulates a covalent modification of the 5' cap structure that is, methylation of the first and second ribose moieties that are immediately 3' of the phosphate bridge, that enhances translational activity (Kuge and Richter, 1995).

Repressive role of CPEB involves *maskin* protein. *Xenopus* CPEB interacts with *maskin*, which in turn binds the initiation factor eIF4E. This interaction may preclude the binding of eIF4E with eIF4G and thereby causes repression prior to oocyte maturation (Stebbins-Boaz et al, 1999). *Maskin* protein from *Xenopus* has a

motif that is reminiscent of an eIF4E-binding domain with which eIF4G and eIF4E-BPs interacts (Gingras et al, 1999).

Poly A polymerase (PAP), one of the factors required for cytoplasmic polyadenylation of mRNAs, is multiply phosphorylated by cdc2 and is consequently inactivated (Colgan et al, 1996; 1998). CPSF, cleavage and polyadenylation specificity factor, a heterotetramer (sizes of 160, 100, 70 and 30 kD) is another factor that is essential in clearing pre-mRNA and for polyadenylation (Zhao et al, 1999). CPSF binds AAUAAA and probably facilitates PAP positioning on the end of the pre-mRNA.

The maternal transcripts of several axis-determining genes are translationally dormant in oocytes but are activated soon after fertilization. This coordinate activation often requires cytoplasmic polyadenylation. mRNAs that encode key regulatory proteins for the anterior, terminal and dorsal-ventral patterning systems respectively-*bicoid, torso and toll* (Driever and Nusslein-Volhard. 1988 a, b; Casanova and Struhl, 1989; Gay and Keith, 1992) undergo polyadenylation concomitant with their activation. Unlike *bicoid* mRNA, translational activation of mRNAs encoding *nanos* and *oskar,* two crucial posterior determinants, do not involve detectable change in poly (A) length upon fertilization (Lie and Macdonald, 1999). However, the ultimate effect of *nanos* protein is to control the poly (A) status and translation of *hunch back* mRNA in the posterior (Wreden et al, 1997).

An unusual case is seen in the developmentally regulated *Caenorhabditis elegans* gene *lin-14*. Translation of this mRNA is inhibited by short (22-nucleotides) RNA transcribed from the lin-4 gene that can base pair with the sequences in the 3' UTR of the *lin-14* mRNA (Lee et al, 1993; Ha et al, 1996; Moss et al, 1997). Thus, structural features in mRNA and transacting factors and their covalent modifications (chiefly phosphorylation) play very important roles in the binding of mRNAs to the 43S preinitiation complex to form functional 48S initiation complexes and eventually in the translation of mRNAs.

S6 is one of the 33 proteins of the 40S ribosome which is located near the mRNA/tRNA binding site, at the interface between the small and the large subunit where it has been shown to make direct contact with the 28S rRNA of the larger 60S subunit (Nygard and Nilsson, 1990). In response to mitogens, S6 is phosphorylated when quiescent cells re-enter the cell cycle (Thomas et al, 1979). This phenomenon

has been reported *in vivo*, in the liver during regeneration (Gressner and Wool, 1974) or following fasting and refeeding. S6 phosphorylation is seen generally in response to signals that induce cell growth. S6 phosphorylation is correlated with the synthesis of a number of proteins like eEF1α (Thomas and Thomas, 1986), which belongs to the 5' TOP (oligopyrimidine tract at the 5' transcription start site) mRNA family (Jefferies et al, 1994), without involving the transcription. Phosphorylation sites in this ribosomal protein are mapped to ser-235, ser-236, ser-240, ser-244 and ser-247 which are located at the carboxy terminus of the protein (Krieg et al, 1988). Phosphorylation occurs in an ordered fashion with ser-236 being the first residue phosphorylated followed by ser-235, 240, 244 and 247 residues. A 70 kD kinase called S6K1 phosphorylates the protein (Jeno et al, 1988; Kozma et al, 1989). $p70^{S6K}$ and $p85^{S6K}$ (Banerjee et al, 1990; Reinhard et al, 1992) are two isoforms of S6K1. The amino terminus, rich in acidic residues is important in conferring rapamycin sensitivity to the enzyme (Cheatam et al, 1995; Dennis et al, 1996). The catalytic domain belongs to the protein A, G and C families (Hanks and Hunter, 1995). Recent studies have shown that ribosomal protein production in metazoans is largely controlled at the translational level in part through the activation of S6 Ser/Thr kinase (Jefferies et al, 1997). These findings suggest that S6K1 plays a pivotal role in the process of cell growth.

L18, another ribosomal protein that belongs to 60S subunit, found frequently over expressed in colorectal cancer tissue may potentiate protein synthesis in the transformed cells (Barnard et al, 1993). A recent study has shown that this ribosomal protein inhibits PKR, a double-stranded RNA-dependent eIF2α kinase activity *in vitro* and *in vivo* (Kumar et al, 1999). It competes with dsRNA for binding to the first double-stranded RNA binding motif of PKR enzyme. Inhibition of PKR results in decreased eIF2α phosphorylation and enhanced translation.

The translational apparatus is a target of signal transduction pathways that are activated by mitogens. Many signaling pathways regulate mRNA translation and protein synthesis. The foregoing account gives a brief idea as to how the perturbations in the pathways can result in metabolic dysregulation and disease. The mammalian phosphoinositide kinase related kinase (PIKK) family of proteins is comprised of FKBP-12-rapamycin-associated protein (FRAP) and DNA-PKs all of which contain a C-terminal kinase domain related to the phosphoinositide kinase domain. The PIKK

members are protein kinases and contain a short segment at their extreme C-terminus not present in PI3 or PI4 kinases (Gingras et al, 2001). FRAP functions to regulate the rate of protein translation in response to mitogenic, signals, allowing progression from the G1 to S phase of the cell cycle (Kuruvilla and Schrieber, 1999). FRAP kinase activity promotes the translation of a subset of cellular mRNAs by phosphorylating its targets $p70^{S6K}$ (S6K1 and S6K2) and 4E-BP1 and 4E-BP2. The phosphorylation of $p70^{S6K}$ promotes the phosphorylation of the S6 ribosomal protein leading to an increase in translation (Dennis et al, 1996). *Drosophila* mutant lacking the p70 S6 kinase is only half the size of the wild-type fly, due to smaller cells, but not fewer cells (Montagne et al, 1999). These findings suggest that cell growth (increase in cell mass) not cell proliferation (increase in cell numbers) is affected by the lack of S6 kinase, an activator of protein synthesis. One class of mRNA targets translated by the FRAP signaling pathway is the 5' TOP messages (ribosomal proteins, eEF1α, eEF2 and Poly A-binding protein etc), which contain a 5' terminal oligopyrimidine tract (Loreni et al, 2000). Additionally, FRAP activity increases the translation of mRNAs such as C-myc and cyclin D1 (Grewe et al, 1999). The translation of these classes of mRNAs is inhibited by rapamycin treatment, a potent inhibitor of FRAP activity.

It is not known if enhanced cancer, or the high translation rate in malignant cells is a necessary consequence of the increased proliferation rate. Over expression of eIF4E induces malignant transformation of immortal NIH-3T3 cells (Lazaris-Karatzas et al, 1990) and activates *ras* oncogene (Lazaris-Kartzas et al, 1992). Over expression of 4E-BPs suppresses eIF4E activity and results in partial level of the transformed phenotype. eIF4E is also implicated in apoptosis. Elevated eIF4E levels prevent apoptosis induced by serum deprivation in NIH-3T3 cells (Tan et al, 2000; Polunovsky et al, 1996). It appears that at least some genes encoding parts of the translational machinery are susceptible to regulation by oncogenes such as C-myc, ras and viral oncogenes. For example, eIF4E and eIF2α are over expressed in immortal cells transformed with C-myc (Rosenwald et al, 1993b). The difference in eIF4E levels between normal and breast cancer cells is so large that eIF4E abundance might be suitable as a prognostic tumor marker (Li et al, 1997; Nathan et al, 1999).

Abrogation of eIF2α phosphorylation by over expressing a nonphosphorylatable form such as eIF2α S51A in which the 51 serine residue is replaced by alanine in NIH-3T3 cells, promoted malignancy (Donze et al, 1995). A

recent study using animal model shows that eIF2α phosphorylation couples protein synthesis with glucose sensing, glucose metabolism and insulin production (Scheuner et al, 2001; Harding et al, 2001). These studies are done by engineering PERK-deficient mice (i.e. mice lacking PERK eIF2α kinase) or mice with a mutation in the phosphorylation site ($Ser^{51} \rightarrow Ala$). Both mutant strains had defects in pancreatic β cells, these defects were apparent in ser^{51} mutant embryos, but only became apparent in PERK-deficient animals several weeks after birth. PERK-deficient and ser51 mutant mice exhibited severe but opposing defects in glucose homeostatis. PERK-deficient animals developed marked hyperglycemia (elevated blood glucose) at 4 weeks of age, whereas the eIF2α mutant mice were normal at birth but died of severe hypoglycemia 18 hours later. One of the gluconeogenic enzymes phosphoenol-pyruvate carboxy kinase is found defective in eIF2α mutant mice.

2.3. Regulation of elongation

Under conditions where the cell needs to conserve energy or divert it to other processes, inhibition of elongation would temporarily serve to reduce the amount of energy. This will save the nucleoside triphosphates that can be utilized for other processes (e.g. muscle contraction). Decreased activity of the elongation factors such as eEF1A (or eEF1B) is found to result in increased fidelity. (Carr-Schmid et al, 1999). Several instances are known where elongation of protein synthesis is regulated. For example, insulin has been shown to increase rapidly (with in times > 1hr) the rate of elongation, as measured from ribosomal transit time in several cell types (Chang and Traugh, 1997). Anoxic or hypertonic stress caused a decrease in overall rate of elongation (Binninger and Weber, 1984). Wounding in plants decreases protein synthesis and increases polysome formation suggesting that elongation is blocked (Davies et al, 1987). Glucagon has been shown to cause similar effect in hepatocytes (Ayuso-Parrilla et al, 1976).

In higher eukaryotes, the mRNA encoding eEF1A possesses a 5' terminal tract of pyramidines (5' TOP) which confers on it regulation through the rapamycin-sensitive mTOR (target of rapamycin) pathway. Serum treatment stimulates eEF1A mRNA synthesis (Jefferies et al, 1994). Insulin with in twenty minutes induces the synthesis of eEF2 by activating the translation of its mRNA (Levenson et al, 1989), which also contains a TOP sequence at 5' end, suggesting that its regulation may be

similar to other TOP mRNAs or eEF1A. eEF1A and the α and β-subunits of eEF1B are phosphoproteins, serve as substrates *in vitro* for protein kinase C (Venema et al, 1991a) and all undergo increased phosphorylation when reticulocytes are treated with phorbol esters (Venema et al, 1991b). Phosphorylation of the purified eEF1B complex *in vitro* by PKC increased its activity in guanine nucleotide exchange and the formation of complexes with charged tRNA. Insulin also brings in increased phosphorylation of eEF1A and the α and γ–subunits of eEF1B (Chang and Traugh, 1998). Rapamycin treatment has no effect on the insulin-induced phosphorylation of subunits of eEF1A/B (Chang and Traugh, 1998). Hence regulation of eEF2 plays major role in modulating elongation rates in response to insulin and phosphorylation of eEF1A/B is not involved (Redpath et al, 1996). CK II also phosphorylates the α and β subunits of eEF1 but the actual physiological significance is not however understood (Chen and Traugh 1995; Sheu and Traugh, 1999). eEF1B subunits are also phosphorylated by $p34^{cdc2}$ cell cycle regulated kinase in *Xenopus laevis* (Belle et al, 1989; Mulner-Lorillon et al, 1992, 1994). However the functional significance of this phosphorylation event is not yet understood.

Phosphorylation of eEF2 on Thr 56 inhibits its activity (Nairn and Palfrey 1987; Ryazanov et al, 1988; Mackie et al, 1989; Carlberg et al, 1990) probably by impairing the interaction with ribosomes. A variety of agents and treatment that raise cytoplasmic Ca^{++} levels have been shown to result in increased phosphorylation. These include serum (Palfrey et al, 1987; Redpath et al, 1996), bradykinin in fibroblasts (Palfrey et al, 1987), thrombin and histamine in endothelial cells (Marino et al, 1996). Glutamate treatment of primary neuronal cultures causes rapid and pronounced increase in eEF2 phosphorylation, which correlates with changes in intracellular Ca^{+2} levels and with inhibition of protein synthesis.

eEF2 kinase is not closely related to the main ser/thr tyr kinase super family or to the histidine kinase/mitochondrial kinase family either (Ryazanov et al, 1997). It appears to belong to myosin heavy-chain kinases (Ryazanov et al, 1999). The activity of eEF2 kinase is completely dependent on Ca^{++} ions and calmodulin at neutral pH (Hovland et al, 1999), but following treatment with CAMP-dependent protein kinase (PKA), which phosphorylates eEF2 kinase at multiple sites (Redpath and Proud, 1993), it acquires Ca^{++} CaM-independent activity. ADP ribosylation of eEF2 catalyzed by *Diphtheria and Pseudomonas A* toxins also inhibits its activity (Fendrick

et al, 1992). However to date there has not been convincing physiologic response that might trigger this modification.

The range of biological processes that entails translational control is no doubt expanding rapidly. As our understanding of the molecular details of protein synthesis is increasing, it should be possible to explain better the mechanism and kinetics of translational control. As has been mentioned above, translational control is intimately connected to signal transduction pathways, transformation, apoptosis, growth, development, differentiation and disease.

INTRODUCTION & OBJECTIVES

This laboratory is involved in studying the regulation of translational initiation in plant, mammalian and insect systems. Attention is mainly focused on the a) regulation of protein synthesis chiefly through the phosphorylation of translational initiation factor 2 (eIF2) in wheat germ lysates, a model for translational system for plants; b) expression of human eIF2 subunits (α, β and γ) in insect cells to obtain purified recombinant trimeric eIF2, to understand the structure and function of each of these subunits and to determine the interaction of eIF2 with other proteins, particularly when the alpha-subunit is phosphorylated; c) to understand the importance of eIF2α phosphorylation and the kinase cascade in apoptosis in the ovarian cells of *Spodoptera frugipedra*, a lepidopteran insect which is used as a model system to study the expression of heterologous proteins and apoptosis and d) the translational regulation in ageing rat brain.

Although the basic protein synthesis framework is functionally similar between plants and animal systems, very little is known or understood regarding the translational regulation in plants. For example mammalian eIF2α is phosphorylated in response to several stress conditions such as double-stranded RNA virus infection, amino acid starvation, unfolded protein accumulation, heat shock, heavy metal, oxidative stress, heme-deficiency and other conditions as listed (Sudhakar et al, 1999). A family of eIF2α kinases are cloned and characterized from animal systems as has been reviewed in the chapter 1. Previous studies from Roth's lab has shown a) phosphorylation of plant eIF2α by eukaryotic eIF2α kinases in vitro (Chang et al, 1999), b) that plants encode a dsRNA-dependent kinase (pPKR) (Langland et al, 1995) that is analogous to mammalian pPKR and that addition of high concentrations of poly IC impairs the translation of BMV RNA in wheat germ lysates presumably through the activation of PKR (Langland et al, 1996). The main difficulty, however with their results is that no data were presented to correlate translational inhibition to increased eIF2α phosphorylation or kinase activity in wheat germ lysates treated with dsRNA or purified plant PKR (Langland et al 1996). Infact, we could not obtain dsRNA-mediated translational inhibition in wheat germ lysates (Janaki et al, 1995) like other laboratories (Reijnders et al, 1975; Shaikin et al, 1992). This apparent difficulty in observing dsRNA-stimulated eIF2α phosphorylation has been attributed to the varying levels of a glycosylated protein called p67 that is shown to inhibit PKR-catalyzed eIF2α phosphorylation in plants (Langland et al, 1997; Gil et al 2000b). p67 protein, originally discovered in mammalian systems, has been shown to

inhibit the kinase-catalyzed eIF2α phosphorylation but it does not inhibit the activation or autophosphorylation of the kinase (Datta et al, 1989). Further, wheat germ eIF2 affinity for GDP is only 10-20 fold higher than GTP (Shaikhin et al, 1992). The small difference in the relative binding affinities for GDP and GTP for wheat germ eIF2 and non-identification of eIF2B-like protein in wheat germ so far led to the speculation that the GDP/GTP exchange on eIF2 may occur without eIF2Blike protein and phosphorylation of wheat germ eIF2α may not necessarily regulate protein synthesis in plants (Krishna et al, 1997).

Wheat germ eIF2 appears to be heterotetrameric (Shaikhin et al, 1992) containing 4 polypeptides (p36, p41, p42 and p50), where as, mammalian eIF2 is heterotrimeric and has p38, p51 and p52. Since the p41-42 subunits are not in 1:1 ratio, and are phosphorylated by CKII and also by mammalian eIF2α kinases, we considered them as p41-42 doublet subunit (Janaki et al, 1995). Later studies (Metz and Browning, 1997) have shown that it is equivalent to mammalian eIF2α by sequencing studies. Where as p36 and p50 subunits are found to be equivalent to mammalian eIF2β and γ respectively (Metz and Browning, 1997).

Earlier studies from this laboratory have shown that CKII phosphorylates both the p36 and p41-42 doublet subunits of wheat germ eIF2 (Janaki et al, 1995; Vattem Krishna Ph. D. thesis). Interestingly N-ethylmaleimide (NEM) that is shown to inhibit translation and stimulate eIF2α phosphorylation in translating reticulocyte lysates, is also found to inhibit wheat germ translation and stimulate the phosphorylation of p36 (the β-subunit) and p41-42 (α-subunit) subunits of wheat germ eIF2 in lysates. But, the phosphorylation does not inhibit the dissociation of labeled GDP or exchange of labeled GDP for unlabeled GDP (present in the reaction mixtures) in the preformed labeled eIF2.GDP binary complex in translating lysates (Janaki et al, 1995). These findings thus suggest that NEM-mediated translational inhibition may not be due to phosphorylation of wheat germ eIF2α.

Further experiments from this laboratory have shown that NEM-induced phosphorylation of wheat germ eIF2 and of several other proteins in lysates vary with the translational ability of the lysates. Lysates that are translationally active have

shown higher phosphorylation and addition of NEM-did not make significant difference in these lysates. Lysates that are translationally less active have shown reduced phosphorylation and addition of NEM significantly enhances the phosphorylation of several proteins including eIF2. Heat shocked lysates decrease the phosphorylation of proteins and addition of NEM in such heat-shocked lysates enhances phosphorylation of several proteins (Fig. 2, from Narahari Janaki Ph. D. thesis, 1996 with permission). NEM-induced phosphorylation was analyzed by radio-active labeling and autoradiogram. These studies suggest that NEM activates a kinase(s). It is not known however if it activates a typical eIF2α kinase that can stimulate phosphorylation of serine 51 residue in eIF2α. Unlike NEM, heat treatment of wheat germ lysates decreases phosphorylation of several proteins. Where as in reticulocyte lysates, heat treatment activates HRI kinase activity and stimulates eIF2α phosphorylation (Chen and London, 1995). Further, one of my senior colleagues demonstrated that p41-42 doublet subunit of purified wheat germ eIF2 is phosphorylated by CKII and reticulocyte eIF2α kinases. The phosphopeptide analysis of CKII-mediated phosphorylation is different from eIF2α kinase-mediated phosphorylation (Fig. 3, from Ph. D. thesis of Vattem Krishna, 1997 with permission).

Based on these observations, in the present study we have

a) characterized the NEM-induced eIF2 phosphorylation in wheat germ lysates (Chapter I).

b) Studied the importance of serine 51 phosphorylation on translational inhibition in wheat germ lysates (Chapter II).

Apart from these two major objectives, we have also studied

a) the translational ability of interferon γ-mRNA and mutants in wheat germ lysates and their interaction with wheat germ eIF2 (Chapter III) and

b) identified caspase-like activity in wheat germ lysates that inhibits wheat germ translation (Chaper IV).

Fig. 2. Phosphoprotein profiles of different lysates in the presence of NEM and or heat treatment.

Three batches of wheat germ lysates with different translational abilities (WG lysate I: moderate; WG lysate II: strong; WG lysate III: weak) were used for the experiment. Protein synthesis was carried out in standard 25ml reaction mixtures at 25°C and 40°C (heat treatment) for 10 min, with or with out NEM (1mM) as shown. 10µl aliquots of proten synthesis reactions were then supplemented with 5 µl of Tris-HCl buffer (20 mM, pH 7.8) containing [γ-^{32}P] ATP (10 µCi). The final reaction mixtures containing 2.5 mM Mg^{2+} were incubated at 25°C for 5 min. Aliquots of 7.5 µl were withdrawn and separated by 10% SDS-PAGE. (A) A coomassie-stained gel and (B) an autoradiogram (from Narahari Janaki Ph. D. thesis, 1996 with permission).

Fig. 3. Cleveland partial peptide digestion of phosphorylated wheat germ eIF2 subunits.

The phophorylated bands of wheat germ eIF2 subunits were obtained from a 10% SDS-PAGE. 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. The dried gel pieces containing the phosphorylated subunits were equilibrated and made ready for SV8 protease as described under "Materials and Methods" The protease-treated labeled subunit was then separated on 15% SDS-PAGE An autoradiogram is shown. All lanes represent the SV8 protease-digested radio labeled products.

Lanes. 1, SV8 digest of p41-42 subunit of wheat germ eIF2 phosphorylated by CKII, 2, SV8 digest of p36 subunit of wheat germ eIF2 phophorylated by CKII, 3, SV8 digest of p41-42 subunit of wheat germ eIF2 phosphorylated by HRI (from Ph. D thesis of Vattem Krishna, 1997 with permission)

Fig. 2

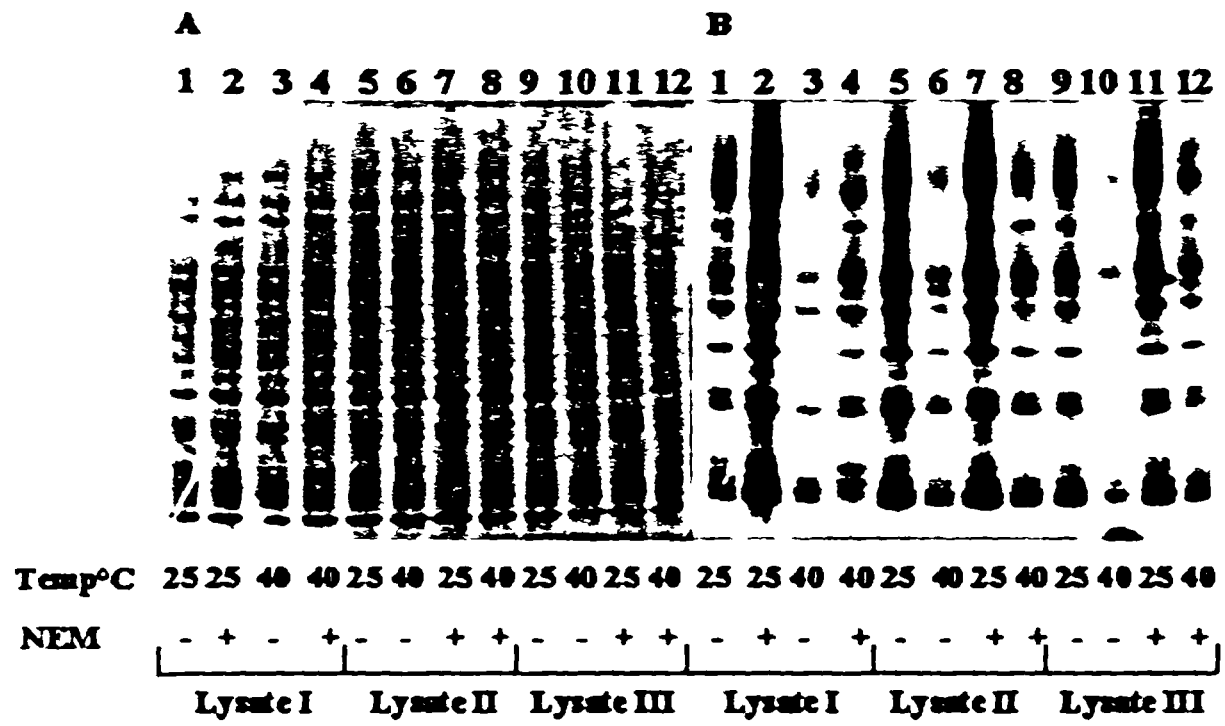

A
B
1 2 3 4 5 6 7 8 9 10 11 12 1 2 3 4 5 6 7 8 9 10 11 12
Temp°C 25 25 40 40 25 40 25 40 25 40 25 40 25 25 40 40 25 40 25 40 25 40 25 40
NEM - + - + - - + + - - + + - + - + - - + + - - + +
Lysate I
Lysate II
Lysate III
Lysate I
Lysate II
Lysate III

Fig. 3

Mr
1
2
3
66-
45-
31-
21-
14-
WG eIF-2 p41-42
CK-II
WG eII-2p36
HRI
WG eIF-2 p41-42

MATERIALS AND METHODS

1. METHODS

1.1.Preparation of wheat germ lysate

1.2.Wheat germ lysate protein synthesis

1.3. Preparation of heme-deficient reticulocyte lysate

1.4. Measuring reticulocyte lysate protein synthesis

1.5. Protein phosphorylation in translating wheat germ and reticulocyte lysates

1.6. Autoradiography/Phosphorimage

1.7. Western blotting

1.8. Protein estimation

1.9. Purification of wheat germ eIF2

1.10. PKR-GST over expression and purification

1.11. *In vitro* transcription

1.12. Electrophoretic mobility shift assay

1.13. *In vitro* caspase assays

1.14. Isolation of rat liver nuclei

1.15. DNA laddering

1.16. Phosphopeptide analysis in one dimension

1.17. Sulfhydryl content estimation

2. CHEMICALS AND MATERIALS

1.1. Preparation of wheat germ lysate

Wheat germ lysate was prepared as described (Roberts and Paterson, 1973; Ramaiah and Davies, 1985). All the necessary glassware and solutions were autoclaved (except Hepes and DTT which were prepared in autoclaved double distilled water). About 20 g of wheat germ was floated on carbon tetrachloride and cyclohexane mixture in the ratio of 2.5:1. The floated wheat germ was vacuum dried (1hr in a hood) before processing further. The floated and dried wheat germ (3 g) was removed with the help of a spatula, powdered in liquid nitrogen and made into a paste with extraction buffer (40 mM Hepes-KOH, pH 7.6, 100 mM KOAc, 1 mM $Mg(OAc)_2$, 2 mM $CaCl_2$ and 1 mM DTT) on ice. Extraction and subsequent steps were done as quickly as possible at 3° C. The paste was spun in a high speed refrigerated centrifuge at 15,000 rpm for 15 min. The top $3/4^{th}$ supernatant was collected and clarified again at 15,000 rpm for 15 min. The above material was then loaded on a 50 x 2.5 cm Sephadex G-25 column that was pre-equilibrated with the column buffer containing 40 mM Hepes-KOH, pH 7.6, 100 mM KOAc, 5 mM $Mg(OAc)_2$. Elution was also carried out using column buffer and 2 ml fractions were collected. Highly turbid fractions were pooled and spun at 15,000 rpm for 20 min. The top $3\,4^{th}$ supernatant was collected and stored as 0.1 ml aliquots in liquid nitrogen.

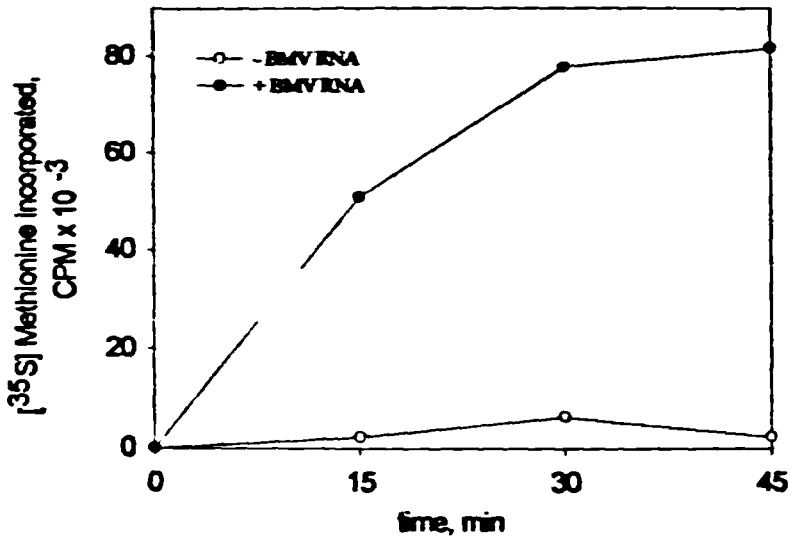
1.2. Wheat germ lysate protein synthesis

Wheat germ lysate protein synthesis was performed as described (Janaki et al 1995) with the following modifications. Since the endogenous message (mRNA) in wheat germ lysate is almost absent, an exogenous message, brome mosaic virus (BMV) mRNA (20 µg/ml) is used as template RNA in all the translation experiments. Typically, the reaction mixture, in a 25 µl volume, contained amino acid mix without methionine (25 µM each), 1 mM ATP, 100 µM GTP, 8 mM creatine phosphate, 30 µg/ml spermine, 14 mM Hepes KOH, pH 7.6, 60 µg/ml of creatine phospho kinase, 0.1mM KOAc, 1 mM $Mg(OAc)_2$ 1 µCi of [^{35}S] methionine (1000 Ci/mmole), 0.5 µg of BMV RNA and 30% wheat germ lysate. 1 mM DTT was added in some experiments as mentioned in the respective figure legends.

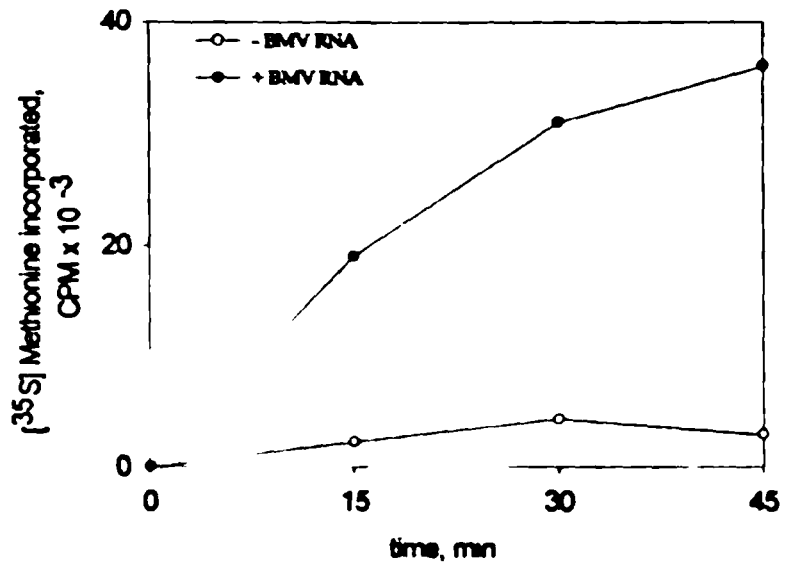
Fig. 4. Protein synthesis in different batches (I, II and III) of wheat germ lysates.

Standard lysate protein synthesis assays (25 µl) were carried out as described in Materials and Methods in the presence of BMV RNA (20 µg/ml) at 25°C for 45 minutes. Protein synthesis was assessed by the incorporation of [^{35}S] methionine into acid precipitable protein in a 5 µl aliquot of the reaction mixtures, taken at different time intervals (15, 30 and 45 min), as described in 'Materials and Methods'. A control reaction without the addition of BMV RNA was also carried out to assess the endogenous lysate protein synthesis.

I


- BMV RNA
+ BMV RNA
[35S] Methionine incorporated, CPM x 10 -3
0
20
40
60
80
0
15
30
45
time, min

II


- BMV RNA
+ BMV RNA
[35S] Methionine incorporated, CPM x 10 -3
0
20
40
0
15
30
45
time, min

III

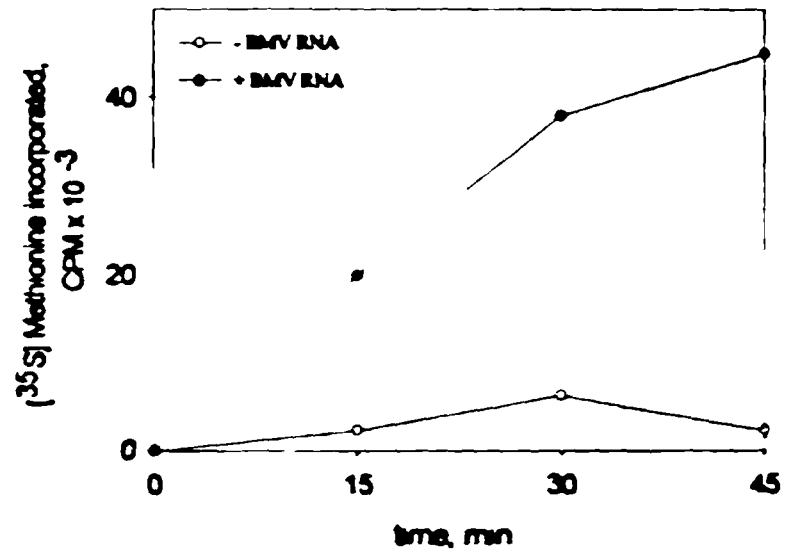
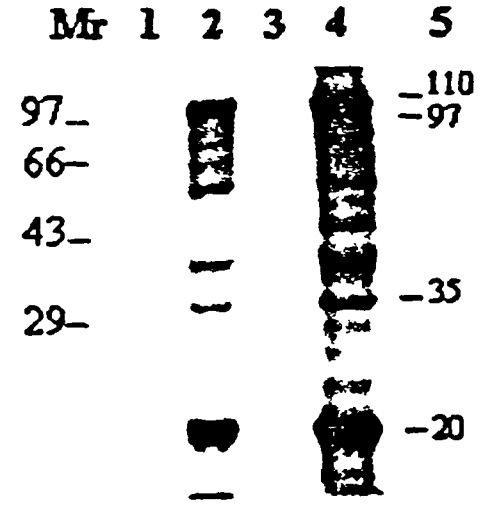

- BMV RNA
+ BMV RNA
[35S] Methionine incorporated, CPM x 10 -3
0
20
40
0
15
30
45
time, min

Fig. 5. Protein products of BMV RNA translated in two different batches of wheat germ lysate.



Standard lysate protein synthesis assays (25 µl) were carried out in the presence of BMV RNA (20 µg/ml) at 25°C for 45 minutes. The reaction was terminated by addition of 100 µl of 1X sample buffer and the labeled proteins were separated on 10% SDS-PAGE. The gels were coomassie stained, dried and analyzed by phosphorimage.

The figure is a phosphorimage showing [^{35}S] labeled protein products of BMV RNA translated in two different batches of wheat germ lysates that reflect the ability of lysates to carry out protein synthesis.

Lanes: Mr, protein molecular weight marker (in kD); 1, wheat germ lysate 1 with out BMV RNA; 2, wheat germ lysate 1 + BMV RNA; 3, wheat germ lysate 2 with out BMV RNA; 4, wheat germ lysate 2 + BMV RNA; 5, Masses of the major protein products of BMV RNA (in kD).

The reactions were incubated at 25° C to carry out protein synthesis. The performance of lysates in 5 µl aliquots was assessed with time by their ability to incorporate labeled methionine into acid precipitable protein. Samples of 5 µl were removed at different time intervals and spotted on a Whatman #1 filter paper disc. Proteins in the samples were precipitated by immersing the filters in 10% cold TCA for 1 hour. Afterwards, the filters were washed with 5% hot TCA (3-5) min. and 5% TCA at room temperature (3-5min) to remove any non-specific radioactivity. Later, the filters were washed with ethanol and acetone and finally air-dried. Radioactivity of the filters was determined in a liquid scintillation counter.

Performance of the various lysates prepared from different batches of wheat germ is shown in Fig. 4. The incorporation of labeled amino acid into protein was not very significant without added BMV RNA. Addition of small amount (0.5µg) of BMV RNA in a 25 µl assay stimulated the incorporation of the labeled amino acid into protein quite significantly. In addition to monitoring the incorporation of [^{35}S] methionine into protein using a scintillation counter (Wallac, Model: 409), the translational products were separated on 10% SDS–PAGE and analyzed by phosphorimager (Molecular Dynamics, Storm 840 Model) as shown in the Fig. 5. The translation of BMV RNA yields prominently four proteins of the following sizes: 110 kD, 97 kD, 35 kD and 20 kD respectively.

1.3. Preparation of heme-deficient reticulocyte lysate

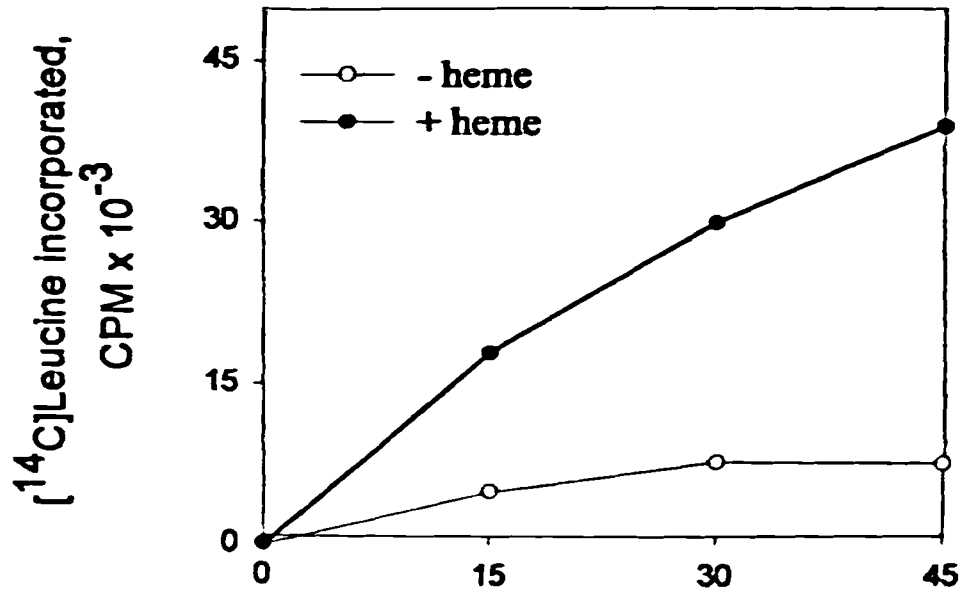
Heme-deficient rabbit reticulocyte lysates that can respond to added hemin *in vitro*, have been prepared from New Zealand white male anemic rabbits as described (Hunt et al., 1972). Rabbits (approximately 2.5 kg in weight) were made anemic by injecting subcutaneously 2 ml of 1 % acetyl phenyl hydrazine consecutively for five 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 2,000 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 KCl and 5 mM $Mg(OAc)_2$) While removing

Fig. 6. Protein synthesis in different batches (I, II and III) of rabbit reticulocyte lysates.

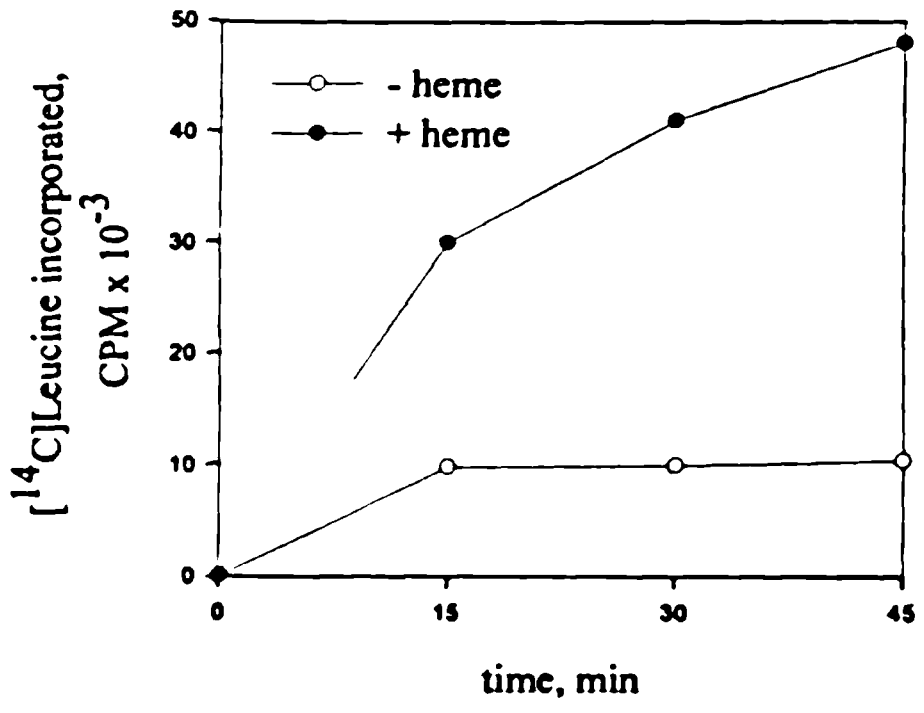
Standard reticulocyte lysate protein synthesis assays (25µl) were carried out at 30°C for 45 minutes as described in 'Materials and Methods' in the presence and absence of 20 µM hemin.

Protein synthesis was assessed by the incorporation of [^{14}C] leucine into acid precipitable protein in a 5µl aliquot of the reaction mixtures, taken at different time intervals (15, 30 and 45 minutes), as described in 'Materials and Methods'.

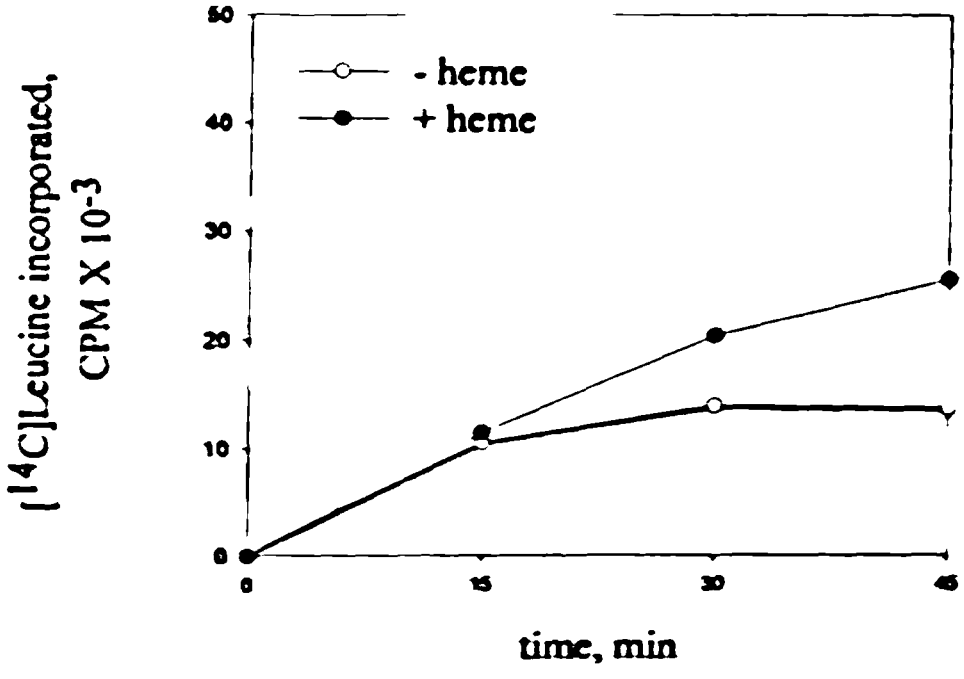
I


- heme
+ heme
[14C]Leucine incorporated, CPM x 10-3
0
15
30
45
time, min

II


- heme
+ heme
[14C]Leucine incorporated, CPM x 10-3
0
10
20
30
40
50
0
15
30
45
time, min

III


- heme
+ heme
[14C]Leucine incorporated, CPM X 10-3
time, min

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, and stored in liquid nitrogen as 0.1 ml aliquots.

1.4. Measuring reticulocyte lysate protein synthesis

Unlike wheat germ lysates, reticulocyte lysates were not gel filtered. A standard incubation mixture for reticulocyte protein synthesis contains the following: 60% reticulocyte lysate, 4 μM creatine phosphate (CP), 250 μg creatine phosphate kinase (CPK), 80 mM KCl, 1 mM $Mg(OAc)_2$, 200 μM GTP, 33 μM amino acid mix without leucine and 33 μM [^{14}C] leucine (340 μCi/mmole) (Ramaiah et al, 1997; Krishnamoorthy et al, 1998). The components of the incubation mixture were mixed together at 0° C and the protein synthesis reaction was carried out at 30° C. The performance of lysates in 5 μl aliquots was assessed with time by their ability to incorporate labeled leucine into acid precipitable protein. Samples of 5 μl were removed at different time intervals and spotted on a Whatman No.1 filter paper discs. The proteins were precipitated by placing the filter discs in ice-cold 10% trichloroacetic acid (TCA) for 1 hour. The filters were then washed with 5% boiling TCA (3-5 min) and 5% TCA at room temperature (3-5 min). The filters were then washed with ethanol and acetone. After wards, these were air dried and transferred to H_2O_2 solution (1:1 diluted with water) for 10 min. to bleach the red color present on the filters. This is 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 air dried filters were read in a liquid scintillation counter to determine the protein synthesizing capacity of the extracts.

Performance of the heme-deficient reticulocyte lysates in the presence and absence of added hemin is shown in Fig 6.

1.5. Protein phosphorylation in translating wheat germ and reticulocyte lysates

Protein synthesis in wheat germ and reticulocyte lysates was carried out (in 25 μl) as described in the earlier section, by supplementing unlabeled amino acids.

Protein synthesizing lysates were treated with various agents (as mentioned in the figure legends) added at the start of the protein synthesis incubation, for 10 min. at 25°C (wheat germ lysates) or at 30°C (reticulocyte lysates). The lysates were then pulsed with 20 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) for 10 minutes. The reaction mixture was concentrated by pH 5.0 precipitation as described (Janaki et al, 1995; Babu and Ramaiah, 1996) by the addition 0.8 ml of NaF and EDTA (50 mM and 5 mM final respectively) followed by the addition of 12 μl of 0.5 M glacial acetic acid. The reaction mixtures were then left on ice for 1 hour for the proteins to precipitate and then centrifuged at 10,000 rpm for 15 minutes. The supernatant obtained after centrifugation was discarded. The pellet was suspended in 50 μl of 1X SDS – sample buffer (62.5 mM Tris-HCl, pH 6.8, 40 % glycerol, 5 % SDS, β-mercaptoethanol and bromophenol blue of 0.1 %) and heated in boiling water bath for 3 min. Proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gels as described (Ramaiah et al., 1997) and the phosphoproteins were analyzed by autoradiography or phosphorimage. Details and modifications (if any) are mentioned in the figure legends.

1.6. Autoradiography

The labeled proteins were separated on SDS-PAGE. The gel was then dried in a bio-rad gel drier equipment and the dried gel was exposed to an X- ray film (Kodak) at –70°C. Alternatively, the labeled proteins were separated on SDS-PAGE and transferred to nitrocellulose membrane. The nitrocellulose membrane was then exposed to X- ray film 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 manufacturer's instructions. Alternatively, phosphorimager is used to scan the labeled dried gels or the dried nitrocellulose membranes.

1.7. Western blotting

After separation of proteins on SDS-PAGE, the proteins were transferred eletrophoretically on to nitrocellulose membrane. Transfer of proteins was carried at 70 volts for 4hrs at 4°C in transfer buffer (25 mM Tris buffer and 195 mM Glycine in 40% methanol) Afterwards, the membrane was 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 membrane, free of proteins were blocked with TBST (Tris-HCl, pH 8.0, 150 mM NaCl and 0.05% Tween-20) containing 5% milk powder for 1 hr at room temperature. The blocking solution was decanted and the membrane was rinsed once with TBST solution. The membrane was then incubated with a primary antibody for overnight at 4°C with gentle shaking. The nitrocellulose membrane was washed with TBST for three times (x 10 min) to remove unbound antibodies. The nitrocellulose membrane was then incubated with alkaline phosphatase conjugated secondary antibody. Incubation was done for 1hr at room temperature. Primary and secondary antibodies were stored for reuse. The nitrocellulose membrane was washed with TBST for three times (x 10 min) to remove unbound secondary antibodies. Then membrane was then treated with the color developing solution (66µl of NBT, 33µl 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 bands and the blot was thoroughly washed with water, properly dried and scanned.

1.8. Protein Estimation

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

1.9. Preparation of wheat germ eIF2

Wheat germ eIF2 was prepared as described earlier by Lax et al , (1986) with some minor modification to enhance the purity of the preparation. All the steps in the procedure were carried out at 4° C unless otherwise indicated. The scheme of eIF2 purification from wheat germ is shown in Fig. 7 and various steps in the purification procedure are as follows.

Preparation of post-ribosomal supernatant 500 g of wheat germ was divided into four batches, ground into a fine powder in liquid nitrogen and mixed with buffer A containing 20 mM Hepes-KOH, pH 7 6, 120 mM KCl, 1 mM $Mg(OAc)_2$, 2 mM $CaCl_2$, 6 mM β-mercaptoethanol and with protease inhibitors like soya bean trypsin inhibitor (0.1 mg ml) and PMSF (0 5 mM). For every gram of wheat germ 1.2 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 spatula and top 3/4th content was drawn out and passed through 1litre G-25 column. The protein elute after the void volume was collected and spun at 15000 rpm for 20 min. The 15 K supernatant was centrifuged at 40 K rpm for 4.5 hr in a Ti70 beckman rotor. The supernatant thus obtained is referred as post-ribosomal supernatant (PRS).

Ammonium sulphate fractionation of the post-ribosomal supernatant (PRS): The entire PRS (1lit) 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 2 hrs at 4°C and centrifuged at 12 K rpm for 30 min. The 12 K supernatant was brought to 70% saturation by the gradual addition of 200 g of ammonium sulphate. The contents were stirred and centrifuged at 12 K rpm again. The 12 K pellet obtained in this step was resuspended in about 50 ml of buffer B containing 40 mM KCl (buffer B containing 20 mM Tris-HCl, pH 7.6, 1 mM DTT, 0.1 mM EDTA and 10% glycerol). The suspension was then dialyzed against 100 volumes of buffer B in 40 mM KCl and clarified by centrifugation at 10 K rpm for 10 min prior to storage.

Separation of 40-70% ammonium sulphate fraction of PRS on DEAE – 52: The 40-70% ammonium sulphate fraction of PRS , about100 ml containing 12 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 above buffer B. The column was washed with same buffer until the absorbance 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 KCl 10 ml fractions, collected and those fractions whose absorbance was 0 4 and above were pooled and concentrated by 0-80% ammonium sulphate cut

Purification of eIF2 on phosphocellulose and CM sephadex C–50 column

The 120 mM KCl concentrated fraction of DEAE-52 was fractionated and concentrated by 0-50% and 50-80% ammonium sulphate. The fractions were dialyzed with buffer B containing 100 mM KCl The dialyzed fraction of 50-80% ammonium sulphate cut (25 ml containing 1 0 g of total protein) was applied to a 50 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 KCl. 3 ml

SCHEME FOR WHEAT GERM eIF2 PURIFICATION

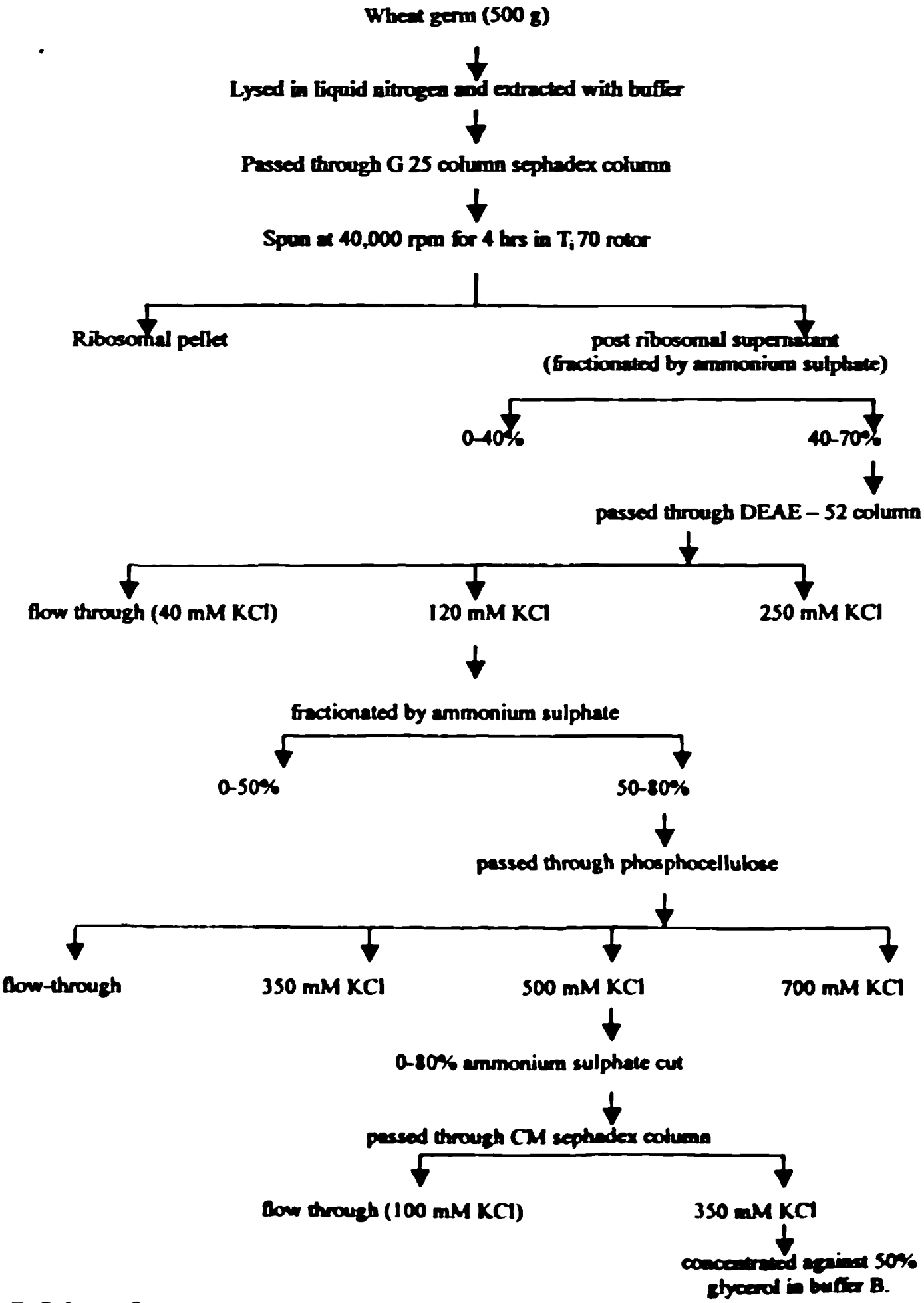


Fig. 7. Scheme for wheat germ eIF2 purification.

eIF2 was purified from post ribosomal supernatant by ion-exchange chromatography as described in "Materials and Methods".

Fig. 8A. Purification profile of wheat germ eIF2.

Different fractions that were obtained during the purification of wheat germ eIF2 were separated on 10% SDS-PAGE as described in 'Materials and Methods'. The figure is a coomassie stained gel.

Lanes: Mr, molecular weight marker (kD); 1, Post ribosomal supernatant (PRS); 2, PRS 40-70% ammonium sulphate cut; 3, Diethyl amino ethyl cellulose (DEAE), 120 mM KCl elute; 4, 50-80% ammonium sulphate cut of DEAE-120; 5, Phosphocellulose (P11), 500 mM KCl elute; 6, CM sephadex, 350 mM elute.

Fig. 8B. Identification of purified wheat germ eIF2 by a polyclonal antibody.

Wheat germ eIF2, eluted in the 350 M KCl fraction of CM sephadex, was separated on 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was probed with a polyclonal anti wheat germ eIF2α antibody raised by Vattem Krishna (Ph.D. thesis, 1997) from this laboratory. The figure is a western blot.

Lanes: Mr, Molecular weight marker (kD), 1, purified WG eIF2 CM sephadex, 350mM KCl elute (1μg), 2, purified WG eIF2 - CM sephadex, 350mM KCl elute (2μg)

Fig. 8A

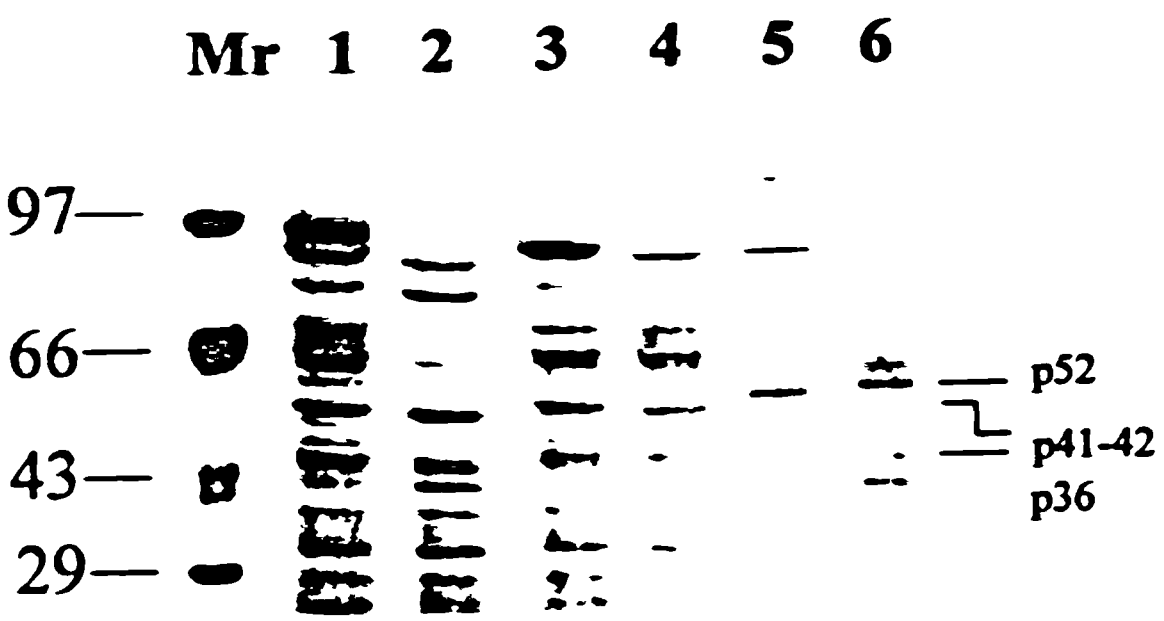

Mr
1
2
3
4
5
6
97—
66—
43—
29—
p52
p41-42
p36

Fig. 8B

Mr
1
2
97 –
66 –
43 –
28 –
WG eIF2α (p41-42)
WG eIF2β (p36)

fractions were collected, pooled and concentrated by 0-80% ammonium sulphate fractionation.

The fractions were dialyzed against buffer B containing 100 mM KCl. The concentrated 500 mM KCl elute of phosphocellulose column (3 ml containing 6 mg of protein) was further chromatographed on a CM sephadex column. The column was pre-equilibrated with 100 mM KCl in buffer B. After washing the column with 100 mM KCl, the bound proteins were eluted with 350 mM KCl. The eIF2 fractions of the CM sephadex column were dialyzed against buffer B containing 50 mM KCl and concentrated against buffer B containing 50% glycerol. Purification profile of different fractions is shown in Fig. 8, Panel A.

The purified eIF2 was tested by

1) a polyclonal antibody that was raised in this laboratory (Vattem Krisha Ph. D. thesis, 1996) as shown in Fig 8, Panel B.
2) the protein was further characterized by its ability to serve as a substrate for eIF2α kinases. Phosphorylated form of WG eIF2α was identified by an auto radiogram or by a phosphospecific antibody that recognizes specifically serine 51 phosphorylation in eIF2α.

1.10. Recombinant mutant mammalian PKR-GST over expression and purification

Competent cell preparation A primary culture of *E coli* BL-21 cells was prepared by inoculating a single colony into 3 ml of LB broth, pH 7.5 (for 1 lit : 10 g NaCl, 5 g yeast extract and 10 g bactotryptone) and incubated at 37° C for 10-12 hrs 1ml of primary culture was inoculated into 100 ml of LB and incubated at 37° C in an orbital shaker at 300 rpm for 2 hrs. Turbidity was measured using spectrophotometer and an O D of 0 4 to 0 6 at 595 nm indicated the growth to be in log phase. Then the culture was harvested by centrifuging at 4000 rpm for 10 min. and the pellet was suspended in 40 ml of chilled 0 1 M $MgCl_2$ and spun at 4000 rpm for 10 min. The pellet was resuspended in 40 ml of 0 1 M $CaCl_2$ and incubated on ice for 30 min and was then spun at 4000 rpm for 10 min. The pellet was resuspended in 10 ml of chilled 0 1M $CaCl_2$ and incubated on ice for 60 min. To this, chilled glycerol 15°• v v was added and incubated on ice overnight in cold room. It was aliquoted and stored at – 70°C.

Fig. 9. Purification of mammalian recombinant PKR-GST fusion protein.

PKR-GST, fusion protein was over expressed in BL21 *E. coli* cells supplemented with IPTG as described in 'Materials and Methods'. The cells were harvested, sonicated and the crude lysate was passed through glutathione sepharose 4B column. The bound protein (PKR-GST) was eluted with 10 mM GSH in 20 mM Tris-HCl, pH 8.0.

Panel A: Coomassie stained gel

Panel B: Western blot probed with anti-PKR monoclonal antibody, which gives single band of 97 kD indicating PKR-GST fusion protein.

Lanes:Mr, protein molecular weight marker (kD); 1, crude lysate of BL21 *E. coli* cells supplemented with IPTG (~10 μg); 2, protein eluted with buffer containing 10 μM GSH on Glutathione sepharose 4B column.

Fig. 10. Phosphorylation of wheat germ and reticulocyte eIF2 in the presence of purified PKR-GST protein.

Wheat germ or reticulocyte eIF2 (~ 500 ng) was phosphorylated by the addition of mammalian recombinant PKR-GST (~ 40ng) (purified as described in legend of Fig 18) in the absence or presence of poly IC, in a reaction mixture containing 20 mM Tris-HCl (pH 7 8), 2 5 mM Magnesium acetate, 1μM DTT, 80 mM KCl and 50 μM ATP at 25°C f r 10 minutes. Reactions were separated on 10% SDS-PAGE and analyzed by western blotting using a phosphospecific anti-eIF2α antibody as described in 'Materials and Methods' The figure is a western blot.

Lanes. 1, wheat germ eIF2 without added PKR-GST, 2, wheat germ eIF2 + PKR-GST, 3, wheat germ eIF2 + PKR-GST + poly IC 0 5 μg ml, 4, wheat germ eIF2 + PKR-GST + poly IC 1 0 μg ml; 5 wheat germ eIF2 + PKR-GST + poly IC 2.5 μg/ml; 6, reticulocyte eIF2 without added PKR-GST, 7, [illegible] eIF2 + PKR-GST, 8 [illegible] eIF2 + PKR-GST + poly IC 0.25 μg ml.

Panel A **Fig. 9** **Panel B**

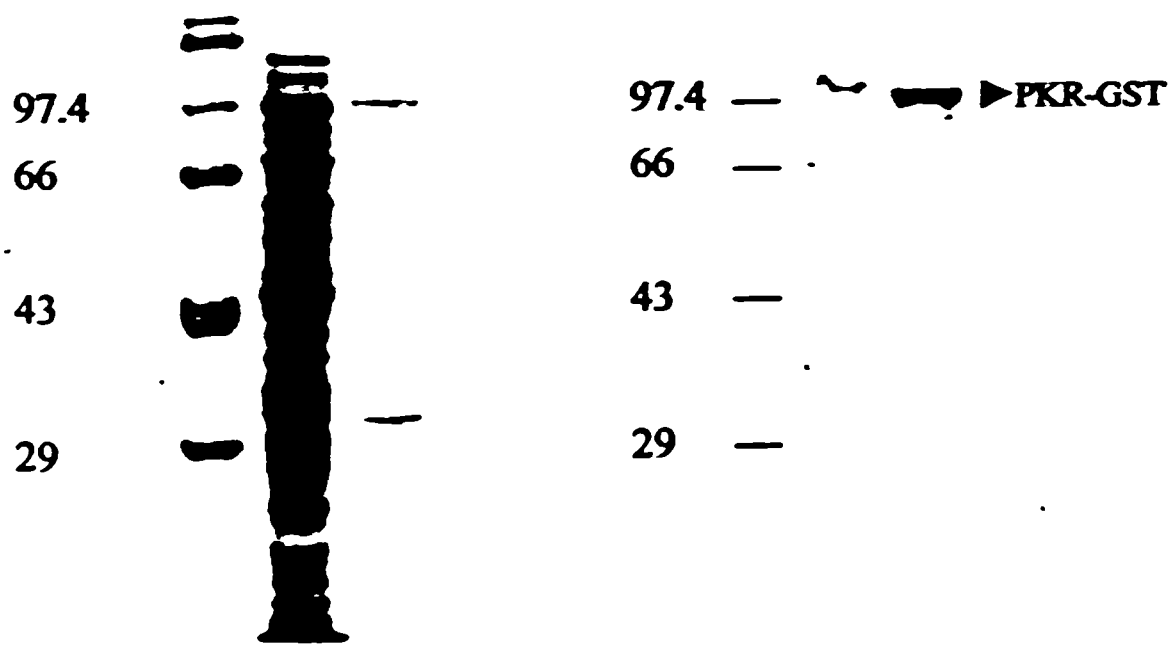
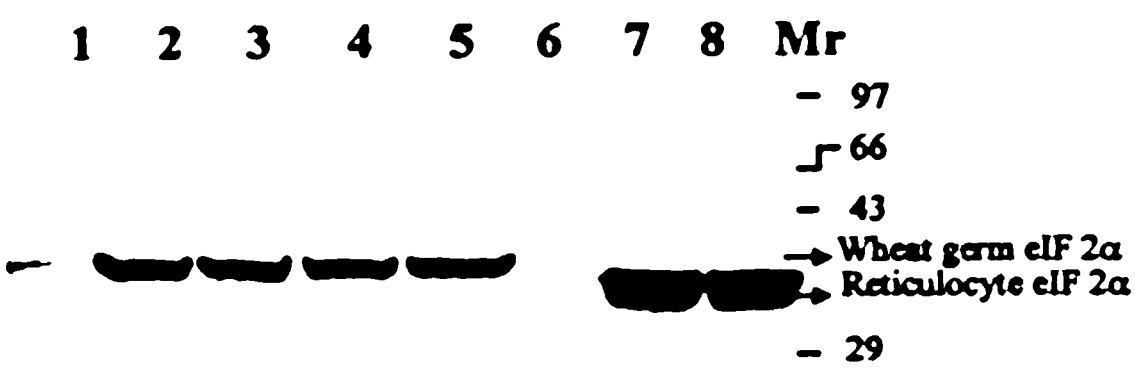


Fig. 10



Transformation: The plasmid DNA (10 ng) was added to *E. coli* BL21 (DE3) competent cells (100 µl) and incubated on ice for 30 min followed by heat shock which was given for 45 sec at 42°C and transferred immediately on to ice. To the cells, 500 µl of L.B.broth containing ampicillin (100 µg /ml) was added and incubated at 37°C in an orbital shaker at 150 rpm for 45 min. The suspension was later spun at 4000 rpm for 1-2 min. Most of the supernatant was discarded and the cell pellet was suspended in 50 µl of supernatant. The cell suspension was added to LB agar plate containing ampicillin (100 µg/ml) and was spread evenly by a spreader. The plates were then incubated at 37°C for 10-12 hrs.

Over expression and purification of PKR-GST: 5ml culture of LB containing ampicillin (100 µg/ml) was inoculated with transformed *E. coli* BL21 (DE3) cells for overnight growth at 37°C. Next day it was diluted to 50 ml with fresh LB containing ampicillin in 250 ml flask. Culture was grown to 0.8 OD at A 600 nm. Then IPTG was added to 2mM and incubated for 3-4 hr at 37°C. The culture was harvested by centrifuging at 6,500 rpm at 4°C for 15 min. The pellet was resuspended in NETN (150 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0 and 1% NP-40) with protease inhibitors PMSF (0.5 mM and pepstatin 1 µg/ml). Pellet from the 50 ml culture was resuspended in 1.6 ml of NETN buffer and sonicated 4 x 20 s on ice. Lysate was centrifuged at 4°C for 15 min and the supernatant was filtered through 0.45 µm filter. The filtered sample was loaded on GST column, which was prewashed with NETN. After loading the filtered lysate extract, the column was washed with NETN until the O.D came down to 0.01 and the PKR-GST was eluted with Tris-HCl, pH 8.0 containing 5 mM reduced glutathione (Fig. 9, Panel A). Purified PKR-GST was subjected to 4hr dialysis to eliminate the reduced glutathione.

The purified mutant recombinant PKR is identified using anti-PKR monoclonal antibody (Fig 9, Panel B) The PKR-GST does not require polyIC for its activation as suggested by Prof. Bryan G Williams (Personal communication) Accordingly, here we observed that recombinant PKR-GST (as shown in Fig 10) phosphorylates wheat germ eIF2 in the absence of added polyIC (lane 2) and also in the presence of increasing concentrations of polyIC (lanes 3, 4 and 5) without any significant difference as analyzed by a phosphospecific anti-eIF2α antibody Similarly, the kinase phosphorylates also purified reticulocyte eIF2 (lane 7) and the

presence of polyIC does not make any difference (lane 8). Absence of added kinase does not result in any significant phosphorylation of purified wheat germ eIF2 (lane 1) or reticulocyte eIF2 (lane 6).

1.11. *In vitro* transcription

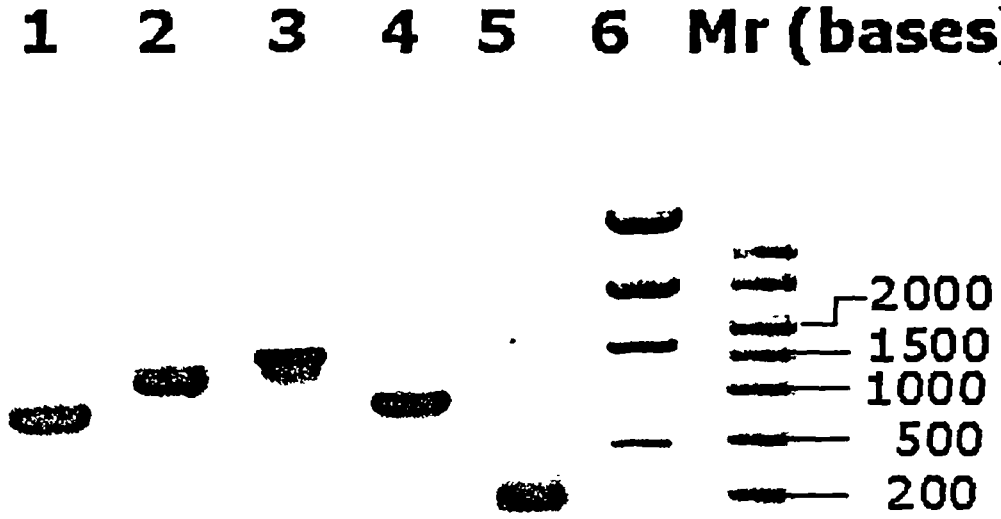
In vitro transcription kit was (Promega Corporation, USA) utilized to transcribe the above constructs to synthesize the respective chimaeric mRNAs as shown in Fig. 11. Standard 100 µl assay mixtures consists of 500 ng of DNA, 20 µl of 5X Transcription buffer (Promega), 10 mM DTT, 0.5 mM rATP, 0.5 mM rUTP, 0.5 mM rGTP, 0.5 mM rCTP, 2µl of RNASIN (RNAse inhibitor, Promega) and 3 µl of T7 RNA Polymerase or SP 6 RNA Polymerase. The final volume was adjusted with nuclease-free water. The reaction mixtures were incubated for 1hr at 37°C. To the reaction mixture, further 3µl of T7 RNA Polymerase or SP6 RNA Polymerase was added followed by 1hr incubation at 37° C. Then 10 µl of Dnase (1unit/µl) was added and incubated 20 min at 37° C. The reaction mixture was then passed through 1ml of G-50 sephadex column that was pre-equilibrated with TE buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl and 1 mM EDTA). To the filtrate, 10 µl of 10 M ammonium acetate and 1ml of absolute ethanol were added and incubated for 20 min. The reaction mixture was centrifuged at 14 K rpm for 30 min at 4° C. To the pellet 500 µl of 70% ethanol was added followed by centrifugation at 14 K rpm for 10 min at 4°C. The pellet was resuspended in 30 µl of DEPC treated redistilled water.

Preparation of ^{32}P-RNA 203γ IFN: Uniformly labeled T7 transcripts were synthesized using [α-^{32}P] UTP (0.8 Ci/mmol) and 10 nM UTP for labeling (as shown in Fig. 11, lane 5).

1.12. Electrophoretic Mobility Shift Assay

Uniformly labeled T7 5'-terminal 203 nt IFN-γ mRNA transcript was synthesized using [α-^{32}P] UTP (0.8 Ci/mmol) and 10 nM UTP for labeling. Wheat eIF2 was purified as described in the earlier section. Complex formation between

Fig. 11. Synthesis of chimaeric RNAs and labeled IFN-γ mRNA, 203 nt by *in vitro* transcription.



RNA products are transcribed *in vitro* using sp6 RNA polymerase (for chimeric mRNAs) and T7 RNA polymerase (for IFN-γ labeled mRNA, 203 nt), separated on 1.3% agarose gel and viewed under UV as described in 'Materials and Methods'.

Lanes :

1, pHST 106 RNA (Alpha mosaic virus (AMV) leader and rabbit α-globin mRNA);

2, pHST 215 (Alpha mosaic virus leader sequence with Interleukin1 mRNA);

3, pHST 301 (plant α amylase leader and α amylase RNA);

4, pHST 400 (AMV viral leader and AMV RNA);

5, IFN-γ labeled RNA, 203nt;

6, RNA mass ladder (Novogen Co.);

Mr, RNA marker (Novogen Co.).

wheat germ eIF2 and labeled 203nt mRNA was assayed by elctrophoretic mobility shift assay. The reaction mixture (20µl) contained [^{32}P] mRNA (2.2 X 10^6 c.p.m./pmol), unlabeled competitor RNA as shown and wheat germ eIF2 in binding buffer (50 mM KCl, 20 mM Tris-HCl, pH 7.8, 2 mM Mg acetate, 1 mM dithiothreitol). After incubation for 15 min at 30° C followed by incubation for 10 min on ice, samples were run for 5 h at 100 V and 4° C through 4% native polyacrylamide gels in 90 mM boric acid, 25 mM EDTA, 90 mM Tris base. The native gel was dried and subjected to autoradiography.

1.13. *In vitro* caspase assay

100 µl of wheat germ cell free system was diluted to 1 ml with 20 mM Hepes-KOH, pH 7.5. To this 4 µl of 50 mM solution of Ac-DEVD-AFC was added to obtain a final concentration of 200 µM and incubated in a quartz 1 cm square cell. Fluorescence was measured in a spectrofluorometer (excitation: 400 nm, and emission: 505 nm) as described in Bhuyan et al 2001.

1.14. Isolation of rat liver nuclei

Preparation and purification interphase mouse liver nuclei was carried out as described (Blobel and Potter, 1966). Rat liver was obtained from the male albino rats. The livers were removed quickly and chilled immediately in several volumes of ice-cold 0.25 M sucrose in TKM (50 mM Tris-HCl, pH 7.5, 25 mM KCl and 5 mM $MgCl_2$). All subsequent operations were performed at temperatures near 0-3°C. Livers were blotted and minced with scissors in two volumes of ice-cold 25mM sucrose in TKM. They were homogenized in a Potter-Elehjem homogenizer with a motor-driven Teflon pestle (clearance 0.025 cm) with 10 to 15 strokes at 1700 rev/min. The homogenate was centrifuged at 2000 rpm for 10 min to obtain loose pellet consisting of crude nuclei. Crude nuclei were then resuspended in TKM buffer.

1.0 ml of crude nuclei, prepared as described above, was pipetted into polyallomer tube of the SW 28 rotor; 2.0 ml of 2.3M sucrose in TKM was then added by means of a syringe with a 13-guage needle (rather than with a pipette, because of the high viscosity of the solution) and thoroughly mixed with the 250 mM sucrose homogenate by inversion. Sucrose concentration of the homogenate was thereby

raised to approximately 1.62 M, the density of which is just sufficient to float mitochondria and rough endoplasmic reticulum. The mixture was then under laid by 1.0 ml 2.3 M sucrose in TKM with a syringe and 13-guage needle: tip of the needle was placed at the bottom of the tube and the heavy sucrose solution was introduced, forcing the lighter homogenate upward. After centrifugation for 45 minutes at 28,000 rpm in a Beckman SW 28 rotor at 0° to 3°C, the supernatant was removed. Material adhering to the wall of the tube was removed with a spatula and added to the supernatant; the tube wall was then wiped dry with tissue paper wrapped around a spatula. The white nuclear pellet was taken up in TKM buffer. The nuclei were suspended in nuclei storage buffer (10 mM PIPES, 80 mM KCl, 20 mM NaCl, 250 mM sucrose, 5 mM EGTA, 0.5 mM spermidine, 0.2 mM spermine, 50% glycerol) stored in a freezer at -70°C.

1.15. DNA laddering

About 1 x 10^5 rat liver nuclei were introduced into 50 µl of wheat germ cell-free extract and the system was incubated at 25°C for 8 to 10 hrs. Later, the samples were suspended in 10 volumes of buffer D (100 mM Tris-HCl, pH 8.0, 5mM EDTA, 0.2 mM NaCl, 0.4% SDS, 0.2 mg/ml proteinase K), incubated overnight at 37°C, then extracted with 1:1 phenol:chloroform, and precipitated by 0.6 volumes of isopropanol and followed by precipitation by 2 volumes of ethanol. DNA extracts were electrophoresed in 2% agarose gels in TBE (0.089 M Tris-borate, 0.089 M boric acid and 0.002 M EDTA). DNA was visualized by ethidium bromide staining (Zhao et al, 2001).

1.16. Phosphopeptide mapping in one dimension

Peptide mapping in one dimension by limited proteolysis in SDS-polyacrylamide gels was done as described (Cleveland et al, 1976). p41-42 sub unit of WG eIF2 were phosphorylated by CKII *in vitro* or in NEM-treated wheat germ lysate. The bands corresponding to the phosphorylated p41-42 subunits of wheat germ eIF2 were cut out of the dried gel through the X-ray film. The dried gel pieces containing the phsophorylated subunits were processed for SV8 (200 ng/lane) protease digestion. 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 contaminants like gel drying filter paper were removed.

The above samples (gel slice containing labeled phosphoproteins) were incubated in the wells of a 15% gel (1.5 mm thick with 3-5 cm long stacking gels) for 15 min in the presence of SV8 protease (200 ng) and gel slice overlaying solution that contains 20% glycerol.

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, 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 subjected to autoradiography.

1.17. Determination of protein sulfhydryls in wheat germ lysates

Protein sulfhydryls were determined in WG lysates obtained from different batches having low to high translational abilities, heat- treated lysates and lysates treated with different SH- reactive agents such as DTT, GSH, GSSH and diamide using 5, 5' –Dithiobis-2-nitrobenzoic acid (DTNB) as described (Habeeb, 1972). The -SH content was estimated in a total reaction mixture of 50 µl containing 45 µl of WGL (3mg/ml) and 5 ml of the respective -SH reactive agent or redistilled water as indicated in the legend to Fig. The mixtures were incubated for 20 min at 25° C or at 40° C wherever indicated. To this reaction, 33 µl of DTNB solution containing 2% SDS, 0.08 M sodium phosphate buffer, pH 8.0 and 0.5 mg/ml EDTA were added. After 15 minutes, the sample was read at 410 nm against lysate mixture in SDS to give apparent absorbance. A reagent blank was subtracted from the apparent absorbance to give the net absorbance. For calculation of sulfhydryl content, the net absorbance is employed with a molar absorptivity value of 13, 600 M^{-1} cm^{-1}.

2. CHEMICALS AND MATERIALS

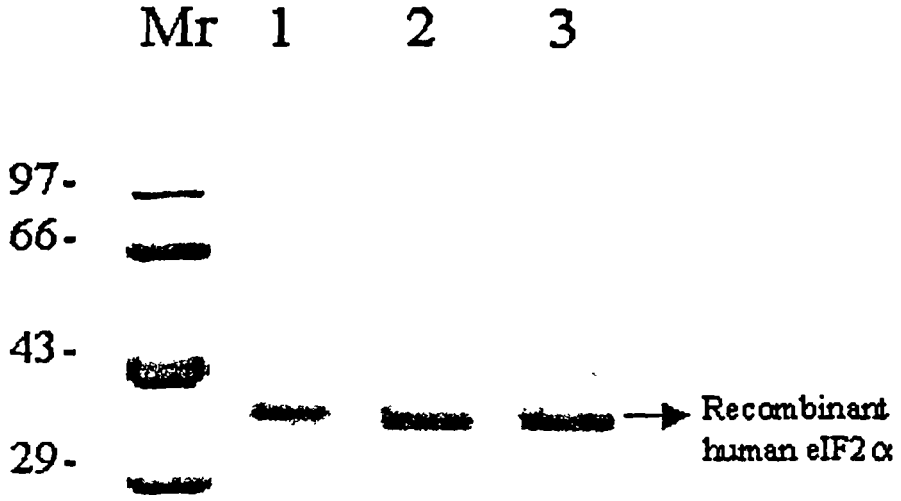
ATP, CP, CPK, DTT, poly IC, NEM, SV 8 protease, Casein Kinase II, CM Sephadex C-50 were obtained from Sigma, USA. [^{14}C] Leucine (340 mCi/mmol) was obtained from Amersham Pharmacia, UK. [γ-^{32}P] ATP (3500 Ci/mmol) and [^{35}S] Methionine (1100 Ci/mmol) were obtained from Jonaki Center, BRIT, Hyderabad and BRIT, Mumbai, India respectively. Ion exchange resins DE-52 (DEAE cellulose), P-11 (phosphocellulose) and nitrocellulose membrane were obtained from Whatmann Company, UK. Phosphospecific antibody for eIF2α was obtained from Research Genetics, Huntsville, AL. Hepes buffer, BMV RNA, RNAse inhibitor, *In vitro* transcription kit, western blot color developing agents NBT and BCIP, secondary antibodies were obtained from Promega Corporation, USA. X-ray films were purchased from Kodak, India. Acrylamide, bis-acrylamide and SDS were purchased from Boehringer and Mannheim, Germany. Ac-DEVD-AFC and Ac-DEVD-CHO were obtained from Pharmingen, USA. And all other chemicals required for routine work, were purchased from Sigma, USA.

SP6 transcripts of plant mRNAs as well as chimaeric forms of mammalian and plant mRNA were prepared using DNA vectors provided by Dr. Lee Gehrke at MIT. These are (construct number / 5'-UTR leader / coding region); 106/ AMV / rabbit-α-globin; 416 / rabbit-α-globin / AMV; 215 / AMV / IL-1l 301 / α-amylase / α-amylase; 302 / AMV/ α-amylase; 400 /AMV/ AMV (AMV: alfalfa mosaic virus; IL-1, human interleukin-1).

WG eIF2 polyclonal antibody that was raised in this laboratory (Krishna Ph. D. thesis, 1996) was used in this study. pGEX-PKR construct was obtained from Prof. Bryan Williams, Cleveland Clinic Foundation, USA. Chimaeric mRNAs were obtained from Prof. Raymond Kaempfer, Hebrew University, Israel. Purified PKR, K3L were obtained from Dr. Krishna, Prof. R. Wek laboratory, Indiana University School of Medicine, Indianapolis, IN. Recombinant human eIF2α and mutants (as shown in Fig.12) that were over expressed in this laboratory (Sudhakar Ph. D. thesis, 2000) were also used in the present study. Wheat germ was obtained from Sigma, and also locally from Krishna Mills, Bangalore, India. New Zealand white male rabbits were procured locally from National Institute of Nutrition, Hyderabad.

Fig. 12. Recombinant human eIF2α wt, 51A and 51D.

Panel A



Panel B

The recombinant human eIF2α wt, 51A and 51D proteins that are used in the present study were over expressed earlier in this laboratory using baculovirus expression system in Sf9 cells and partially purified using Sephacryl S-300 and DE-52 column (Sudhakar et al, 2000).

Panel A is a coomassie stained gel and **Panel B** is a western blot probed with anti-eIF2α antibody.

Lanes: Mr, protein molecular weight marker, kD; 1, ~2 μg of recombinant human eIF2α wild type (wt); 2, ~2 μg of recombinant human eIF2α 51A; 3, ~2 μg of recombinant human eIF2α 51D.

CHAPTER I

TRANSLATION AND PHOSPHORYLATION OF WHEAT GERM (WG) LYATE: PHOSPHORYLATION OF WG INITIATION FACTOR 2 (eIF2) BY CASEIN KINASE II AND IN N-EHYLMALEIMIDE- TREATED LYSATES.

1. Abstract

2. Results

2.1. Translation in WG Lysates: Effect of Heat Shock and –SH Reactive Agents.

2.2. Phosphorylation of WG eIF2 in NEM, NEM and CK-II, and, Heat-Treated Lysates.

2.3. Effect of –SH Reactive Agents on WG eIF2α Phosphorylation in Control and Heat-treated Lysates and Estimation of Protein-SH content.

2.4. Phosphopeptide analysis of WG eIF2

3. Discussion

1. Abstract

Previously, we observed that N-Ethylmaleimide (NEM), a thiol-alkylating agent was found to stimulate the phosphorylation of several proteins in translating wheat germ (WG) lysates including the phosphorylation of α, the p41-42 doublet subunit, and β, the p36 subunit of the WG initiation factor 2 (eIF2). Further, it was shown that NEM increases phosphorylation of several proteins significantly in lysates, which are moderate or low in their translation than in optimally active lysates. In the present study, we show that heat treatment that stimulates oxidation of protein sulfhydryls, decreases the translation and phosphorylation ability of WG lysates. The decrease in phosphorylation, but not translation, that occurs in heat-treated lysates, is prevented very efficiently by NEM, and partially by reducing agents such as DTT and GSH. DTT however prevents completely the loss in sulfhydryl content of heat-treated WG lysates and does not at all prevent heat-induced inhibition in translation. In contrast, DTT prevents completely the diamide-induced translational inhibition and also the loss in sulfhydryl content. These findings therefore suggest that in addition to the maintenance of sulfhydryl groups, heat labile proteins and their interaction with other proteins play an important role in the overall translation and phosphorylation. A phosphospecific anti-eIF2α antibody recognizes the WG eIF2α(P) that is phosphorylated by an authentic eIF2α kinase such as double stranded-RNA dependent protein kinase (PKR), but it is unable to recognize the eIF2α that is phosphorylated in NEM-treated lysates. These findings therefore suggest that phosphorylation of WG eIF2α in NEM-treated lysates occurs on a site different from serine 51 residue that is phosphorylated by authentic eIF2α kinases. In addition, it also suggests that WG eIF2α, unlike reticulocyte eIF2α, is phosphorylated by eIF2α kinases and also by other kinases. Consistent with the idea, it has been observed that casein kinase II (CKII) phosphorylates WG eIF2α and the phosphorylation is enhanced by NEM *in vitro* and in lysates. The phosphopeptide analysis suggests that NEM-induced phosphorylation in WG lysates resembles CKII - mediated phosphorylation.

2. Results

2 1. Translation in WG Lysates: Effect of Heat Shock and –SH Reactive Agents. To understand the importance of –SH groups in WG translation, the lysate translation was studied in the presence of NEM, DTT and during heat treatment. While addition of DTT does not stimulate translation any further, NEM is found to be inhibitory (Table 1, Expt. 2 & 3). Addition of NEM or DTT does not prevent the translational block caused by heat treatment (Table 1, Expt. 2 & 3). In contrast, DTT is able to mitigate the translational inhibition caused by oxidizing agents such as diamide (Table 1, Expt. 4) and GSSG (data not shown). These findings therefore suggest that the oxidation of protein sulfhydryls and also the denaturation of heat labile proteins may contribute to the poor performance of certain batches of lysates and for the translational inhibition observed in heat-treated lysates

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2.2. Phosphorylation of WG eIF2 in NEM, NEM and CK-II, and, Heat-Treated Lysates: Earlier, we reported that NEM-treated WG lysates showed increased phosphorylation of the p41-42 doublet that is considered to be the alpha subunit. However, it did not inhibit the exchange of guanine nucleotides on WG eIF2 (Janaki et al, 1995). Also, it is known that NEM or heat-treatment enhances eIF2α phosphorylation in reticulocyte lysates (Chen et al, 1989; Panniers, 1994; Gross et al, 1994 and Matts et al, 1993). Hence, we studied the phosphorylation of WG eIF2α in NEM and heat-treated lysates using a phosphospecific anti-eIF2α antibody. Heat shock facilitates the phosphorylation of reticulocyte eIF2α in hemin-supplemented reticulocyte lysates that is recognized by the above phosphospecific anti-eIF2α antibody. The latter recognizes specifically eIF2α that is phosphorylated on its 51serine residue by eIF2α kinases (Fig. 13 compare lanes 2 vs. 3). In contrast, a similar heat treatment however does not affect the basal phosphorylation status of wheat germ eIF2α (Fig. 13 lanes 4 vs. 5).

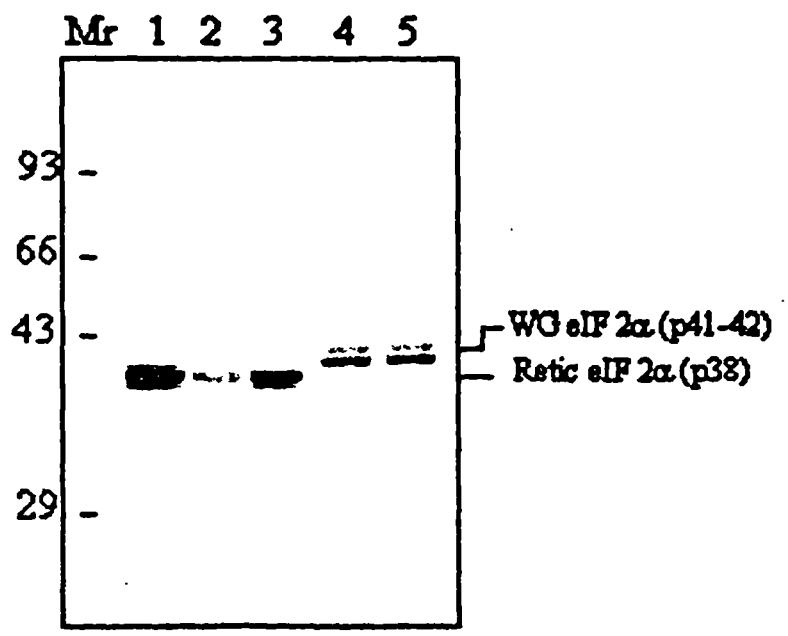
NEM-induced WG eIF2α phosphorylation in lysates is not recognized by the phosphospecific antibody in the western blot (Fig.14 Panel C, lanes 1 vs. 2) but can be detected with [γ–^{32}P] ATP (Fig. 14, Panel A lanes 1 vs. 2). These findings therefore suggest that WG eIF2α is phosphorylated by more than one kinase. Hence, the phosphorylation of WG eIF 2α in lysates treated with CK II or CK II and NEM (Fig.

Table I. Protein synthesis in wheat germ lysate.

Sample	Experimental conditions	Protein synthesis ([^{35}S] methionine incorporated, cpm)		
		15 min	30min	45min
Expt 1				
1	WGL 1	14,763	30,434	30,436
2	WGL 2	30,208	55,646	70,182
3	WGL 3	9,766	31,798	45,880
Expt 2				
1	lysate + 25°C	28,021	33,186	40,219
2	lysate + NEM 0.5 mM + 25°C	24,175	30,879	37,142
3	lysate + NEM 1.0 mM + 25°C	25,714	27,802	31,428
4	lysate + 40oc	23,296	21,868	21,648
5	lysate + NEM 0.5 mM + 40°C	22,637	20,769	21,538
6	lysate + NEM 1·0mM + 40°C	23,186	18,901	18,131
Expt 3				
1	lysate + 25°C	30,027	36,821	41,755
2	lysate + DTT 0.5 mM + 25°C	29,479	36,273	39,239
3	lysate + DTT 1.0 mM + 25°C	28,054	36,164	39,232
4	lysate + 40°C	23,452	23,343	25,534
5	lysate + DTT 0.5 mM + 40°C	23,342	22,465	21,917
6	lysate + DTT 1.0 mM + 40°C	20,273	22,024	21,150
Expt 4				
1	lysate	18,131	31,758	41,758
2	lysate + diamide 1mM	11,208	12,747	14,285
3	lysate + diamide 1mM + DTT 1mM	17,802	32,197	44,725
4	lysate + DTT mM	16,153	32,637	40,879

Note: Standard lysate protein synthesis was carried out in 25µl reaction mixtures in the presence of BMV RNA at 25°C for 45 min and was determined by measuring the incorporation of labeled [^{35}S] methionine into acid-precipitable protein in 5µl of the reaction mixtures with time as described in "Materials and Methods". Expt 1, translational ability of three different batches of lysates (WGL 1: weak; WGL 2: Strong; WGL 3: moderate). Expt. 2, 3, and 4, effect of SH-reactive agents on translation in control and heat-treated lysates.

Fig. 13. eIF2α phosphorylation in translating heat-treated reticulocyte and wheat germ lysates.



Protein synthesis was carried out in 25 µl reaction mixtures in heme-sensitive reticulocyte lysate (with or without added hemin) and in wheat germ extract (25 µl) at 30°C or 25°C respectively for 15 min as mentioned in 'Materials and Methods'. Heat treatment was given to samples at 40°C for 15 min. The reactions were then terminated and subjected to pH 5.0 precipitation as described in 'Materials and Methods'. The samples were separated by 10% SDS-PAGE. The figure is a western blot probed with a phosphospecific anti-eIF2α antibody.

Lanes: Mr, protein molecular weight marker (kD); 1, - heme; 2, + heme; 3, + heme + heat treatment at 40°C; 4, + wheat germ extract; 5, + wheat germ extract + heat treatment at 40°C.

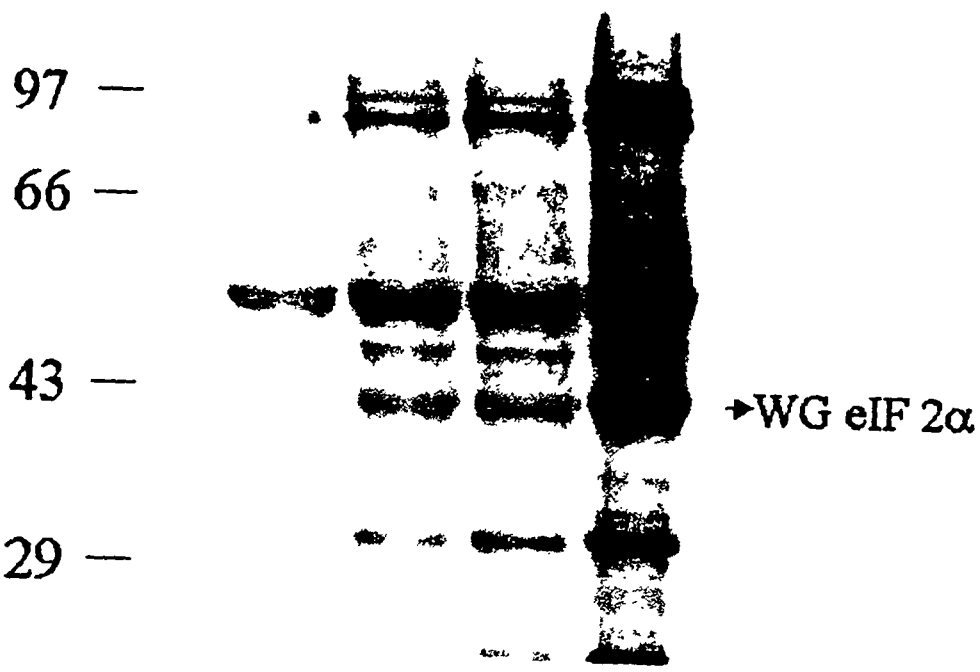
Fig. 14. Phosphoprotein profile of NEM and/ or CKII-treated wheat germ lysate.

Protein synthesis was carried out in 50 µl of wheat germ lysate in the presence or absence of NEM (1mM) and or CK II (~ 40 ng) at 25° C for 10 min. An aliquot of 20 µl protein synthesizing lysate was then incubated with a 5 µl reaction mixture of purified wheat germ eIF2 (500 ng), KCl (100 mM) 20 µCi [γ-^{32}P] ATP, and Mg^{2+} (2.5 mM) for 5 min. at 25°C. The reactions were then terminated and subjected to pH 5.0 precipitation. The samples were separated by 10% SDS-PAGE and then transferred to a nitrocellulose membrane.

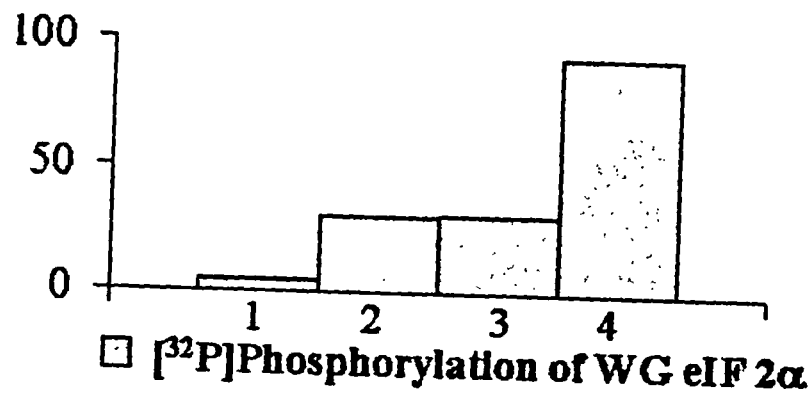
Panel A, is an autoradiogram/ phosphor image. **Panel B.** The bar diagram represents the band intensities of the corresponding figure in panel A for eIF2α phosphorylation. The arbitrary values were divided by 10, 000. The eIF2α phosphorylation in the control extract (lane 1) was 49, 646 au as measured by UVI band supplied by UVI tech. **Panel C** is a western blot probed with a phosphospecific anti-eIF2α antibody.

Lanes: Mr, protein molecular weight marker (kD); 1, WG lysate + eIF2; 2, WG lysate + NEM + eIF2; 3, WG lysate + CK II + eIF2; 4, WG lysate + NEM + CK II + eIF2.

(A)


Mr
1
2
3
4
97
66
43
29
WG eIF 2α
NEM - + - +
CK II - - + +
WG eIF2 + + + +

(B)


100
50
0
1
2
3
4
[32P]Phosphorylation of WG eIF 2α

(C)

WG eIF 2α

14) has also been assessed. It is observed that CKII phosphorylates WG eIF2α (Fig. 14 Panel A and B, lanes 1 vs. 3) and it is enhanced by the addition of NEM (lanes 3 vs. 4). NEM-induces a six-fold increase in the phosphorylation of proteins in lysates as judged from the quantification (lane 1 vs. 2 and also the corresponding bar diagram in Panel B). Also NEM increases the phosphorylation of lysate proteins by added CKII at least by one fold (lanes 3 and 4 and the corresponding bar diagram in Panel B). The CKII-mediated (Fig. 14, lane 3) and, or, the NEM-induced WG eIF2 phosphorylation in lysates (Fig. 14, lanes 2 and 4) is not recognized by the phosphospecific antibody. These findings therefore suggest that NEM or CKII or NEM and CKII-mediated phosphorylation of WG eIF2α occurs at a site different from the 51serine residue. While these above studies are done in lysates, we have also carried out similar studies *in vitro* with the purified components to determine if CKII–mediated WG eIF2 phosphorylation is enhanced by NEM (Fig. 15). Our findings indicate that purified WG eIF2 is not phosphorylated in the absence of any added CKII (Fig. 15 A and B, lane 1). Addition of NEM, however, increases the phosphorylation of this band by two fold (Fig. 15 A and B, lane 1 vs. 3) suggesting that the WG eIF2 preparation contains some CKII-like activity. Addition of CKII causes 3.5 fold increase in WG eIF2α phosphorylation (lane 1 vs. 2), where as, addition of NEM and CKII causes approximately a 16 fold increase in its phosphorylation (lane 1 vs. 4). However, there is no such increase in the phosphorylation status of WG eIF2α when the western blots are probed with phosphospecific antibody (Panel C). Thus both *in situ* and *in vitro* studies indicate that NEM stimulates WG eIF2 phosphorylation that is mediated by CKII.

Further, we have shown that CKII-mediated phosphorylation is different from the phosphorylation caused by known eIF2α kinases such as PKR (a double-stranded RNA-dependent kinase). Both PKR and CKII enzymes are able to phosphorylate the p41-42 subunit of WG eIF2 as has been shown in the autoradiogram (Fig. 16A, lanes 1 and 3). However the PKR-mediated WG eIF2α phosphorylation is different from CKII-mediated phosphorylation which is evident from the fact that the phosphospecific antibody recognizes the PKR-mediated eIF2α phosphorylation but not CKII- mediated phosphorylation (Fig. 16B).

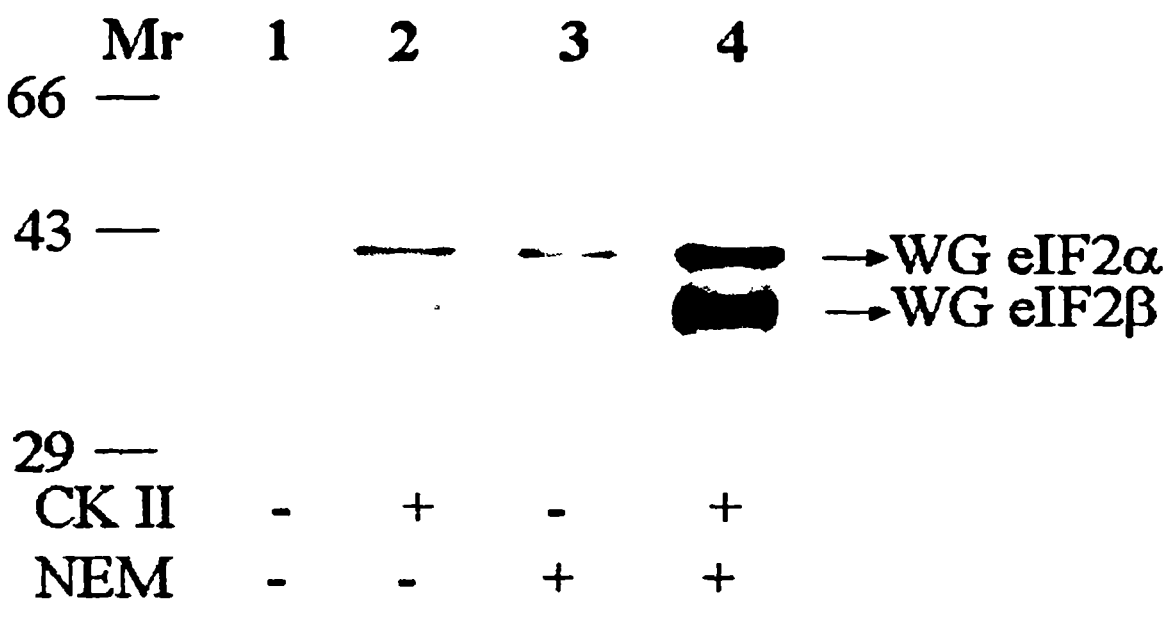
Fig. 15. Phosphorylation of WG eIF2 by NEM and/or CKII *in vitro*.

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 unlabelled ATP and 10 µci of [γ-^{32}P]ATP (3000 Ci/mmol), purified WG eIF2 (500 ng) with or with out pure CKII (~ 40 ng) and NEM (1.0 mM). Reaction mixtures were incubated at 25°C for 10 min and then separated by 10% SDS-PAGE. The gel was then transferred to a nitrocellulose membrane.

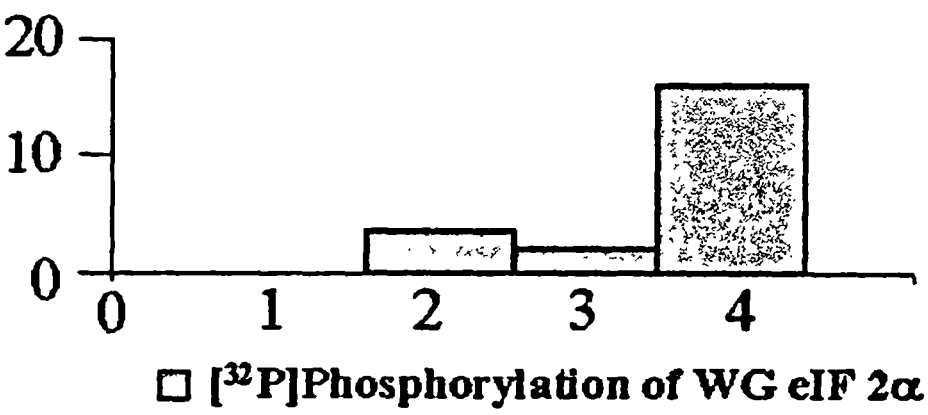
Panel A is an autoradiogram showing labeled protein bands. **Panel B.** The bar diagram represents the band intensities of the corresponding figure in panel A for eIF2α phosphorylation. The arbitrary values were divided by 10, 000. The eIF2α phosphorylation in the control extract (lane 1) was nil as measured by UVI band supplied by UVI tech. **Panel C** is a western blot probed with a phosphospecific anti-eIF2α antibody.

Lanes: Mr, protein molecular weight marker (kD); 1, WG eIF2; 2, WG eIF2 + CKII; 3, WG eIF2 + NEM; 4, WG eIF2 + CKII + NEM.

(A)


Mr 1 2 3 4
66 —
43 —
→WG eIF2α
→WG eIF2β
29 —
CK II - + - +
NEM - - + +

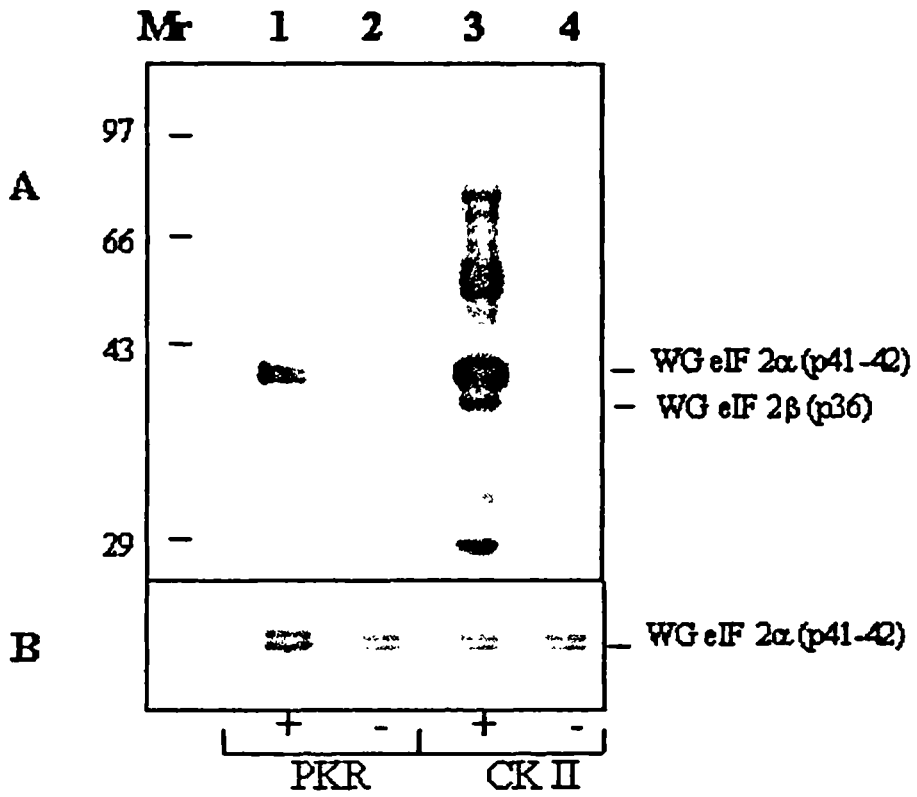
(B)


20
10
0
0 1 2 3 4
□ [32P]Phosphorylation of WG eIF 2α

(C)

→ WG eIF2α

Fig. 16. Phosphorylation of wheat germ eIF2 *in vitro* by PKR or CK II.



Phosphorylation reactions were carried out in standard 20 µl reaction mixtures containing 20 mM Tris-HCl, pH 7.8, 2.5 mM Mg $(OAc)_2$, 30 µM unlabeled ATP, 10 µCi [γ-^{32}P] ATP (3000 Ci/mmole), purified wheat germ eIF2 (~500 ng) with or with out CK II (~ 40ng) or PKR (~ 40ng). Reaction mixtures were incubated at 25°C for 10 minutes, separated by 10% SDS-PAGE and then transferred to a nitrocellulose membrane. The membrane was analyzed by autoradiography **(Panel A)** and was also probed with a phosphospecific anti-eIF2α antibody **(Panel B)**.

Lanes: Mr, protein molecular weight marker (kD); 1, WG eIF2 + PKR; 2, WG eIF2 (substrate control); 3, WG eIF2 + CK II; 4, WG eIF2 (substrate control).

2.3. Effect of –SH Reactive Agents on WG eIF2α Phosphorylation in Control and Heat-treated Lysates and Estimation of Protein-SH content: To determine the importance of protein sulfhydryls in the phosphorylation of WG eIF2α in lysates, we have studied the phosphorylation of lysate proteins in the presence of NEM, DTT, GSH, GSSG, diamide and heat treatment (40° C for 10 min) (Fig. 17A). While DTT, GSH and NEM stimulated the phosphorylation of several lysate proteins including WG eIF2α (Fig. 17A lanes 2, 3, 4 vs. lane 1 respectively), oxidizing agents such as diamide (lane 6), GSSG (to a lesser extent, lane 5) and heat treatment (lane 7) decreased the same. The increase in phosphorylation is found to be higher in NEM-treated lysates and is followed by DTT and GSH. However, there is no increase in eIF2α phosphorylation as judged by the phophospecific antibody (data not shown). This finding therefore suggests that none of these reagents activate eIF2α kinase-like activity in WG lysates and is thus different from what has been reported earlier in reticulocyte lysates (London et al, 1987; Jackson et al, 1991).

To determine the importance of –SH groups in phosphorylation and in translation, the phosphorylation of WG lysate proteins was studied in heat-treated lysates in the presence of –SH reactive agents as mentioned above. While heat treatment causes a profound decrease in the phosphorylation of proteins (Fig. 17B lane 1vs 2) as mentioned in the earlier figure, inclusion of NEM, DTT or GSH (Fig. 17B lanes 3, 4 and 5 vs. lanes 1 and 2) prevents the decrease in phosphorylation. However, NEM prevents almost completely but DTT and GSH prevent partially the heat-induced inhibition in phosphorylation. These agents however do not mitigate the heat-induced translational inhibition as shown in Table-1.

The lysate-SH content is also measured using DTNB (Table 2), in order to relate the changes in phosphorylation and translation to -SH content of the lysate. Total -SH content measured in the presence of SDS is found to be 47-50% more than available –SH (fifth column vs. third column in the table 2). The latter has been measured in the absence of SDS. Lysates with high translational ability have showed more-SH than lysates with low translational ability (see # 12 vs. 13 and 14). Heat Shock decreases the –SH content (# 8 vs. 1). NEM presence has also showed low –SH content (# 4 vs. 1) but this is due to alkylation of –SH groups. Alkylated –SH groups are not accessible for DTNB reaction. Alkylation however causes stabilization of –SH groups and protects them from further oxidation. Oxidizing agent like diamide (# 6)

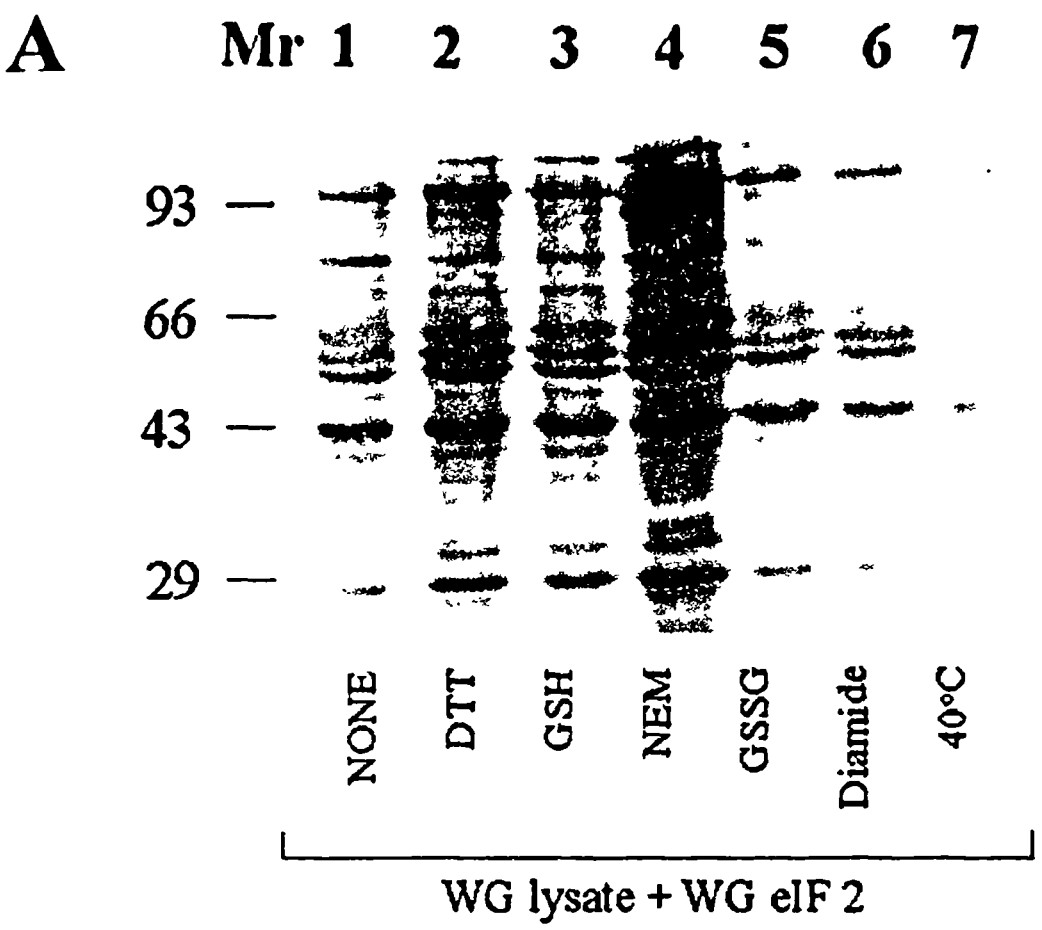
Fig. 17. WG lysate phosphorylation in the presence of –SH reactive agents and during heat treatment.

Panel A: Phosphorylation of the lysate proteins was carried out and the samples were processed as described in "Materials and Methods". The lysates were treated with DTT, NEM, GSH, diamide (1.0 mM each) or exposed to heat treatment for 10 min at 40°C. Figure is an autoradiogram/ phosphor image.

Lanes:1, WG lysate + eIF2; 2, WG lysate + eIF2 + DTT; 3, WG lysate + eIF2 + GSH; 4, WG lysate + eIF2 + NEM; 5, WG lysate + eIF2 + GSSG; 6, WG lysate + eIF2 + diamide; 7, WG lysate + eIF2 + heat treatment at 40^0 C.

Panel B: Effect of -SH reactive agents on the phosphorylation proteins in heat-treated lysates. WG lysates were subjected to heat treatment at 40°C for 10 min in the presence and absence of NEM, DTT and GSH. Figure is an autoradiogram/phosphorimage.

Lanes:1, WG lysate + eIF2; 2, WG lysate + eIF2 + 40°C; 3 WG lysate + eIF2 + 40°C + NEM; 4, WG lysate + eIF2 + 40°C + DTT; 5, WG lysate + eIF2 + 40°C + GSH.


A
Mr 1 2 3 4 5 6 7
93 —
66 —
43 —
29 —
NONE
DTT
GSH
NEM
GSSG
Diamide
40°C
WG lysate + WG eIF 2

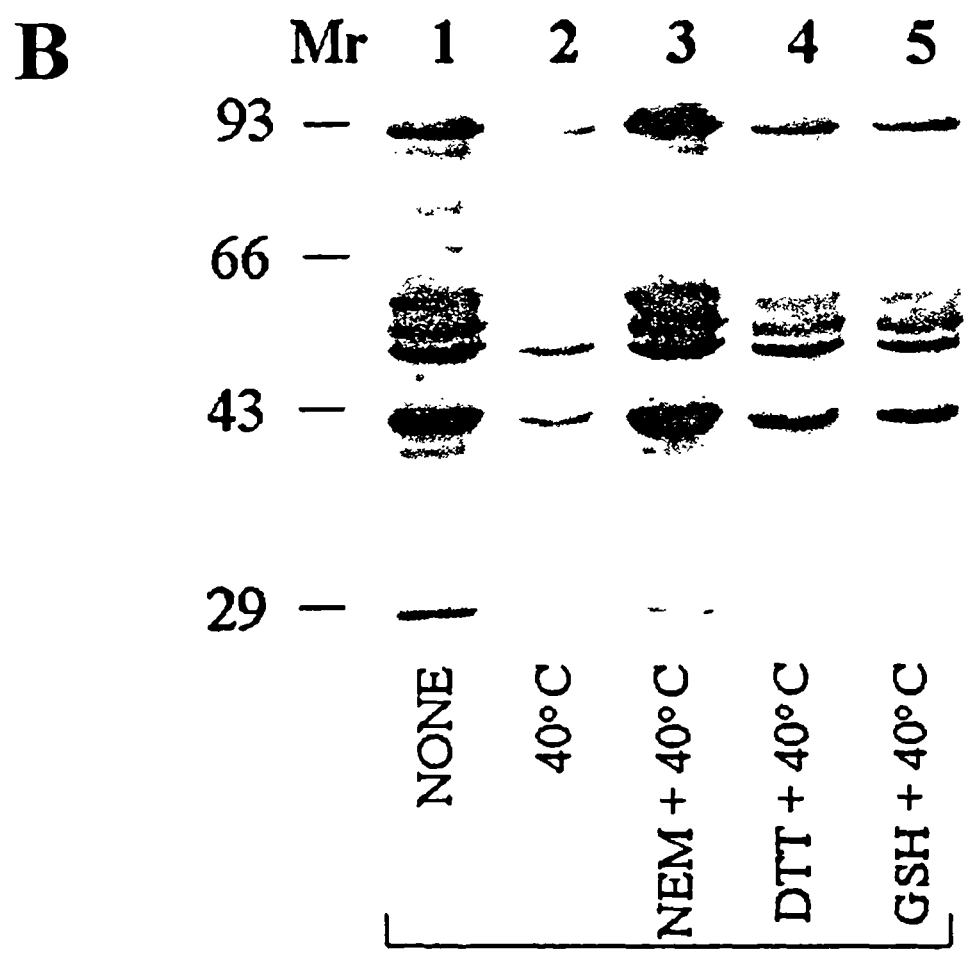

B
Mr 1 2 3 4 5
93 —
66 —
43 —
29 —
NONE
40°C
NEM + 40°C
DTT + 40°C
GSH + 40°C
WG lysate + WG eIF 2

Table II. Sulfhydryl content of wheat germ lysate.

Sample No.	Reaction sample	Total –SH (mM)	+/- SD	Available –SH (mM)	+/-SD
1	WG lysate (WGL)	1.09	0.06	0.58	0.03
2	WGL + DTT	2.55	0.12	1.17	0.06
3	WGL + GSH	1.94	0.10	1.23	0.06
4	WGL + NEM	0.48	0.07	0.35	0.01
5	WGL + GSSG	0.94	0.05	0.51	0.03
6	WGL + diamide	0.56	0.04	0.40	0.02
7	WGL + diamide + DTT	1.10	0.07	0.57	0.04
8	WGL + 40°C	0.81	0.03	0.42	0.05
9	WGL + 40°C + DTT	2.76	0.12	1.15	0.08
10	DTT	2.33	0.12	2.33	0.08
11	GSH	0.88	0.04	0.87	0.02
12	WGL strong	1.20	0.06	0.64	0.01
13	WGL moderate	1.09	0.06	0.58	0.02
14	WGL weak	0.89	0.04	0.46	0.01
15	Diamide + DTT	0.0		0.0	

Note: Sulfhydryl content of wheat germ was measured as described in "Materials and Methods". The lysate was treated with and without the various redox agents (1.0 mM) as indicated. The –SH content was estimated in a 50 µl reaction volume containing 45 µl of WGl (3mg/ml) and 5 ml of the respective –SH reactive agent as indicated. To this reaction mixture, 33 µl of DTNB solution (40mg) DTNB in 10 ml of 0.1 M sodium phosphate buffer, pH 8.0) and 0.1 ml solution containing 2% SDS, 0.08 M sodium phosphate buffer, pH 8.0, and 0.5mg/ml EDTA were added. The color developed for 15 min and was measured at 410 nm against the lysate mixture in SDS to give apparent absorbance. A reagent blank was subtracted from the apparent absorbance to give the net absorbance. For calculation of sulfhydryl content, the net absorbance was employed with a molar absorptivity value of 13,600 M^{-1} cm^{-1}. Based on [^{35}S] methionine incorporation in 5µl of translational lysate, the lysates were characterized as strong, moderate and weak. Strong lysate showed an incorporation of 70,000 cpm, where as the moderate and weak lysates showed an incorporation of 35,000-45,000 or 20,000-30,000 cpm, respectively, in 5 µl aliquots after 45 min of a translational assay. Available protein –SH content was measured in the same manner in the absence of SDS and the absorbance of SDS and the absorbance was monitored at 410 nM.

causes more decrease in the lysate –SH than GSSG (# 5). The decrease in SH content in diamide–treated lysates and in heat-treated lysates is completely prevented by DTT (# 6vs 1 with 7vs 1; 8vs 1 with 9 vs. 2 respectively). However, DTT prevents partially the decrease in phosphorylation (Fig. 17B), but not translation, that is caused by heat treatment (Table 1, Expt. 3). Also, the presence of DTT does not improve the translational performance of a moderate lysate in the absence of heat treatment as has been shown (Table 1, Expt. 3). The addition of reducing agents like GSH and DTT to a lysate did not give an additive effect (Table 2). The total amount of –SH decreases significantly in DTT supplemented lysates than in GSH supplemented lysates when compared to their independent values (that is lysate alone or DTT or GSH alone). This suggests that the reducing ability of these agents is utilized by the system and DTT is used up perhaps more efficiently than GSH.

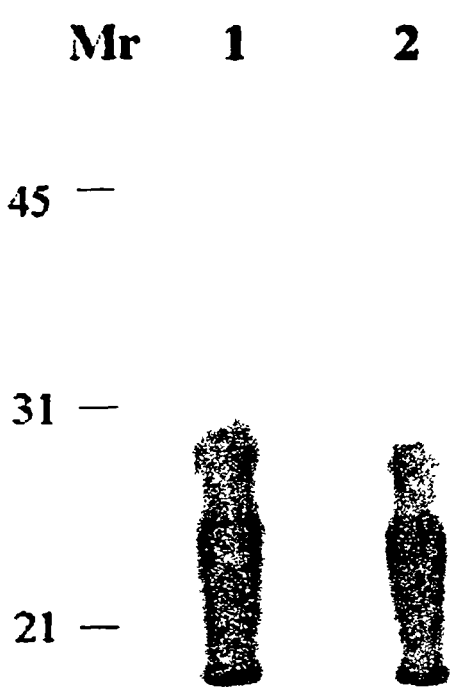
Overall, these findings suggest that in addition to maintenance of –SH groups, heat labile proteins may also play an important role in translation and phosphorylation abilities of a lysate.

2.4. Phosphopeptide analysis of WG eIF2: Limited proteolytic digestion of phosphorylated wheat germ eIF2α subunit carried out using SV8 protease and the peptide maps were analyzed as described in 'Materials and Methods'. SV8 digestion of the p41-42 doublet-subunit phosphorylated by CKII *in vitro* yielded three strongly labeled bands corresponding to 28, 26 and and 19 kD, which are similar to the labeled bands obtained for WG eIF2α phosphorylated in NEM-treated lysate (Fig. 18). This further confirms that NEM stimulates CKII activity in WG lysate.

3. Discussion

Phosphorylation of serine 51 residue in eIF2α inhibits the guanine nucleotide exchange activity of eIF2B (Hinnebusch et al, 2000; Sudhakar et al, 1999; Sudhakar et al, 2000; Pavitt et al, 1998; Clemens et al, 1982; Matts and London, 1984 and Ramaiah et al, 1994). This in turn inhibits the recycling of eIF2.GDP to eIF2.GTP and protein synthesis (Ramaiah et al, 1992). This is major regulatory mechanism in gene expression at the translation level in many eukaryotes studied to date. Previously, we have shown that the p41-42 doublet subunit and the p36 subunit of WG eIF2 are phosphorylated in NEM-treated lysates and also by CK-II (Janaki et al, 1995). This

Fig. 18. Partial peptide digestion of phosphorylated wheat germ eIF2α subunit.



Phosphorylation of the p41-42 subunit of WG eIF2 in NEM-treated lysates (50 µl lysate) and by CK II was carried out *in vitro*. The phosphorylated bands of wheat germ eIF2 subunits were obtained from a 10% SDS-PAGE. The bands were identified by superimposing the autoradiogram on dried gel and were then cut out through the X-ray film. The dried gel pieces containing the phosphorylated subunits were equilibrated and made ready for SV8 protease as described in Materials and Methods. The protease treated labeled subunit was then separated on a 15% SDS-PAGE. The figure is an autoradiogram.

Lanes: Mr, molecular weight marker (kD); 1, WG eIF2 + CKII; 2, WG eIF2 + NEM-treated (1.0 mM) lysate.

phosphorylation did not affect the exchange of guanine nucleotides on eIF2. Subsequent studies have shown that the p41-42 doublet subunit of WG eIF2 is equivalent to mammalian eIF2α (Langland et al, 1995) and the p36 is equivalent to mammalian eIF2β (Metz and Browning, 1997). Addition of higher concentrations of poly IC stimulated probably plant PKR-like activity in WG lysates and facilitated phosphorylation of the p41-42 subunit of WG eIF2. Phosphorylation of the p41-42 doublet subunit under those conditions is found associated with inhibition in protein synthesis in translating wheat germ lysates, although the mechanism underlying this inhibition of protein synthesis is not yet understood (Langland et al, 1996).

Further, studies from this laboratory (Fig. 2, from Narahari Janaki, Ph. D. thesis, 1996) shows that the ability of NEM to stimulate the phosphorylation was found to be dependent on the translational ability of the lysates. NEM stimulated efficiently the phosphorylation of lysates that were relatively low in their translational ability than in optimally active lysates. The active lysates may have their protein-sulfhydryl groups well maintained and addition of NEM alkylates the –SH groups and thereby prevents the formation of disulfide bonds. Hence, no significant difference in phosphorylation is observed under those conditions. In contrast, addition of NEM to weak or moderate lysates enhances the phosphorylation of proteins much more significantly.

In the present study, we have studied the effect of different –SH reactive agents on wheat germ lysate translation and phosphorylation (Laxminarayana et al, 2002). We have observed that diamide treatment causes reduced phosphorylation (Fig 17A), and, the heat-induced decrease in phosphorylation is prevented significantly by the addition of NEM, DTT or GSH (Fig. 17B), support the hypothesis that oxidation of protein-SH groups can lead to a decrease in phosphorylation. However, NEM decreases the lysate sulfhydryl content (either by alkylation of protein-SH, and, or lysate GSH) (Table 2) and inhibits translation (Table 1, Expt. 2), where as, diamide causes oxidation of protein-SH groups. In contrast, DTT and GSH maintain protein – SH groups, do not inhibit protein synthesis but stimulate phosphorylation, albeit, to a lesser extent than NEM. Neither DTT nor NEM can prevent heat-induced inhibition in protein synthesis (Tables 1 & 2 and Fig 17). On the contrary, DTT prevents diamide (Table-1) and GSSG–induced (data not shown) inhibition in protein

synthesis. These findings therefore suggest that in addition to the maintenance of sulfhydryl groups, heat labile proteins or denatured proteins, and, their interaction with other proteins play an important role in the overall translation and phosphorylation. This conclusion is supported by other studies wherein HSP70 (heat shock protein 70) has been shown to restore the inhibition in protein synthesis that occurs due to the activation of heme-regulated kinase (HRI) in heme-deficient reticulocyte lysates (Gross et al, 1994). It is suggested that this is a general mechanism for translational control in response to cellular stress where in the denatured proteins formed in response to heat or other stress may bind to heat shock proteins in competition with HRI and thus leading to the activation of HRI (Matts and Hurst, 1992; Matts et al, 1993). In addition, heat shock protein 72 (Hsp72) has been implicated in preventing the activation of kinases such as JNK (Jun N-terminal kinase) and p38 through the maintenance of a cellular phosphatase activity during heat shock, ethanol, oxidative stress and other stress conditions that cause protein damage in lymphoid tumor cell lines (Gabai et al, 1997 and Yaglom et al, 1999).

Further, our findings indicate that heat treatment stimulates the phosphorylation of reticulocyte eIF2α, but not the wheat germ eIF2α, in their respective translational lysates. This finding suggests that wheat germ lysates probably do not carry HRI-like protein. The eIF2α phosphospecific antibody, as mentioned above, has been used to distinguish the phosphorylation of p41-42 doublet of WG eIF2 caused by eIF2α kinases and a multipotential serine/threonine kinase like CKII. The findings presented in Fig. 16 suggest that these enzymes phosphorylate WG eIF2α at different sites. Also we observed that NEM stimulates CKII-mediated phosphorylation of WG eIF2α both in lysates (Fig. 14) and *in vitro* system (Fig. 15). These studies suggest that NEM stimulates CKII-like activity in wheat germ lysates. This is further supported by the evidence that phosphopeptides of WG eIF2α in NEM-treated lysates resemble to the phosphopeptide map of WG eIF2α that is phosphorylated by CKII *in vitro* (Fig. 18) (Laxminarayana et al, 2002). CKII is a multipotential kinase with several substrates. Consistent with this idea, addition of NEM stimulates phosphorylation of several lysate proteins. While it is known that reticulocyte eIF2α is phosphorylated so far exclusively by eIF2α kinases, WG eIF2α is phosphorylated by eIF2α kinases and CKII.

The physiological significance of CKII-mediated WG eIF2α phosphorylation or the mechanism of activation of CKII in the presence of NEM is not yet understood. Although mammalian eIF2α may not contain any phosphorylation site other than the serine 51 residue that is phosphorylated by eIF2α kinases, yeast eIF2α has been found to be phosphorylated both by eIF2α kinases and CKII. The C- terminus of yeast eIF2α contains three additional phosphorylation sites that are constitutively phosphorylated *in vitro* and in vivo by CKII. Mutations in these CKII phosphorylation sites of yeast eIF2α are found to affect the GDP/GTP exchange on eIF2, presumably by affecting the eIF2B activity (Feng et al, 1994). Recent studies by Le et al (1998) have shown that phosphorylation status of eIF2α varies considerably during wheat seed development and germination. However, the authors find it difficult to explain how phosphorylation at each site of wheat eIF2α affects its activity. This is because plant eIF2α has more than one phosphorylation site and that is consistent with our observations.

Phosphorylation of eIF4 factors (4E, 4A, 4B, 4G) is also known to alter translational rates (Raught et al, 2000). Earlier studies have shown that mammalian eIF4B is phosphorylated *in vitro* by several kinases including CKI and CKII (Morely and Traugh, 1989 and 1990) but the physiological importance of this phosphorylation is not yet understood. However in plants, eIF4B is found to be dephosphorylated in wheat embryos, but it is well phosphorylated in leaves and is also found to be dephosphorylated during heat shock (Gallie et al, 1997). Thus eIF4B phosphorylation correlates with increased rates of protein synthesis. Based on several results it is suggested that eIF4E phosphorylation in animal systems enhances its affinity for mRNA (Minich et al, 1994) but the role of this phosphorylated factor in plant systems is not clear. Phosphorylation of other eIF4 factors like eIF4A has also been shown to occur during a variety of environmental stress conditions and is known to decrease and also increase protein synthesis both in plant and mammalian systems thereby suggesting that phosphorylation occurs at different residues on these proteins, some of which are inhibitory and some of which are stimulatory (Raught et al, 2000). In addition, the interaction of heat shock proteins with the kinases that phosphorylate some of these substrates may also modulate the activity of these initiation factors as has been illustrated for HRI kinase activity (Matts and Hurst, 1992; Matts et al, 1993).

Our observations suggest that there is a general increase in phosphorylation of several proteins including eIF2α in active wheat germ cell free translational system and appears to be because of the presence of CKII-like activity in the system. The mechanism through which CKII is activated and the CKII-mediated eIF2 phosphorylation or the phosphorylation of other initiation factors improves the translational performance of lysates that has to be established by future studies.

CHAPTER II

PHOSPHORYLTON OF THE SERINE 51 RESIDUE IN WHEAT GERM INITIATION FACTOR 2 ALPHA INHIBITS WHEAT GERM LYSATE TRASLATION.

1. Abstract

2. Results

2.1. Phosphorylation of purified wheat germ eIF2α in reticulocyte lysate and by purified recombinant PKR.

2.2. PKR-mediated translational inhibition of BMV RNA in wheat germ lysates.

2.3. Translation of different chimaeric RNAs in PKR-treated wheat germ lysates.

2.4. Addition of purified recombinant human S51D eIF2α inhibits BMV RNA translation.

2.5. Human S51A eIF2α and K3L proteins mitigate PKR-mediated translational inhibition in wheat germ lysates

3. Discussion

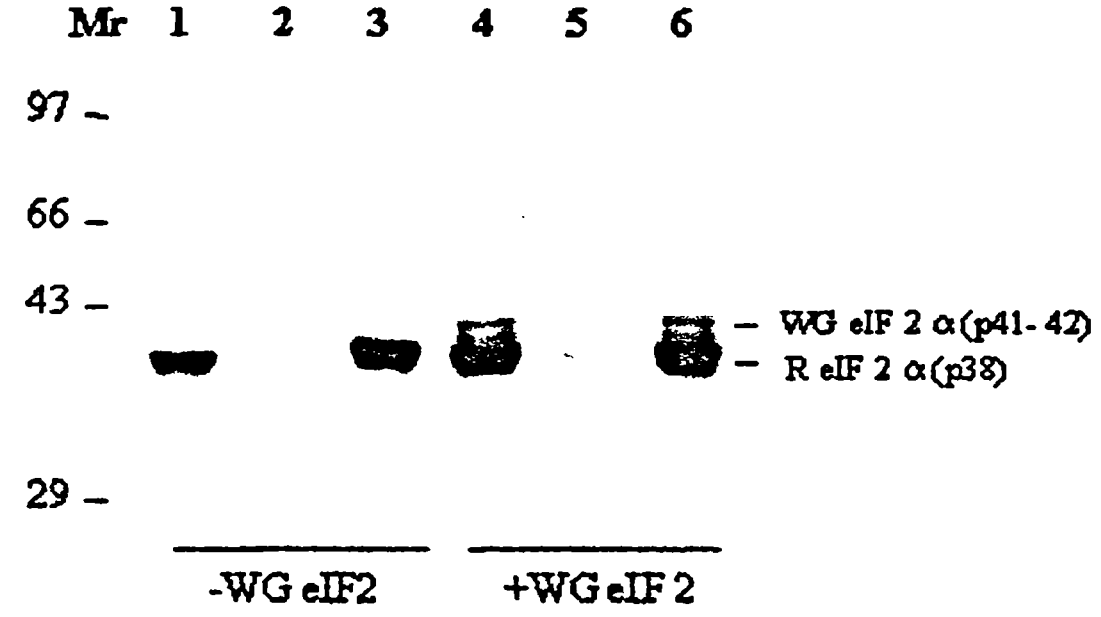
1. Abstract

In the second phase of the work, we have studied the importance of serine 51 residue phosphorylation in wheat germ eIF2α on the translational ability of wheat germ lysates. Since, we have been unable to stimulate an endogenous PKR-like activity in wheat germ lysates by the addition of ds RNA, we used purified recombinant PKR-GST and also purified mutants of human eIF2α protein in which the serine 51residue is replaced by alanine or aspartic acid (S51a or S51D). Although both these mutants are not phosphorylated by mammalian kinases (Sudhakar et al, 1999, 2000), S51D mutant behaves like phosphorylated form. In addition, we have used a phosphospecific anti-eIF2α antibody that recognizes specifically the S51 phosphorylation in eIF2α. Using these probes, a positive correlation was observed between decrease in protein synthesis and increase in eIF2α phosphorylation as judged by a phosphospecific anti-eIF2α antibody in PKR-treated wheat germ lysates. Phosphomimetic mutant of human eIF2α, S51D, also inhibited wheat germ translation. The importance of serine 51 phosphorylation in wheat germ eIF2α on translation inhibition is further complemented by observations indicating that PKR-mediated translational inhibition is partially mitigated a) in the presence of a recombinant nonphosphorylatable mutant of human eIF2α, S51A, in which 51 serine residue is replaced by alanine and b) also in the presence of K3L protein which is encoded by vaccinia virus and has some homology with the eIF2 to serve as a pseudosubstrate. These findings thus suggest that phosphorylation of serine 51 residue in wheat germ eIF2α is inhibitory to protein synthesis.

2. Results

2.1. Phosphorylation of purified wheat germ eIF2α in reticulocyte lysate and by purified recombinant PKR: Previously, we have shown that wheat germ eIF2α (the p41-42) doublet subunit was phosphorylated in hemin-deficient or heme and poly IC-treated reticulocyte lysates in which reticulocyte HRI or PKR was activated (Janaki et al, 1995). [γ-^{32}P] ATP was used in these studies to determine or identify the phosphorylated form of wheat germ eIF2. Since, reticulocyte lysates also contain casein kinase II and can phosphorylate wheat germ eIF2, these studies were unable to distinguish the WG eIF2 phosphorylation caused by these two enzymes. Here, the

Fig. 19. Phosphorylation of reticulocyte and wheat germ eIF2 in reticulocyte lysate.



Translating heme-deficient, hemin-supplemented or heme and poly IC-treated reticulocyte lysates were treated with or without added purified wheat germ eIF2 (1 μg) at 10 minutes of protein synthesis reactions. Reactions were carried out in 10 μl samples for 5 minutes at 30°C and then precipitated at pH 5.0 as described in 'Materials and Methods'. The samples were separated on 10% SDS-PAGE and transferred to nitrocellulose membrane. The membrane is probed with phosphospecific anti-eIF2α antibody that recognizes specifically the serine 51 phosphorylation in eIF2α.

Lanes 1, 2 & 3 are with out added wheat germ (WG) eIF2

Lanes: Mr, molecular weight marker (kD); 1, - heme; 2, + heme; 3, + heme + poly IC 250 ng/ml

Lanes 4, 5 and 6 are reticulocyte lysates that are supplemented with wheat germ eIF2.

Lanes: 4, - heme + WG eIF2 (1 μg); 5, + heme + WG eIF2 (1 μg); 6, + heme + WG eIF2 (1 μg) + poly IC 250 ng/ml.

phosphorylation is evaluated by a phosphospecific anti-eIF2α antibody (Fig. 19). In the absence of added WG eIF2, endogenous reticulocyte eIF2 is efficiently phosphorylated in heme-deficient lysates (Fig. 19, lane 1) and in heme and polyIC-treated lysates (lane 3). In the presence of heme, the endogenous reticulocyte eIF2α is not phosphorylated (lane 2). This is because heme inhibits HRI eIF2α kinase activity but not PKR activity that is stimulated by polyIC (Chen and London, 1995). Purified wheat germ eIF2α is also phosphorylated but not as efficiently as reticulocyte eIF2 in heme-deficient or hemin and polyIC-treated reticulocyte lysates (lanes 4 and 6). These findings suggest that mammalian eIF2α kinases can phosphorylate wheat germ eIF2 but not as efficiently as reticulocyte eIF2. These findings also suggest that mammalian eIF2α kinases phosphorylate wheat germ eIF2α on its 51 serine residue.

As we have been unable stimulate any endogenous PKR-like eIF2α kinase in wheat germ lysates by the addition of polyIC, we have obtained a mutant PKR-GST construct from Bryan G. Williams laboratory, Cleveland Clinic Foundation, USA, in order to determine the effects of purified PKR on wheat germ translation. The PKR-GST construct was expressed in *E. coli* BL21 cells and over expressed PKR was purified using glutathione sepharose 4B column as shown in Materials and Methods (Fig. 9). The purified mutant recombinant PKR does not require polyIC for its activation. Accordingly, we observed that recombinant PKR-GST (as shown in Fig. 10) phosphorylates wheat germ and also reticulocyte eIF2 in the absence of added polyIC. Addition of polyIC did not make any significant difference in PKR-GST catalyzed eIF2α phosphorylation as analyzed by a phosphospecific anti-eIF2α antibody.

2.2. PKR-mediated translational inhibition of BMV RNA in wheat germ lysates:

Wheat germ translation supported by the addition of commercially available BMV RNA is inhibited in PKR-treated wheat germ lysates (Fig. 20). As shown, in the absence of added RNA, wheat germ lysates cannot support any protein synthesis (Panel A, lane 1). Presence of BMV RNA stimulates protein synthesis as evidenced from the labeled products analyzed by a phosphor image (lane 2). Of the four polypeptides produced by BMV RNA, the 110 and 20 kD peptides are distinctly visible, the 97 and 35 kD peptides are faintly observed. The intensity of the labeled

Fig. 20. Protein synthesis in wheat germ lysate in the presence of purified PKR-GST.

The translation of BMV RNA (20 μg/ml) in wheat germ lysate was studied in the presence and absence of purified recombinant mammalian PKR-GST (40 ng/ml) as described in 'Materials and Methods'. The reaction mixtures (20 μl) were incubated at 25°C for 45 minutes and the proteins were separated on 10% SDS-PAGE and analysed by western using a phophospecific anti-eIF2α antibody. The dried nitrocellulose membrane was subjected to phosphorimage to visualize the [^{35}S] methionine labeled protein products of BMV RNA. Endogenous lysate protein synthesis was measured by omitting BMV RNA in the reaction mixture (as shown in lane1).

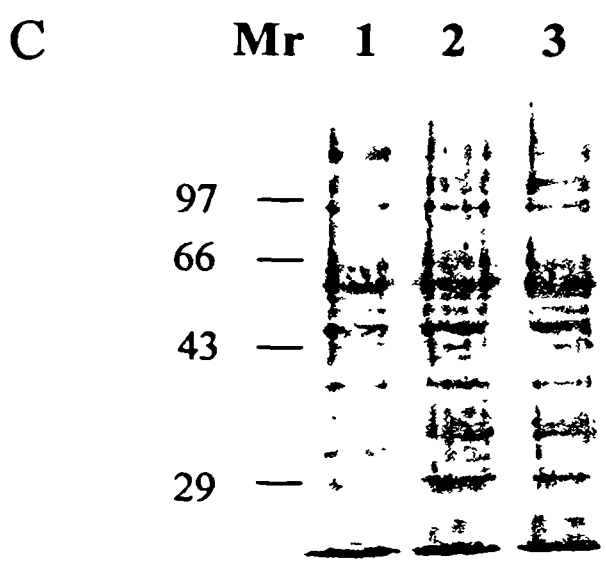
Panel A is a phosphorimage showing labeled protein products of BMV RNA.

Panel B is a western blot analyzed by a phosphospecific anti eIF2α antibody.

Panel C is a ponceau stained copy of the western blot.

Lanes: Mr, protein molecular weight marker, kD; 1, WGL; 2, WGL + BMV RNA; 3, WGL + BMV RNA + PKR-GST.

A
Mr
1
2
3
4
97
66
43
29
110
97
35
20
B
WG eIF 2α


C
Mr
1
2
3
97
66
43
29

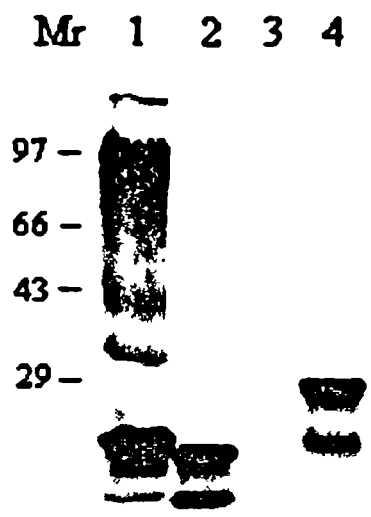
products however is decreased in the presence of purified PKR-GST (lane 3). The same reaction mixtures were also analyzed by a phosphospecific antibody to determine the phosphorylation of lysate wheat germ eIF2 (Fig. 20, Panel B). In the absence of added PKR, one can see a small but significant level of endogenous eIF2α phosphorylation (lanes 1 and 2), which is however increased in the presence of added PKR (lane 3). This increase in eIF2α phosphorylation in PKR-treated lysates is not due to any increase in the amount of lysate proteins as has been judged by a ponceaus stain (Fig 20, Panel C).

2.3. Translation of different chimaeric RNAs in PKR-treated wheat germ lysates: The following chimaeric mRNAs were tested i) pHST 106 mRNA (with alpha mosaic viral (AMV) leader and rabbit globin mRNA), ii) pHST 215 (AMV leader with Interleukin-1 (IL-1) mRNA, iii) pHST 301 (Barley alpha amylase leader and alpha amylase mRNA), iv) pHST 400 (AMV leader and AMV RNA). The constructs for this mRNAs were prepared by Joblings and Gherke as described (1987) to evaluate the effect of AMV leader sequence on mRNA translation. These mRNAs have been used to study the importance of AMV leader sequence on the translation in wheat germ lysates and also to determine the importance of eIF2α phosphorylation on the translation of these mRNAs in wheat germ lysate.

The above constructs were transcribed as described in 'Materials and Methods'. The RNAs were gel filtered (Fig. 11). Translation was carried out using equal amounts of these gel filtered RNAs. Consistent with the original observations of Jobling and Gherke (1987), we observed that the translation of RNAs in general is significantly higher with AMV leader sequence as compared to without the leader sequence (RNAs 106, 215 and 400 vs. 301) (Fig. 21 and Fig. 22, Panel A). However, the mRNAs 106, 215 and 400 without the viral leader sequence could not be traced by the original investigator and we were unable to prepare these constructs due to practical limitations.

In the presence of purified PKR (~ 40 ng/ml), it is observed that the translation of BMV RNA is significantly inhibited (Fig. 22, Panel A, lane 2 vs. 3) but not the translation of any of the chimaeric mRNAs described above (Panel A, lanes 4, 6, 8, 10 vs. 5, 7, 9, 11).

Fig. 21. Protein products of BMV RNA and different chimeric mRNAs translated wheat germ lysate.



Standard lysate protein synthesis assays (25 µl) were carried out in the presence of BMV RNA or chimeric mRNAs viz., 215 (Alpha mosaic virus leader sequence with Interleukin 1 mRNA), 301 (plant α amylase leader and α amylase RNA) or 400 (AMV viral leader and AMV RNA) (20 µg/ml) at 25°C for 45 minutes. The reaction was terminated by addition of 100 µl of 1X sample buffer and the labeled proteins were separated on 10% SDS-PAGE. The gels were coomassie stained, dried and subjected to phosphorimage to visualize the [^{35}S] labeled protein products. The figure is an autoradiogram/phosphorimage.

Lanes:

1) WGL + BMV RNA
2) WGL + 215 (Alpha mosaic virus leader sequence with Interleukin 1 mRNA)
3) WGL + 301 (plant α amylase leader and α amylase RNA) or 400 (AMV viral leader and AMV RNA)
4) WGL + 400 (AMV viral leader and AMV RNA)

Fig. 22. Translation of mRNAs in wheat germ lysate (WGL) in the presence of purified PKR-GST and analysis of eIF2α phosphorylation.

Standard lysate protein synthesis assays (25 µl) were carried out using BMV RNA or chimeric mRNAs viz., 106 (Alpha mosaic virus (AMV) leader and rabbit α-globin mRNA), 215 (Alpha mosaic virus leader sequence with Interleukin 1 mRNA), 301 (plant α amylase leader and α amylase RNA), or 400 (AMV viral leader and AMV RNA) (20 µg/ml) in the absence and presence of purified PKR-GST (~ 40 ng/ml) at 25°C for 45 minutes as described in 'Materials and Methods'. The reactions were terminated by the addition of 100 µl of 1X sample buffer and the labeled proteins were separated on 10% SDS-PAGE. The gels were coomassie stained, dried and subjected to phosphorimage to visualize the [^{35}S] labeled protein products.

Panel A is a phosphorimage showing labeled protein products of BMV RNA.
Panel B is lower half of western blot analyzed by phosphospecific anti-eIF2α antibody.
Panel C is upper half of western blot analyzed by anti-PKR antibody.
Panel D is a ponceau stained copy of the western blot.

Lanes:

Mr, molecular weight marker, (kD); 1, WGL; 2, WGL + BMV RNA; 3, WGL + BMV RNA + PKR-GST; 4, WGL + 106 RNA; 5, WGL + 106 RNA + PKR-GST; 6, WGL + 215 RNA; 7, WGL + 215 RNA + PKR-GST; 8, WGL + 301 RNA; 9, WGL + 301 RNA + PKR-GST; 10, WGL + 400 RNA; 11, WGL + 400 RNA + PKR-GST.

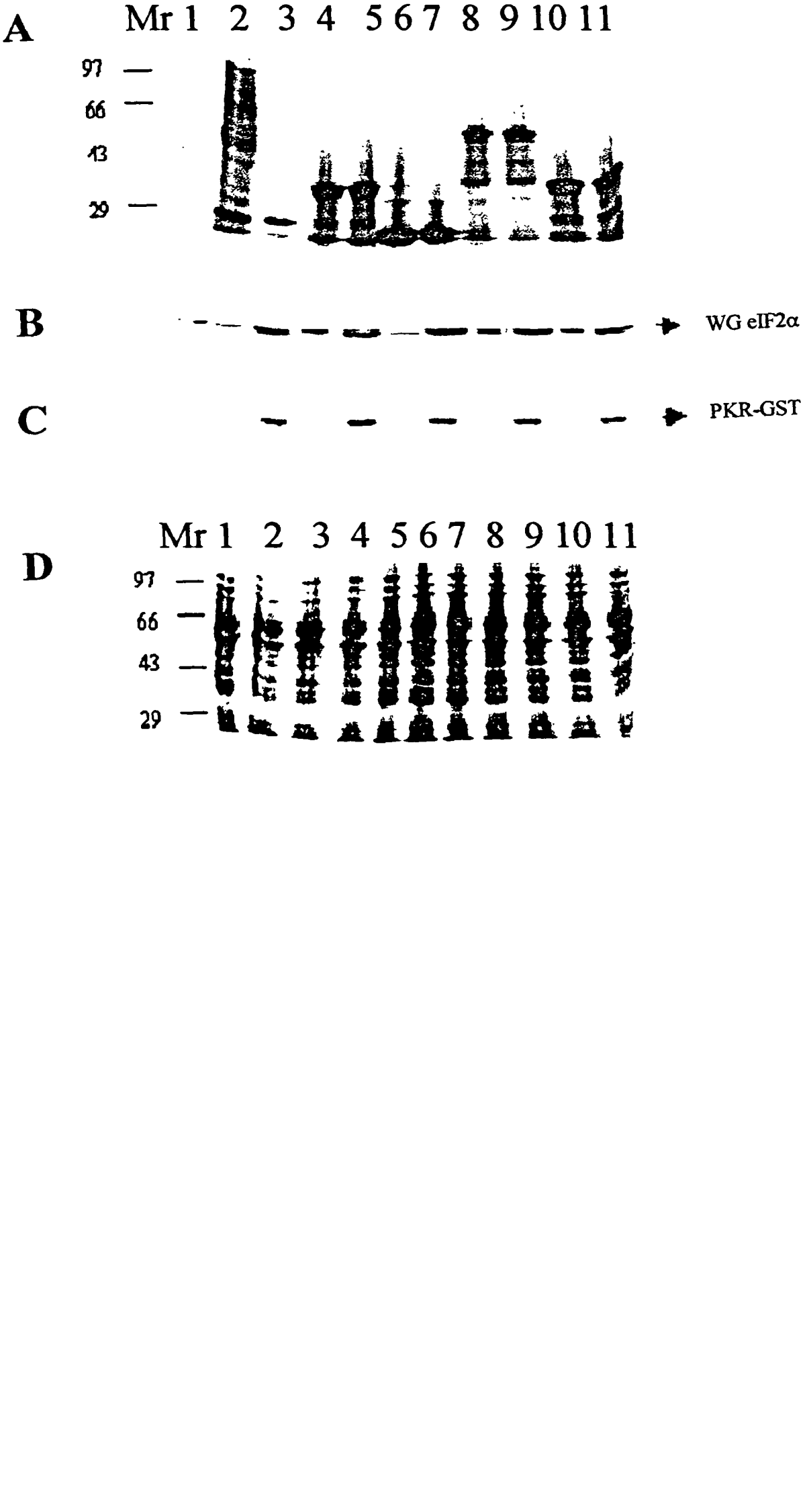

A
Mr 1 2 3 4 5 6 7 8 9 10 11
97
66
43
29
B
WG eIF2α
C
PKR-GST
D
Mr 1 2 3 4 5 6 7 8 9 10 11
97
66
43
29

Fig. 23. Translation of chimeric mRNAs (106 and 215) in wheat germ lysate (WGL) in the presence of increasing concentrations of purified PKR-GST and analysis of eIF2α phosphorylation.

Standard lysate protein synthesis assays (25 µl) were carried out in the presence of chimaeric mRNAs viz., 106 (Alpha mosaic virus (AMV) leader and rabbit α-globin mRNA), or, 215 (Alpha mosaic virus leader sequence with Interleukin 1 mRNA) (20 µg/ml) in the absence and presence of increasing concentrations of purified PKR-GST (~ 40, 80 and 120 ng/ml) at 25°C for 45 minutes. The reactions were terminated by the addition of 100 µl of 1X sample buffer and the labeled proteins were separated on 10% SDS-PAGE. The gels were coomassie stained, dried and subjected to phosphorimage to visualize the [^{35}S] labeled protein products.

Panel A is a phosphorimage showing labeled protein products

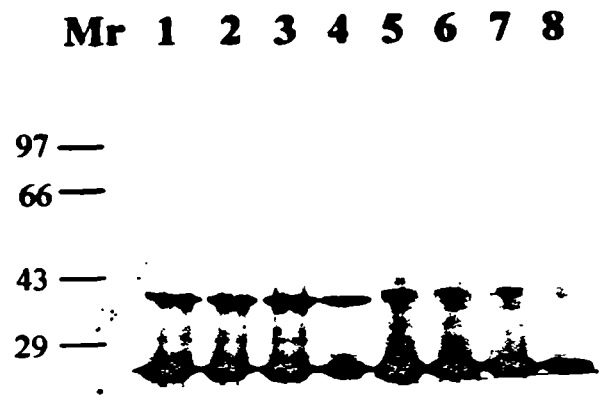
Panel B is lower half of western blot analyzed by phosphospecific anti eIF2α antibody **Panel C** is upper half of western blot analyzed by anti-PKR antibody.

Panel D is a ponceau stained copy of the western blot.

Lanes:

Mr, protein molecular weight marker, kD; 1, WGL + 106 RNA; 2, WGL + 106 RNA + PKR-GST (40 ng/ml); 3, WGL + 106 RNA + PKR-GST (80 ng/ml); 4, WGL + 106 RNA + PKR-GST (120 ng/ml); 5, WGL + 215 RNA; 2, WGL + 215 RNA + PKR-GST (40 ng/ml); 3, WGL + 215 RNA + PKR-GST (80 ng/ml); 4, WGL + 215 RNA + PKR-GST (120 ng/ml).

A


Mr 1 2 3 4 5 6 7 8
97 —
66 —
43 —
29 —

B

43 —
WG eIF 2α

C

97 —
PKR-GST

D

Mr 1 2 3 4 5 6 7 8
97 —
66 —
43 —
29 —

Fig. 24. Translation of chimeric mRNAs (301 and 400) in wheat germ lysate (WGL) in the presence of increasing concentrations of purified PKR-GST and analysis of eIF2α phosphorylation.

Translation of chimaeric RNAs 301 (plant α amylase leader and α amylase RNA) and 400 (AMV viral leader and AMV RNA) in wheat germ lysate was carried out as explained in the legend to Fig 27 in the absence or presence of increasing concentrations of purified mammalian recombinant PKR-GST.

Panel A is a phosphorimage showing labeled protein products

Panel B is lower half of western blot analyzed by phosphospecific anti eIF2α antibody

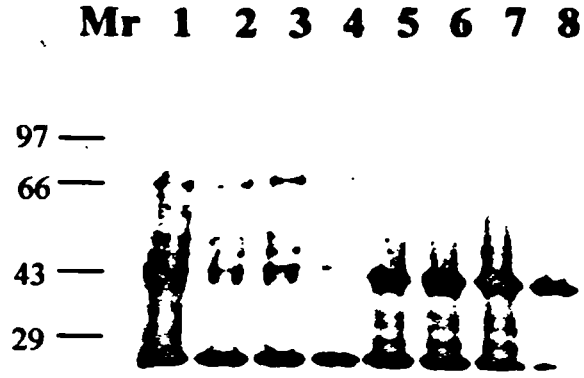
Panel C is upper half of western blot analyzed by anti-PKR antibody.

Panel D is a ponceau stained copy of the western blot.

Lanes:

Mr, protein molecular weight marker, kD; 1, WGL + 301 RNA; 2, WGL + 301 RNA + PKR-GST (40 ng/ml); 3, WGL + 301 RNA + PKR-GST (80 ng/ml); 4, WGL + 301 RNA + PKR-GST (120 ng/ml); 5, WGL + 400 RNA; 2, WGL + 400 RNA + PKR-GST (40 ng/ml); 3, WGL + 400 RNA + PKR-GST (80 ng/ml); 4, WGL + 400 RNA + PKR-GST (120 ng/ml).

A


Mr 1 2 3 4 5 6 7 8
97
66
43
29

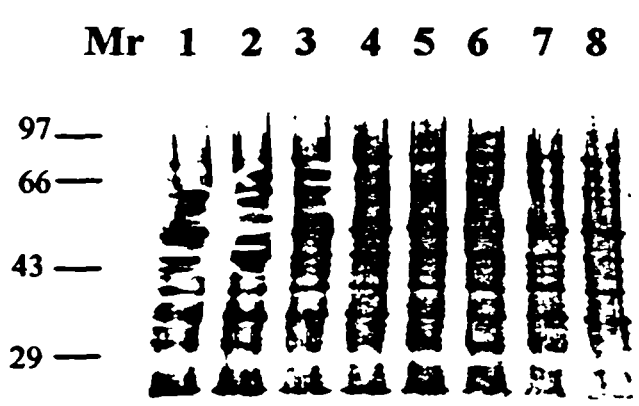
B

43
WG eIF 2α

C

97
PKR-GST

D


Mr 1 2 3 4 5 6 7 8
97
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29

Analysis of eIF2α phosphorylation by a phosphospecific anti-eIF2α antibody (Fig 22, Panel B) shows that addition of PKR stimulates wheat germ eIF2α phosphorylation to the same extent in the presence of BMV RNA (lane 3) and other RNAs (lanes 5, 7, 9 and 11). However, BMV RNA translation is inhibited significantly than any of the other RNAs as mentioned above. This is not due to any difference in the levels of added PKR or wheat germ lysates as has been analyzed by PKR antibody (Panel C) or poncaeus-stained gels (Panel D). Since there are examples that eIF2α phosphorylation can cause gene-specific inhibition and can also up regulate the translation of GCN4 like mRNAs (Hinnebusch, 1994), we have carried out translation of different mRNAs as mentioned above with the increasing concentrations of purified PKR (40, 80 and 120 ng) to determine the translational sensitivity of wheat germ lysate and the relative translational ability of different mRNAs to increasing eIF2α phosphorylation.

In the present study, we observed (Fig. 23 and 24) that the addition of increased PKR levels to a wheat germ lysates causes inhibition of translation of all the four chimaeric RNAs tested (Panel A in Fig. 23 and 24) and this is related to eIF2α phosphorylation (Panel B of the Fig. 23 and 24) and to the levels of PKR as identified in the western blots (Panel C of the Fig. 23 and 24). Interestingly, 301 mRNA (Barley α amylase) that is translated poorly as compared to other RNAs, is inhibited more strongly in the presence of PKR than other mRNAs. It is not known however, if this is due to the absence of AMV leader sequence in this RNA. These findings suggest that while wheat germ eIF2α phosphorylation is inhibitory and the translational sensitivity of different RNAs for eIF2α phosphorylation is somewhat at variance. Some RNAs probably require a higher level of phosphorylation in order to be inhibited, particularly the one with AMV leader. Whereas BMV and barley α amylase mRNAs are inhibited relatively at low levels of eIF2α phosphorylation compared to the other RNAs.

2.4. Addition of purified recombinant human S51D eIF2α inhibits BMV RNA translation: Recently this laboratory has expressed human eIF2α wild type and mutants such as S51A and S51D in which 51 serine residue is replaced by alanine or aspartic acid respectively in insect cells using bacculovirus expression system

Fig. 25. Protein synthesis in wheat germ lysate (WGL) in the presence of partially purified human recombinant human eIF2α (wild type and mutant forms).

The translation of BMV RNA (20 μg/ml) in wheat germ lysate was studied in the absence and presence of purified recombinant human recombinant human eIF2α wt or eIF2α S51A (where serine 51 is replaced with alanine) and eIF2α S51D (where serine 51 is replaced with aspartic acid) (~ 500 ng/ml) as described in 'Materials and Methods'. The reaction mixtures were incubated at 25°C for 45 minutes and the proteins were separated on 10% SDS-PAGE and analysed by western using a monoclonal anti-eIF2α antibody. The dried nitrocellulose membrane was subjected to phosphorimage to visualize the [^{35}S] methionine labeled protein products of BMV RNA.

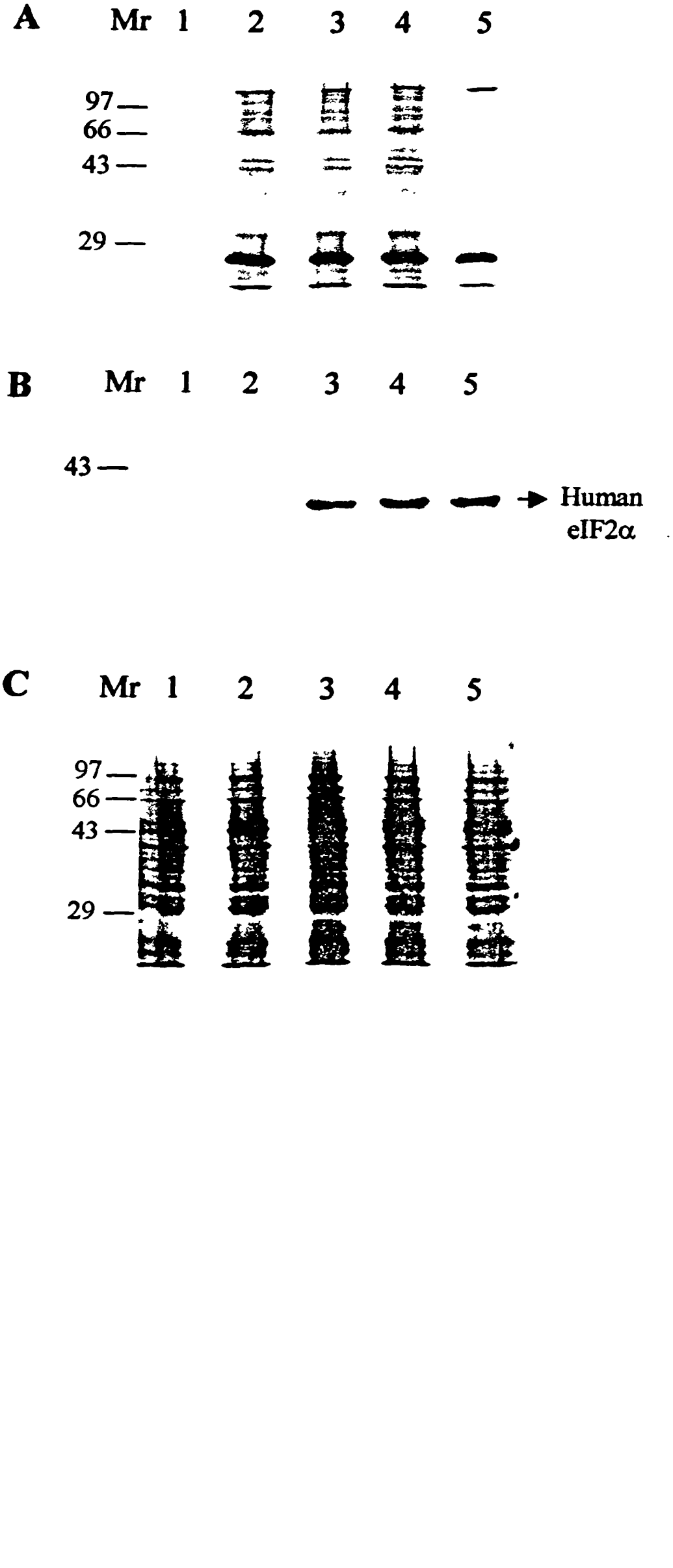
Panel A is a phosphorimage showing labeled protein products of BMV RNA.

Panel B is a western blot analyzed by polyclonal anti-eIF2α antibody.

Panel C is a ponceau stained copy of the western blot.

Lanes:

Mr, protein molecular weight marker, kD; 1, WGL; 2, WGL + BMV RNA; 3, WGL + BMV RNA + recombinant human eIF2α wt; 4, WGL + BMV RNA + recombinant human eIF2α S51A; 5, WGL + BMV RNA + recombinant human eIF2α S51D.


A
Mr
1
2
3
4
5
97
66
43
29
B
Mr
1
2
3
4
5
43
Human
eIF2α
C
Mr
1
2
3
4
5
97
66
43
29

(Sudhakar et al, 1999 and 2000). The purified over expressed proteins are shown in Fig. 12 of 'Materials and Methods'. The mutants of eIF2α, S51A and S51D cannot be phosphorylated by eIF2α kinases due to the absence of 51serine residue. However, S51D eIF2α is found to mimic like the phosphorylated form (eIF2αP) based on several studies (Choi et al, 1992; Ramaiah et al, 1994; Sudhakar et al, 2000). Here it is observed that addition of S51D eIF2α, in the absence of added PKR, inhibits the translation of BMV RNA in wheat germ lysates (Fig. 25, Panel A, lane 5). A similar effect is not observed in the presence of S51A eIF2α (lane 4) or wt eIF2α (lane 3). A polyclonal anti-eIF2α antibody is used to recognise the mutants and wt human eIF2α that are added to the translating lyasates. This antibody recognizes the mammalian eIF2α but not the wheat germ eIF2 (Fig. 25, Panel B). This is carried out essentially to show that uniform amounts of recombinant eIF2α (wt or mutant forms) are added to the lysate. Ponceaus stained gel (Fig. 25, Panel C) is shown to indicate the reaction mixtures consist of equal amount of wheat germ lysate.

2.5. Human S51A eIF2α and K3L proteins mitigate PKR-mediated translational inhibition in wheat germ lysates: In order to trace the inhibition of protein synthesis that is mediated by eIF2α phosphorylation, earlier studies have used S51A mutant of eIF2α. This mutant cannot be phosphorylated and is shown to by pass the protein synthesis inhibition and mitigate the inhibition in eIF2B activity caused by wt or endogenous eIF2α phosphorylation in cells that have been exposed to heat shock or other stress conditions in mammalian systems (Choi et al, 1992, Murtha-Riel et al, 1993; Ramaiah et al, 1994).

K3L protein is another probe that can be used to understand the importance of eIF2α phosphorylation in translational regulation. This protein is coded by vaccinia virus and has been sent as a free gift by one of our former colleagues Dr. Vattem M Krishna from Prof. R. C. Wek laboratory in Indianapolis, USA. This protein has 88 aa residues and has 28% homology to the N-amino terminus of eIF2α (Davies et al, 1992). This homology region is around serine 51 in eIF2α. However, K3L does not have a phosphorylated serine residue at this position. Based on the sequence similarity, it was proposed that it functions as a pseudosubstrate inhibiting PKR by binding the kinase catalytic domain (Beattie et al, 1991, Davies et al, 1992; Carroll et

Fig. 26. Effect of recombinant human eIF2α 51A on protein synthesis in wheat germ lysate treated with recombinant mammalian PKR-GST.

The translation of BMV RNA (20 μg/ml) was studied in the presence and absence of partially purified recombinant human eIF2α 51A (~ 500 ng/ml) in wheat germ lysate that was treated with or without PKR-GST (50 ng/ml) as described in 'Materials and Methods'. In a control reactions, BMV RNA translation in wheat germ lysate were studied by adding recombinant human eIF2α wt (~ 500 ng/ml) in wheat germ lysates that were treated with or without PKR-GST (50 ng/ml). The reaction mixtures were incubated at 25°C for 45 minutes and the proteins were separated on 10% SDS-PAGE and transferred to nitrocellulose membrane. The dried nitrocellulose membrane was subjected to phosphorimage to visualize the [^{35}S] methionine labeled protein products of BMV RNA. After which, the nitrocellulose membrane was cut in to two halves. The upper half of nitrocellulose membrane was treated with monoclonal anti-PKR antibody and the lower half of the membrane was probed with an phosphospecific anti-eIF2α antibody.

Panel A is a phosphorimage showing labeled protein products of BMV RNA.
Panel B is lower half of western blot analyzed by polyclonal phosphospecific anti-eIF2α antibody.
Panel C is upper half of western blot analyzed by a monoclonal anti-PKR antibody.
Panel D is a ponceau stained copy of the western blot.

Lanes: Mr, molecular weight marker (kD); 1, WGL; 2, WGL + BMV RNA; 3, WGL + BMV RNA + PKR-GST; 4, WGL + BMV RNA + PKR-GST + human eIF2α 51A; 5, WGL + BMV RNA + human eIF2α 51A; 6, WGL + BMV RNA + PKR-GST + human eIF2α wt; 7, WGL + BMV RNA + human eIF2α wt.

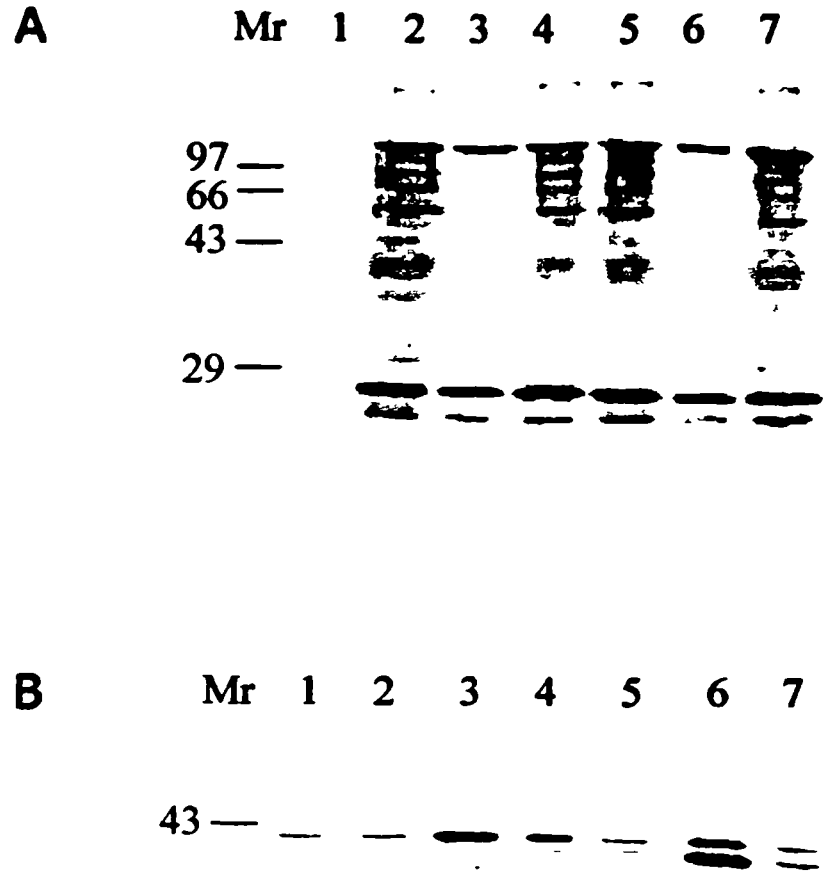

A
Mr 1 2 3 4 5 6 7
97
66
43
29
B
Mr 1 2 3 4 5 6 7
43
WG eIF2α
Human eIF2α
C
Mr 1 2 3 4 5 6 7
97
PKR-GST
D
Mr 1 2 3 4 5 6 7
97
66
43
29

Fig. 27. Effect of purified K3L on protein synthesis in wheat germ lysate treated with recombinant mammalian PKR-GST.

The translation of BMV RNA (20 μg/ml) was studied in the presence and absence of purified K3L protein (500 ng/ml) in wheat germ lysate that was treated with or without PKR-GST (50 ng/ml) as described in 'Materials and Methods'. The reaction mixtures were incubated at 25°C for 45 minutes and the proteins were separated on 10% SDS-PAGE and followed by western blotting. The dried nitrocellulose membrane was subjected to phosphorimage to visualize the [^{35}S] methionine labeled protein products of BMV RNA. After which, the nitrocellulose membrane was cut in to two halves. The upper half of nitrocellulose membrane was treated with monoclonal anti-PKR antibody and the lower half of the membrane was probed with phsophospecific anti-eIF2α antibody.

Panel A is a phosphorimage showing labeled protein products of BMV RNA.

Panel B is lower half of western blot analyzed by a phosphospecific anti-eIF2α antibody.

Panel C is upper half of western blot analyzed by a monoclonal anti-PKR antibody.

Panel D is a ponceau stained copy of the western blot.

Lanes:

Mr, molecular weight marker (kD); 1, WGL; 2, WGL + BMV RNA; 3, WGL + BMV RNA + PKR-GST; 4, WGL + BMV RNA + PKR-GST + K3L; 5, WGL + BMV RNA + K3L.

A
Mr 1 2 3 4 5
97
66
43
29
B
Mr 1 2 3 4 5
43
WG eIF 2α
C
Mr 1 2 3 4 5
97
PKR-GST
D
Mr 1 2 3 4 5
97
66
43
29

al, 1993). Infact, this is a viral trick to overcome host eIF2α phosphorylation (Kaufman, 1999a). Hence, we used both S51A and K3L proteins to determine if they can overcome the translational block in wheat germ lysates caused by PKR (Fig. 26 and 27 respectively). Addition of BMV RNA (Fig. 26, Panel A, lane 2) stimulates translation as can be seen from the protein products. Addition of PKR inhibits BMV RNA translation (lane 3). Addition of purified bacculovirus-expressed recombinant human S51A (lane 5) or wt eIF2α (lane 7) does not inhibit significantly translation in the absence of added PKR. However, PKR-mediated BMV RNA translational inhibition as observed in lane 3, is significantly mitigated by the addition of S51A protein (lanes 4), but not by wt eIF2α (lane 6).

In the same experiment (Fig. 26, Panel B), the phosphorylation status of endogenous wheat germ eIF2α has also been evalutated using phosphospecific anti-eIF2α antibody. This antibody identifies the phosphorylated recombinant human eIF2α and wheat germ eIF2α. Wheat germ eIF2α migrates slightly above the human eIF2α because of the differences in their molecular masses. In the absence of added PKR, wheat germ lysate eIF2α is not significantly phosphorylated (Fig 26, Panel B, lane 2). Addition of PKR stimulates wheat germ eIF2α phosphorylation (lane 3). Addition of S51A mutant of eIF2α reduces PKR-mediated wheat germ eIF2α phosphorylation (lane 4) and thus correlates to the increased translation under those conditions. As expected, the lower band corresponding to S51A eIF2α is not phosphorylated (lane 4). However, the recombinant wt human eIF2α is phosphorylated efficiently (lane 6) in PKR treated lysates. In this reaction, the endogenous lysate wheat germ eIF2α is also phosphorylated (lane 6). In the absence of added PKR, however the phosphorylation of wt eIF2α or wheat germ eIF2α is low and found to be insignificant (lane 1 and 7). These findings therefore suggest that PKR-mediated phosphorylation of wheat germ eIF2α and translational inhibition in lysates is reduced or mitigated partially in the presence of human recombinant S51A, but not by wt eIF2α. Such observations are not due to any difference in the levels of added PKR or wheat germ lysates as has been analyzed by PKR antibody (Fig. 26, Panel C) or poncaeus-stained gels (Fig. 26, Panel D).

The effect of addition of K3L protein in PKR-treated lysates is shown in Fig. 27. Purified K3L protein alone does not inhibit BMV RNA translation (Fig. 27, Panel

A, lane 5) and does not affect wheat germ eIF2α phosphorylation (Panel B, lane 2 vs. lane 5). Addition of K3L protein however reduces PKR-mediated translational inhibition and wheat germ eIF2α phosphorylation (compare lane 3 vs. lane 4 in Panel A & B). This is consistent with the ability of K3L protein to serve as a pseudosubstrate (Carroll et al., 1993).

3. Discussion

Mutants of human eIF2α like S51A, S51D and S48A were used earlier by various investigators to localize or detect the contribution of eIF2α phosphorylation in the translational inhibition caused by heat shock and other stress conditions (Murtha-Riel et al, 1993; Ramaiah et al, 1994). In fact a couple of recent publications from Roth's laboratory have demonstrated that over expression of wheat eIF2α S51A from vaccinia virus vectors inhibits partially the PKR-mediated translational inhibition in BSC-40 cells (African green monkey kidney cells) (Gil et al, 2000a) and also the expression of the above mutant overcomes the growth repression in yeast caused by amino acid starvation and GCN2 activation (Chang et al, 2000). Consistent with these observations, we observed that purified recombinant mammalian PKR-mediated translational inhibition in wheat germ lysates is mitigated by human S51A eIF2α mutant and also by a pseudosubstrate like K3L suggesting that phosphorylation of serine 51 residue in wheat germ eIF2α is inhibitory to translation. Complementing these observations, addition of S51D, a phosphomimetic form of human eIF2α also resulted in translational inhibition in wheat germ lysates. The mechanism of eIF2α phosphorylation-mediated translational inhibition is not yet understood. But, it is most likely that translation inhibition is mediated through the inhibition of eIF2B like activity in wheat germ lysate. This notion is supported by the reports that plants contain the nucleotide sequences of eIF2B subunits ε, α and δ (Chang et al, 2000). These results suggest that plants also carry an eIF2α phosphorylation pathway that appears to be similar to mammalian systems.

CHAPTER III

INTERFERON-γ (IFN-γ) mRNA TRANSLATION IN WHEAT GERM LYSATE AND eIF2 BINDING *IN VITRO*

Ben Asouli et al (2002) from Dr. Kaempfer's laboratory in Hebrew University, Jerusalem, Israel have shown recently that wild type full length IFN-γ mRNA form (1264nt) activates PKR efficiently and facilitates eIF2α phosphorylation as compared to the truncated forms (469 and 203 nt) of IFN-γ mRNA in BHK-21 cells and in reticulocyte lysates. Further, they observed that the 5' terminal 203nt IFN-γ mRNA fragment is sufficient to activate PKR at least as efficiently as dsRNA. The activation of PKR is achieved by a pseudoknot present in the 5' terminal region of IFN-γ mRNA. Mutations that impair the psedoknot stability reduce the ability to activate PKR and strongly increase translation efficiency of IFN-γ mRNA. Apparently, this is the first example of an mRNA that limits its own translation by activating PKR.

This laboratory is collaborating with Prof. Kaempfer's laboratory on certain aspects of eIF2 phosphorylation and I had a chance to work in his laboratory during August, 2000 for a month. I carried out some preliminary experiments during this period to determine the translational ability of wt and mutant forms of IFN-γ mRNAs in wheat germ lysate, their ability to activate wheat germ lysate PKR like activity (if any) and interaction with wheat germ eIF2 *in vitro* (by gel shift analysis).

Translation of wt and mutant forms of IFN-γ mRNA in wheat germ lysates: Wt IFN-γ mRNA and its mutants a4, d1, d2, d3 and d4 that are created by various substitutions or deletions in the 5' UTR as described by Ben Asouli et al (2002) (the sequence information of these mRNAs is shown in Fig 28, Panel B) were translated in wheat germ lysates using labeled methionine. The labeled protein products were analyzed by autoradiography (Fig. 28, Panel A). Translation of wt IFN-γ mRNA is found to be relatively poor compared to the mutant forms of IFN-γ mRNA (compare lane 3 vs. 4-8). A control reaction containing leuciferase mRNA translation is shown in lane 2. In the absence of added mRNA, no translation is observed in wheat germ lysates (lane 1). These results are essentially comparable to what has been observed by Kaempfer's group in cell-free translational systems derived from reticulocyte lysates.

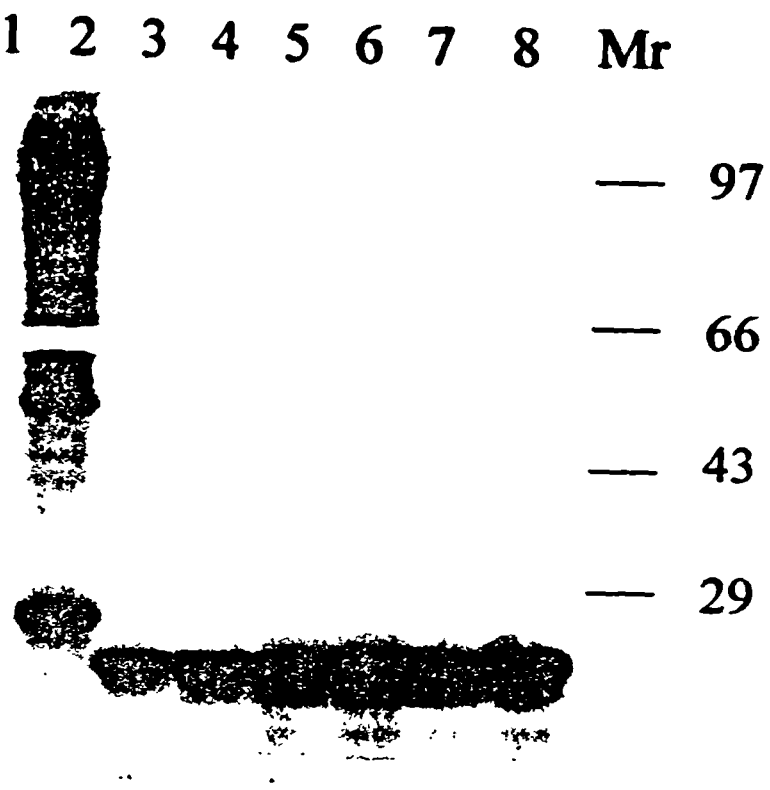
Fig. 28. Translation of IFN-γ mRNAs in wheat germ lysate.

Panel A: Standard lysate protein synthesis assays (25 µl) were carried out in wheat germ lysate in the presence of IFN-γ mRNAs, wild type (wt) and mutants (a4, d1, d2, d3 and d4) (15 µg/ml) corresponding to the lanes 3 – 8 respectively using [^{35}S] Methionine, at 25°C for 20 min. The reaction mixtures were terminated with the addition of SDS sample buffer and the proteins were separated by 10% SDS-PAGE as described in 'Materials and Methods'. The figure is an autoradiogram.

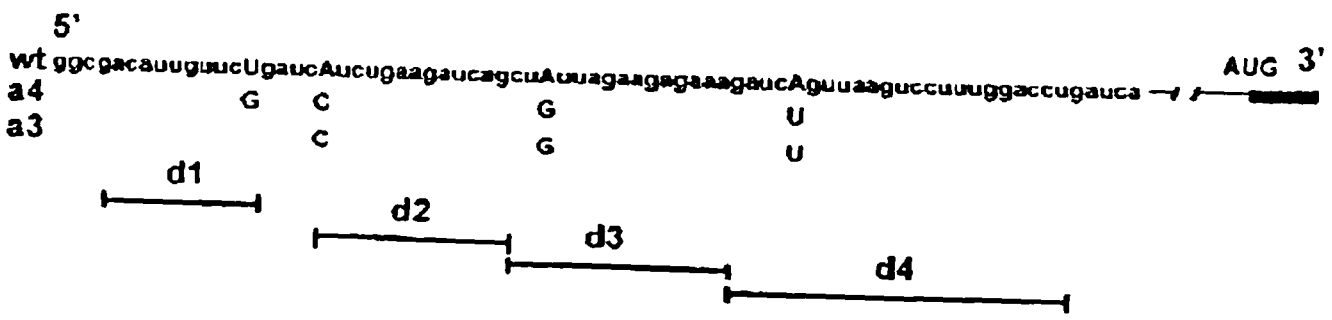
Lanes: Mr, molecular weight marker; 1, WGL; 2, WGL + luciferase mRNA; 3, WGL + wt IFN-γ mRNA; 4, WGL + a4 IFN-γ mRNA; 5, WGL + d1 IFN-γ mRNA; 6, WGL + d2 IFN-γ mRNA; 7, WGL + d3 IFN-γ mRNA; 8, WGL + d4 IFN-γ mRNA .

Panel B: Sequence information of wt IFN-γ mRNA on its 5' UTR, a4 mutant and the deletions made in d1, d2, d3 and d4 mutants (from Dr. Kaempfer's laboratory, Israel).

Panel A



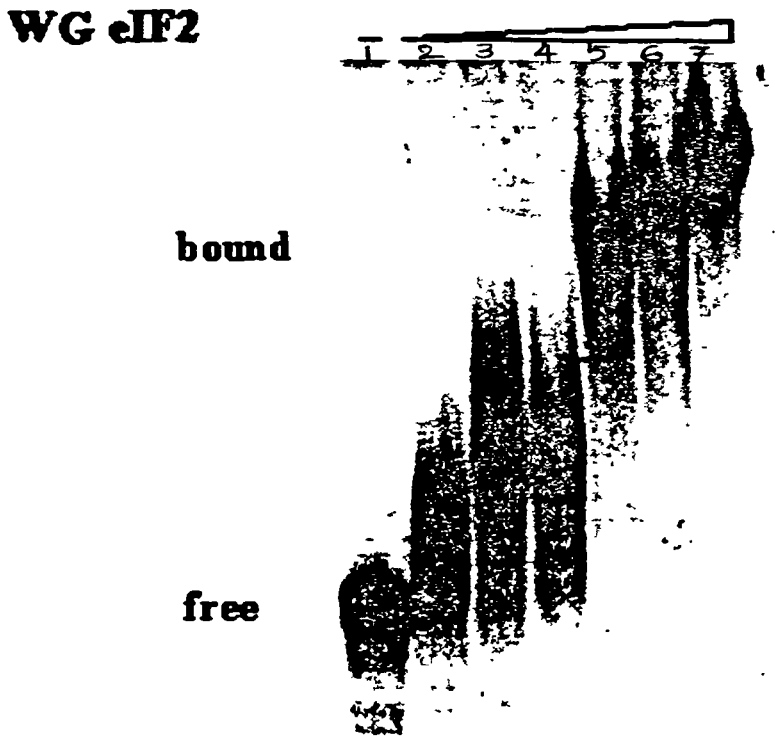
Panel B



Wheat germ eIF2 phosphorylation in translating lysates: Since the reduced translation of IFN-γ mRNA is correlated to increased PKR activation and eIF2α phosphorylation in reticulocyte lysates (Ben Asouli et al, 2002), we have also tested the ability of these RNAs to activate wheat germ lysate PKR (if any). The activation of PKR was monitored by eIF2α phosphorylation. However, addition of these RNAs (wt or mutants) has not increased eIF2α phosphorylation in translating wheat germ lysates (data not shown). This may be because the wheat germ lysates may not have a potent PKR-like kinase. This is consistent with many of our earlier observations wherein we failed to detect any stimulation in wheat germ lysate PKR activity or eIF2α phosphorylation in the presence of double stranded RNA. Hence we think that the requirement of PKR inactivation for augmented translation of the mutant forms of IFN-γ mRNAs is not obligatory in wheat germ system.

eIF2-mRNA binding studies: Because eIF2 from yeast system has been shown to promote AUG initiation codon recognition (Yoon and Donahue, 1992; Pestova et al, 1998) and affinity of an mRNA for eIF2 is found correlating with its ability to compete in translation in reticulocyte lysates (Rosen et al, 1982; Ben-Asouli et al, 2000), we have also evaluated the ability of IFN-γ mRNA binding to purified wheat germ eIF2 *in vitro*. In these experiments, 5'-terminal 203 nt IFN-γ mRNA fragment (203 nt RNA) (which has been shown to act as efficiently as dsRNA to stimulate PKR by Ben-Asouli et al, 2002) was used. The RNA transcript was labeled using [α-^{32}P] UTP during its transcription. Uniformly labeled 203 nt mRNA was incubated with increasing amounts of purified wheat germ eIF2 and the complexes were separated on 4% polyacrylamide gel and analyzed by autoradiography (Fig. 29). The migration of 203 nt mRNA is retarded with increasing concentrations of wheat germ eIF2. This finding suggests that wheat germ eIF2 forms a complex with IFN-γ mRNA. After establishing this point, further studies are carried out to identify the affinity between wheat germ eIF2 and different forms of IFN-γ mRNA. In these assays, the complex formation between labeled 203 nt IFN-γ mRNA and wheat germ eIF2 was studied in the absence and presence of unlabelled wt and mutants (d1-d4 and a4) of IFN-γ mRNAs The replacement of labeled mRNA by unlabeled competitor mRNA suggests that the competitor mRNA has a higher affinity for eIF2. Our observations (Fig. 30)

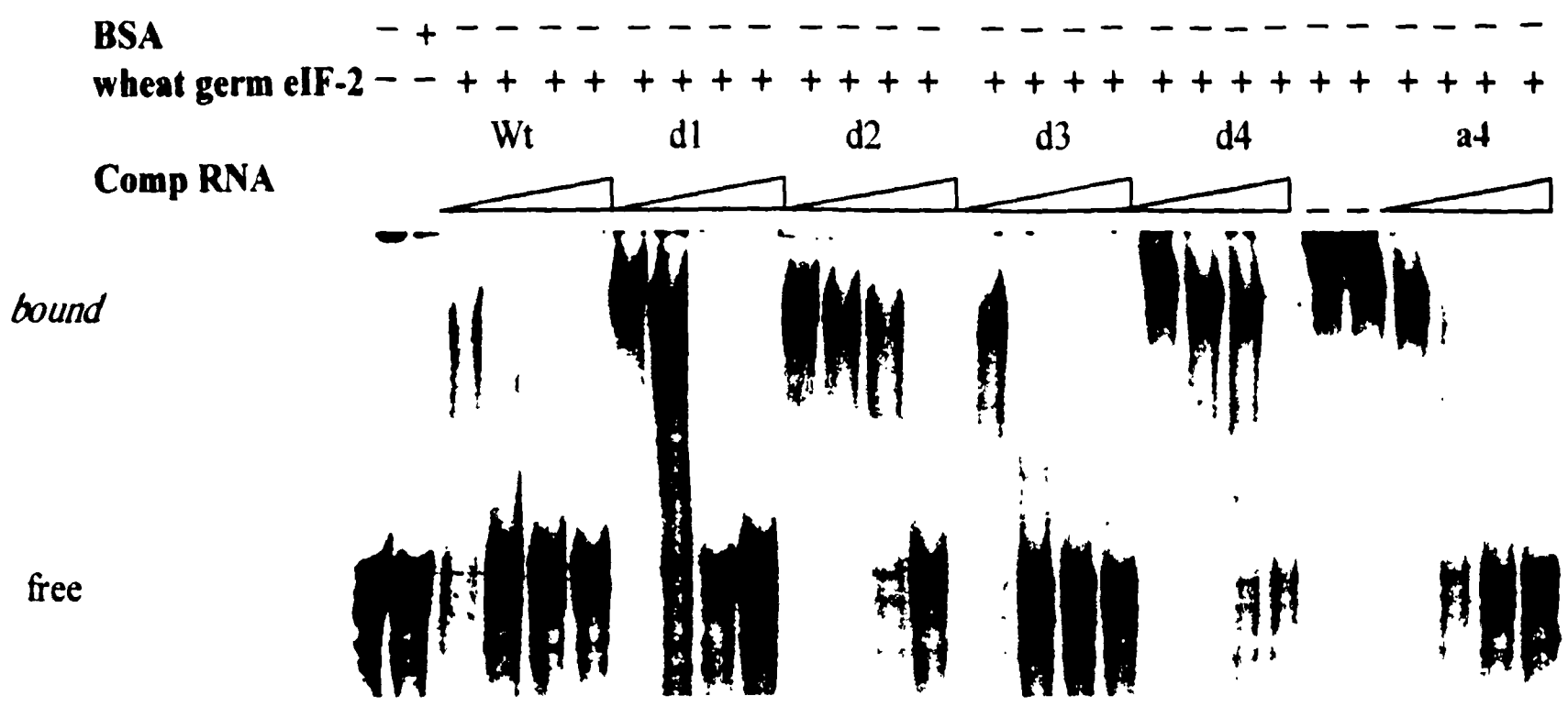
Fig. 29. Binding of wheat germ eIF2 (WG eIF2) to mRNA (human IFN-γ mRNA 5' – terminal 203 nt)



Uniformly [^{32}-P] labeled human IFN-γ mRNA 5' – terminal 203 nt transcript (0.08 pmol, 1.25 x 105 cpm/pmol) was incubated with out eIF2 (lane 1) or with wheat germ eIF2 (50, 100, 200, 400, 700 and 1000 ng corresponding to lanes 2 to 7 respectively). The reaction mixture was subjected to electrophoresis on a native gel to separate free human IFN-γ mRNA 5' – terminal 203 nt from labeled RNA bound to wheat germ eIF2. The figure is an autoradiogram.

Fig. 30. Electrophoretic mobility shift assay using labeled human IFN-γ mRNA 5'-terminal 203 nt and wheat germ eIF2 in the presence and absence of unlabeled wild type and mutants of IFN-γ mRNAs.

Uniformly [^{32}-P] labeled human IFN-γ mRNA 5'-terminal 203 nt transcript (0.08 pmol, 1.25 x 105 cpm/pmol) was incubated with out eIF2 or with 0.3 pico moles of wheat germ eIF2 in the absence or presence of unlabeled wild type (wt) and mutants (a4, d1, d2, d3, and d4 of IFN-γ mRNAs (0.005, 0.01, 0.02 and 0.05 pmol) (comp. RNA). The autoradiogram shows free and bound RNA. Two controls were used. One control was carried out in the presence of BSA (served as a protein control) and the other one is carried out in the absence of the competitor RNA. The samples were subjected to electrophoresis on a native gel to separate free RNA from bound RNAs. The figure is an autoradiogram.


BSA
wheat germ eIF-2
Wt
d1
d2
d3
d4
a4
Comp RNA
bound
free

indicate that the labeled complex formation is reduced efficiently in the presence of low concentrations of (0.01-0.05 p mol) of wt, d3 and a4 IFN-γ mRNAs. However, higher concentration of d1,d2 and d4 mRNAs are required to replace the labeled 203nt mRNA. These results suggest that the deletion mutants (d1, d2 and d4) have reduced affinity for eIF2 as compared to wt and the importance of the deleted regions in eIF2 binding.

These results on the binding between wheat germ eIF2 and different IFN-γ mRNA forms are essentially similar, except for d3 mutant mRNA, to what has been observed by Kaempfer's group with reticulocyte eIF2. This suggests that plant and mammalian eIF2 discriminate different mRNA species essentially in the same manner.

These preliminary data support the idea that plant eIF2 behaves like mammalian eIF2 in its binding to mRNA but the physiological significance of this eIF2-mRNA interaction on the activation of PKR-like kinase or translational ability of mRNAs in plant systems has yet to be understood.

CHAPTER IV

CASPASE-LIKE ACTIVITY IN WHEAT GERM LYSATES

1. Introduction

2. Results

2.1. Caspase like activity in wheat germ lysates: Specific hydrolysis of Ac-DEVD-AFC in wheat germ lysate

2.2. Caspase-specific inhibitor reduces the caspase activity

2.3. DNA laddering in rat liver nuclei

2.4. Physiological significance

3. Discussion

1. Introduction

The caspase family of cysteine proteases was discovered following a search of human cDNA libraries for sequences homologous to ced-3, a cell death gene described in the nematode worm *C. elegans* (Ellis et al, 1991). The first mammalian homologue of CED-3 to be identified was ICE (interleukin-1β converting enzyme) (Yuan et al, 1993). Subsequently numerous mammalian ced-3 homologues have been discovered. The term "caspase" was adopted as a root name for all family members that are known now as cysteinyl-aspartate proteases that have an ability to cleave very specific peptide substrate after aspartic acid residues. Caspases are the executioners of cell death. Caspase activation starts with autoprocessing of procaspase-9 to the active caspase, which then cleaves procaspase-3 to the active caspase-3, the main executioner of the final degradation phase of the animal cell death program (PCD). Caspases cleave a host of cellular substrates such as endonucleases (Enari et al, 1998), protein kinases (Lahti et al, 1995; Saelens et al, 2001), Poly (ADP-ribose) polymerase, PARP (Lazebnik et al, 1994), fodrins, actin and the lamins (Martin et al, 1995; Rao et al, 1996; Orth et al, 1996) and several of the translational factors like eIF2α (Satoh et al, 1999), eIF4G, eIF4B and eIF3 (Clemens et al, 2000) there by affecting the various pathways involved in gene expression, cellular signaling and the components involved in maintaining the cell shape and integrity. The hallmarks of apoptosis include nuclear condensation, cellular blebbing, DNA laddering and caspase activation. Again animal systems are well explored for studying the apoptosis phenomena and relatively very little is known about plant apoptotic phenomena. For example, the cytoplasmic caspase-mediated apoptotic pathway is highly conserved between animal cells but, such a death cascade was not found in plant cells so far. In spite of the fact that sequences of the highly conserved members of the caspase protein family have not been found in most current genomic databases and expressed sequence tags from plant cells yet, a lot of cysteine and aspartate proteinases are described to be involved in plant programmed cell death that occurs due to hypersensitive response (del Pozo and Lam, 1998), senescence (Drake et al, 1996) seed germination (Wang et al, 1996; Schmid et al, 1999), oxidative stress (Solomon et al, 1999; Maccarrone et al, 2000; Amor et al, 1998), pathogen interaction (Che et al, 1999) and heat stress (Balk et al, 1999; Chen et al, 2000; Tian et al, 2000) etc. The typical morphological changes with DNA fragmentation, cytoplasmic condensation or

membrane blebbing (Dangl et al, 2000; Che et al, 1999; Balk et al, 1999) are also connected with the well-known molecular characteristics. These are release of cytochrome c (Balk et al, 1999; Sun et al, 1999), activation of caspase-3-like proteinase (Lam and del Pozo, 2000; Chen et al, 2000; Korthout et al 2000), and subsequent cleavage of lamin-like proteins and poly (ADP-ribose) polymerase (Amor et al, 1998; Chen et al, 2000, Tian et al, 2000, Sun et al, 1999). New synthesis and/or activation of additional proteases were also reported (Delorme et al. 2000, Scarpa et al, 1999). Finally, there is evidence for a role of a membrane-associated Bax protein (Bcl-2 family) as a death-promoting factor and the existence of corresponding Bax inhibitor proteins in plants (Lam and del Pozo, 2000; Sanchez et al, 2000).

Since this is laboratory involved in studying translational regulation of initiation mediated by eIF2 and eIF2α kinases and the recent studies have shown that mammalian eIF2 and eIF2α kinases are substrates for active caspases (Satoh et al, 1999; Marissen et al, 2000; Saelens et al 2000; Aparna et al., 2002), we have undertaken some preliminary studies to determine if wheat germ has any caspase-like activity that affects overall translation. In addition, we always observed wheat germ eIF2α subunit as a doublet (p41 and p42). In contrast mammalian eIF2 has three subunits, which are in 1:1:1 ratio. This is not clearly the case with wheat germ eIF2. The fact that both these subunits are phosphorylated by addition of PKR in our experiments, it is suspected that they may have resulted because of some kind of a proteolytic activity. Hence we carried out assays to determine if wheat extract has any caspase-like activity. Our findings, though preliminary, suggest that wheat germ has a caspase-like activity based on its ability to cleave Ac-DEVD-CHO, a caspase 3-inhibitor but not by other proteinase inhibitors. Further, it has been observed that wheat germ extract can promote DNA laddering in rat liver nuclei and wheat germ lysate treated with the caspase-inhibitor has shown improved translation.

2. Results

2.1. Caspase like activity in wheat germ lysates: Specific hydrolysis of Ac-DEVD-AFC in wheat germ lysate: Caspase activity of wheat germ extracts (2 mg protein)

Fig. 31. Ac-DEVD-AFC hydrolysis in wheat germ extract.

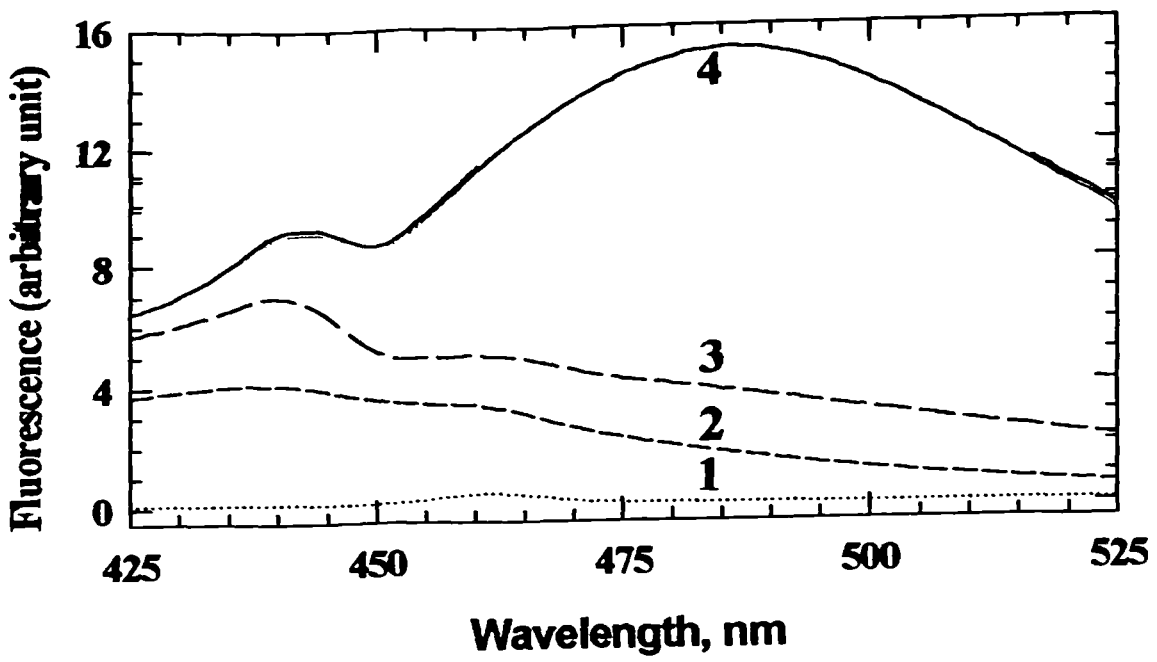
In an *in vitro* caspase assay system (1ml), 100 µl of wheat germ extract was diluted to 1 ml with 20 mM Hepes-KOH, pH 7.5 and was incubated at 25°C for 75 minutes in the presence of 200 µM Ac-DEVD-AFC, a synthetic tetrapeptide that acts as a substrate for caspase-3. The fluorescence was measured in a spectrofluorometer (excitation : 400 nm and emission: 505 nm) at two intervals (5 minutes and 75 minutes) after the addition of Ac-DEVD-AFC. In the control assays only 20 mM Hepes buffer was used instead of wheat germ extract.

Panel A represents the fluorescence emitted (arbitrary units) after incubation of samples at 5 minutes or 75 minutes, due to hydrolysis of Ac-DEVD-AFC against the wavelength range from 425 to 545 nM.

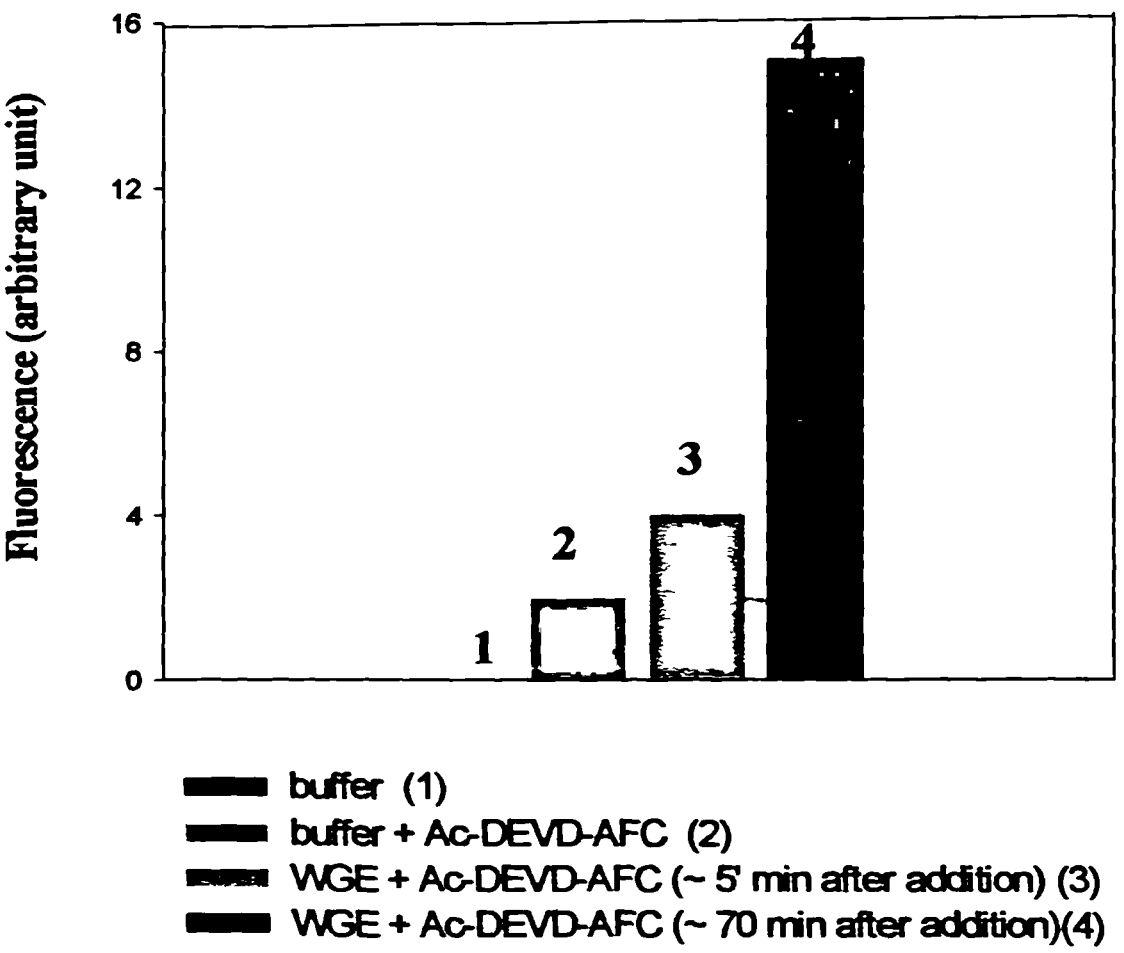
1) Buffer alone
2) Buffer + Ac-DEVD-AFC
3) Wheat germ extract + Ac-DEVD-AFC (5 minutes after addition)
4) Wheat germ extract + Ac-DEVD-AFC (75 minutes after addition)

Panel B, bar diagram, represents the maximum fluorescence emitted at 490 nM in *in vitro* caspase assays of Panel A.

Panel A



Panel B



was measured using Ac-DEVD-AFC, a synthetic substrate for mammalian caspase-3. The fluorescence signal of AFC that was released due to the breakdown of the substrate was monitored by fluorescence emission at 505 nM on excitation at 400 nm for 5 min or for 75 min (Fig. 31). As can be seen from the figure, wheat germ lysate has caspase-3 like activity that can hydrolyse the synthetic substrate was used in these studies. Fractionation of wheat germ lysate by ammonium sulfate precipitation results in the identification of caspase-like activity mostly present in 0-40% ammonium sulfate fraction (Fig. 32).

2.2. Caspase-specific inhibitor reduces the caspase activity: The ability of 0-40% ammonium sulfate fraction of wheat germ extract to hydrolyse Ac-DEVD-AFC was studied in the presence and absence of Ac-DEVD-CHO, a synthetic, specific inhibitor of mammalian caspase-3 (Fig. 33). In the presence of the latter, significant reduction in the hydrolysis of the flourogenic substrate, Ac-DEVD-AFC is observed. However the hydrolysis of Ac-DEVD-AFC is resistant to the protease inhibitors such as aprotenin, leupeptin, pepstatin and PMSF (Fig. 34). Interestingly, the protease inhibitors stimulate the hydrolysis of Ac-DEVD-AFC and the effect is found cumulative in the presence of all the inhibitors. These findings therefore suggest that the ability of wheat germ extract to hydrolyze Ac-DEVD-AFC is due to a caspase-like activity but not due to any protease activity.

2.3. DNA laddering in rat liver nuclei: One of the characteristic features of caspases is to cause DNA degradation that can result in the formation of oligonucleosomal fragments. Hence we have studied the ability of wheat germ extract to induce DNA-laddering in rat liver nuclei (Fig. 35). Rat liver nuclei were incubated with the extract for 8 hrs and the extracted DNA was degraded in to oligonucleosomal fragments (lanes 4, 5). This suggests that extract contains an activity that is typical to the caspases.

2.4. Physiological significance: To determine the physiological significance, translation of wheat germ lysates is carried out in the presence of Ac-DEVD-CHO, a specific caspase inhibitor (50 and 200 ng/ml) (Fig. 36). Interestingly, BMV RNA translation, as analyzed by phosphorimager stimulated in the presence of 50 and 200

Fig. 32. Ac-DEVD-AFC hydrolysis in the presence of ammonium sulphate fractionations of wheat germ extract.

In a standard (1ml) *in vitro* caspase assay system, 1 mg of 0-40% or 40-80% ammonium sulphate fractionated wheat germ extract was incubated in 20 mM Hepes-KOH, pH 7.5 buffer in the presence of 200 μM Ac-DEVD-AFC at 25°C for 30 minutes. The fluorescence was measured in spectrofluorometer (excitation : 400 nm and emission: 505 nm) as described in 'Materials and Methods'.

Panel A represents the fluorescence emitted at the end of incubation period of 30 minutes over a wavelength range of 450 to 550 nM.

1) 0-40% WGE + Ac-DEVD-AFC (30 min after the addition)
2) 40-80% WGE + Ac-DEVD-AFC (30 min after the addtion)

Panel B, bar diagram represents the maximum fluorescence emitted at wavelength 490 nM in *in vitro* caspase assays of Panel A.

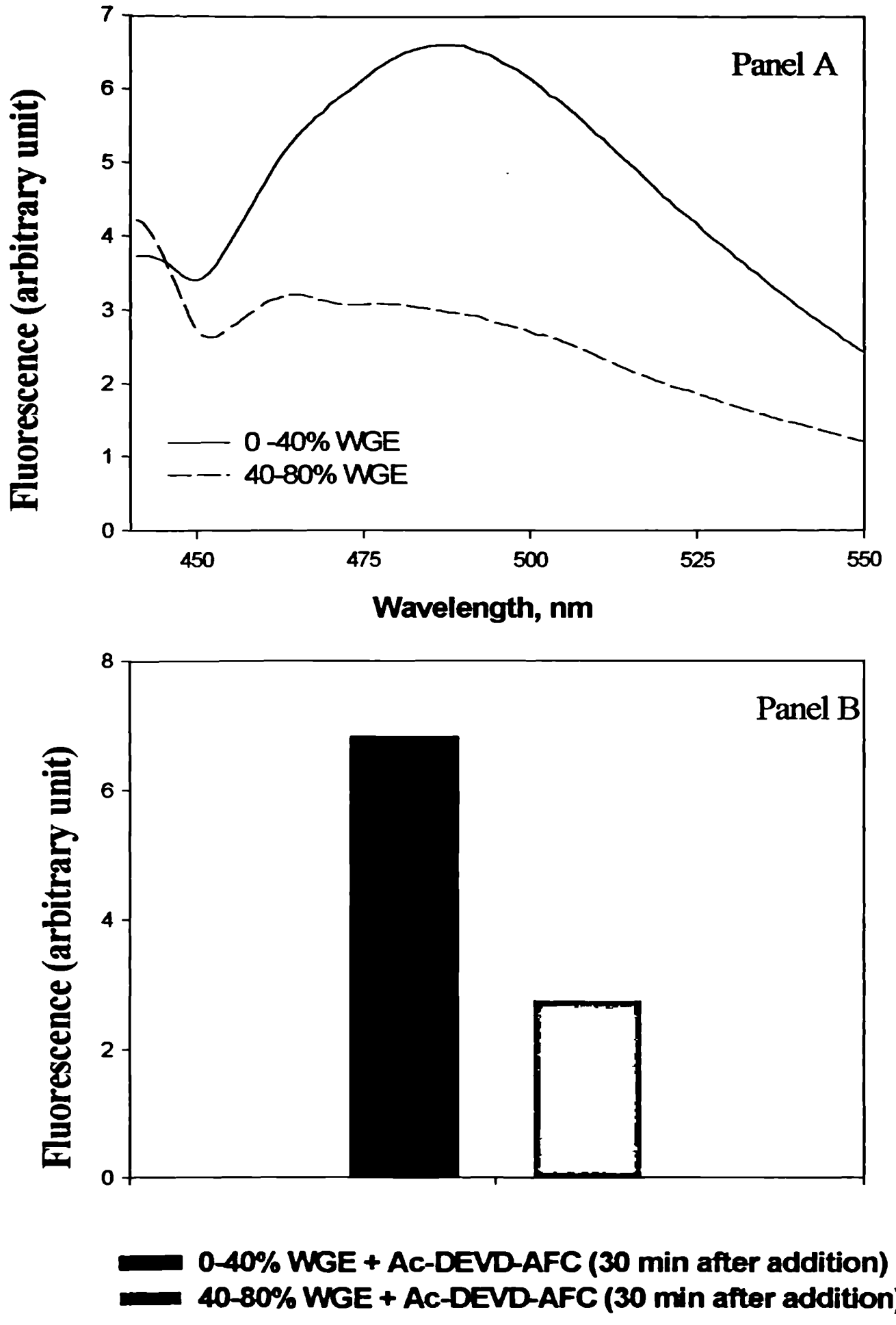

Panel A
Fluorescence (arbitrary unit)
7
6
5
4
3
2
1
0
0 -40% WGE
40-80% WGE
450
475
500
525
550
Wavelength, nm
Panel B
Fluorescence (arbitrary unit)
8
6
4
2
0
0-40% WGE + Ac-DEVD-AFC (30 min after addition)
40-80% WGE + Ac-DEVD-AFC (30 min after addition)

Fig. 33. Effect of caspase-3-specific inhibitor, Ac-DEVD-CHO on the hydrolysis of Ac-DEVD-AFC in the presence of 0-40% ammonium sulphate fractionation of wheat germ extract.

Standard caspase assay system (1ml) as described in 'Materials and Methods' consists of 1mg of 0-40% ammonium sulphate fractionation of wheat germ extract in 20 mM Hepes-KOH, pH 7.5 that was incubated at 25°C for 30 minutes with 200 μM Ac-DEVD-AFC in the absence and presence of caspase-3-specific inhibitor, 400 μM Ac-DEVD-CHO. The fluorescence was measured in spectrofluorometer (excitation : 400 nm and emission: 505 nm) as described in 'Materials and Methods'..

Panel A represents the fluorescence emitted at the end of incubation period of 30 minutes over a wavelength range of 450 to 550 nM.

1) 0-40% WGE
2) 0-40% WGE + Ac-DEVD-AFC
3) 0-40% WGE + Ac-DEVD-CHO + Ac-DEVD-AFC

Panel B, bar diagram, represents the maximum fluorescence emitted at wavelength 490 nM *in invitro* caspase assays of Panel A.

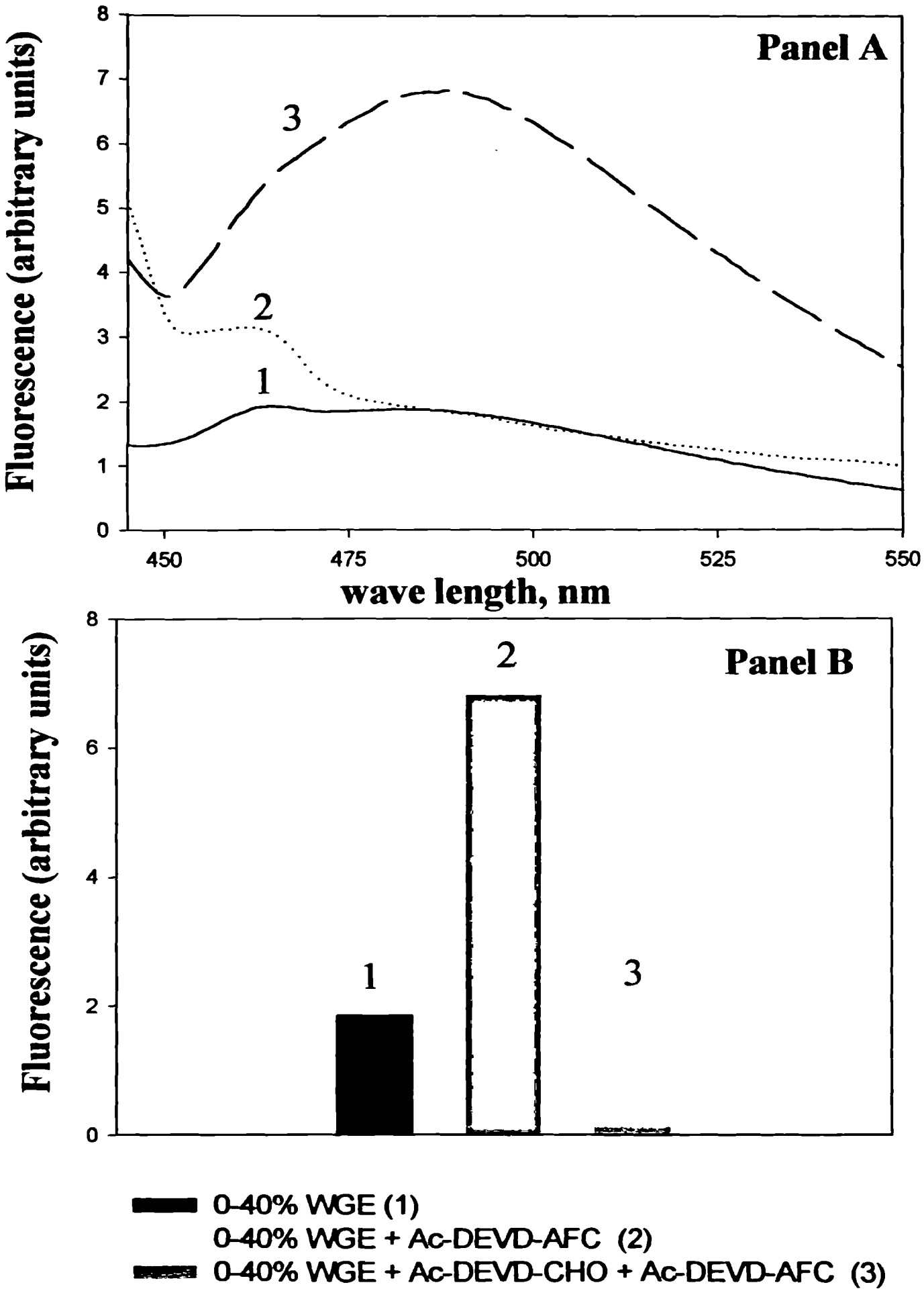

Panel A
Fluorescence (arbitrary units)
8
7
6
5
4
3
2
1
0
3
2
1
450
475
500
525
550
wave length, nm
Panel B
Fluorescence (arbitrary units)
8
6
4
2
0
2
1
3
0-40% WGE (1)
0-40% WGE + Ac-DEVD-AFC (2)
0-40% WGE + Ac-DEVD-CHO + Ac-DEVD-AFC (3)

Fig. 34. Effect of different protease inhibitors on the hydrolysis of Ac-DEVD-AFC in wheat germ extract.

In a standard (1ml) *in vitro* caspase assay system, 100 µl of wheat germ extract was diluted to 1ml using Hepes-KOH, pH 7.5 and incubated with 200 µM Ac-DEVD-AFC at 25°C for 30 min in the absence or presence of protease inhibitors such as aprotenin or leupeptin or pepstatin or PMSF or the mixture of all these protease inhibitors. The fluorescence was measured in spectrofluorometer (excitation : 400 nm and emission: 505 nm) as described in 'Materials and Methods'.

Panel A represents the fluorescence emitted at the end of incubation period of 30 minutes over a wavelength range of 450 to 550 nM.

1) WGE + Ac-DEVD-AFC
2) WGE + Ac-DEVD-AFC + aprotenin
3) WGE + Ac-DEVD-AFC + leupeptin
4) WGE + Ac-DEVD-AFC + pepstatin
5) WGE + Ac-DEVD-AFC + PMSF
6) WGE + Ac-DEVD-AFC + mixture of protease inhibitors

Panel B, bar diagram represents the maximum fluorescence emitted at wavelength 490 nM in *in vitro* caspase assays of Panel A.

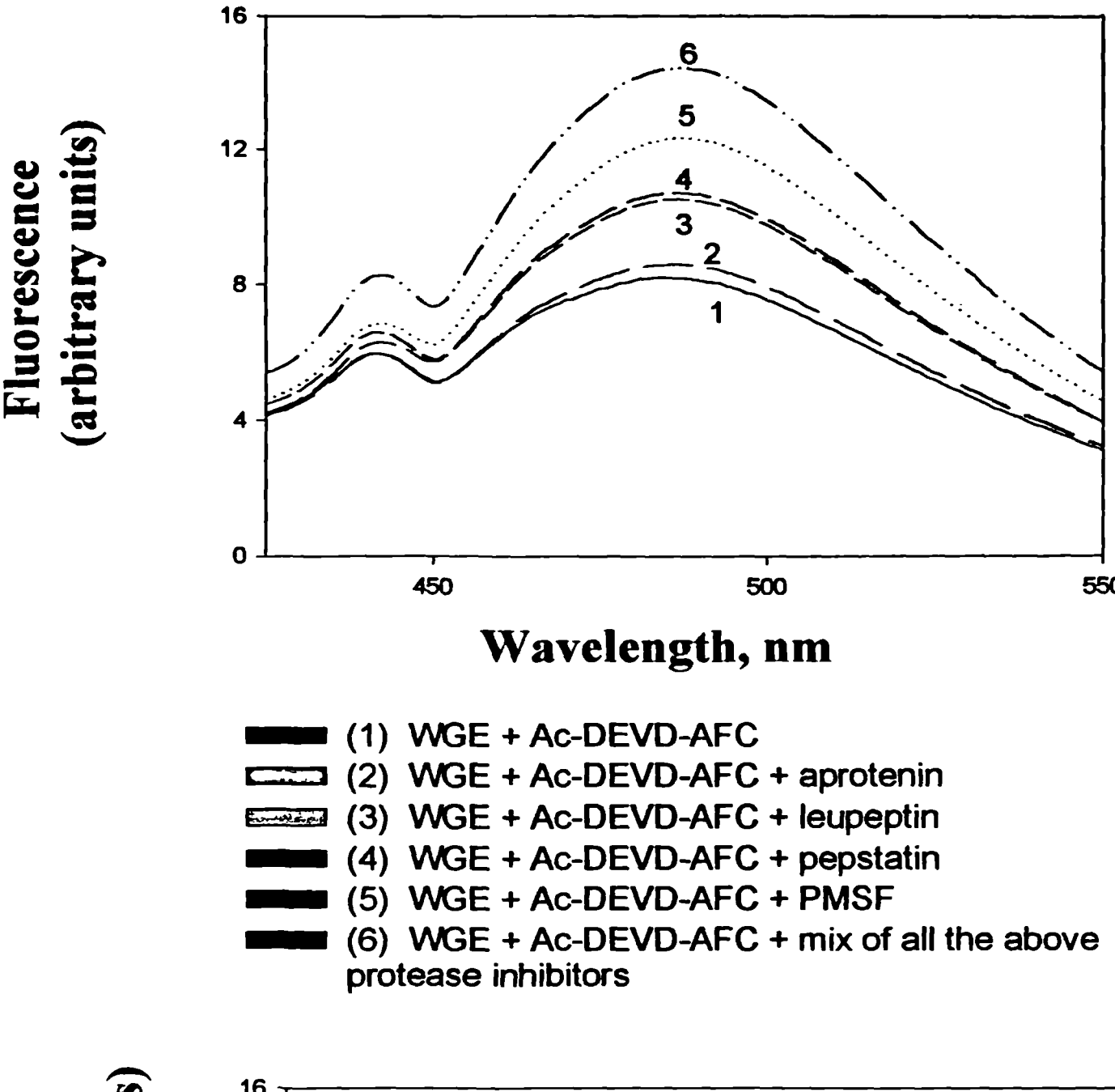
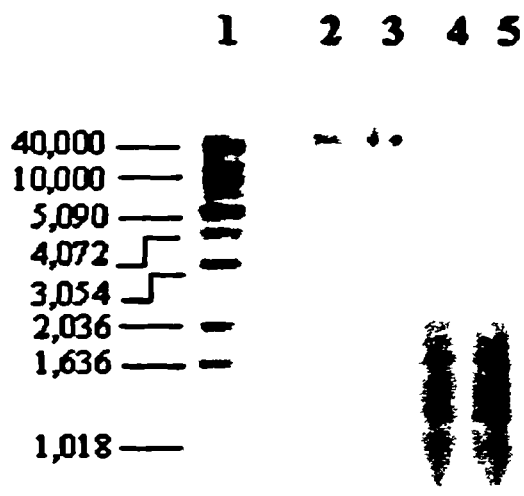

Fluorescence (arbitrary units)
16
12
8
4
0
6
5
4
3
2
1
450
500
550
Wavelength, nm
(1) WGE + Ac-DEVD-AFC
(2) WGE + Ac-DEVD-AFC + aprotenin
(3) WGE + Ac-DEVD-AFC + leupeptin
(4) WGE + Ac-DEVD-AFC + pepstatin
(5) WGE + Ac-DEVD-AFC + PMSF
(6) WGE + Ac-DEVD-AFC + mix of all the above protease inhibitors
Fluorescence (arbitrary units)
16
12
8
4
0
1 2 3 4 5 6

Fig. 35. Specific degradation of DNA into oligonucleosomal fragments in rat liver nuclei that were incubated in wheat germ extract.



Rat liver nuclei were purified as described in 'Materials and Methods'. The purified nuclei (100 µl) were incubated with wheat germ extract for 8 to 10 hrs at 25°C. In a control experiment, rat liver nuclei (100 ml) were incubated in Hepes-KOH, pH 7.5. At the end of incubation the DNA was extracted as described in 'Materials and Methods'. The samples were suspended in 10 vol of buffer D (100 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.2 M NaCl, 0.4% SDS, 0.2 mg/ml proteinase K) and incubated overnight at 37°C, then extrated with 1:1 phenol:chloroform, and precipitated by 2 vol of ethanol. DNA extracts were electrophoresed in 1.2% agarose gels in TBE buffer as described in 'Materials and Methods'. DNA was visualized by ethidium bromide staining. The figure is an agarose gel viewed under UV light.

Lanes:

1) DNA marker (bases)
2) and 3) DNA extracted from rat liver nuclei that were incubated in 20 mM Hepes-KOH, pH 7.5
4) and 5) DNA from extracted from rat liver nuclei that were incubated in 100 µl of wheat germ extrat.

Fig. 36. Effect of caspase-3-specific inhibitor on wheat germ protein synthesis.

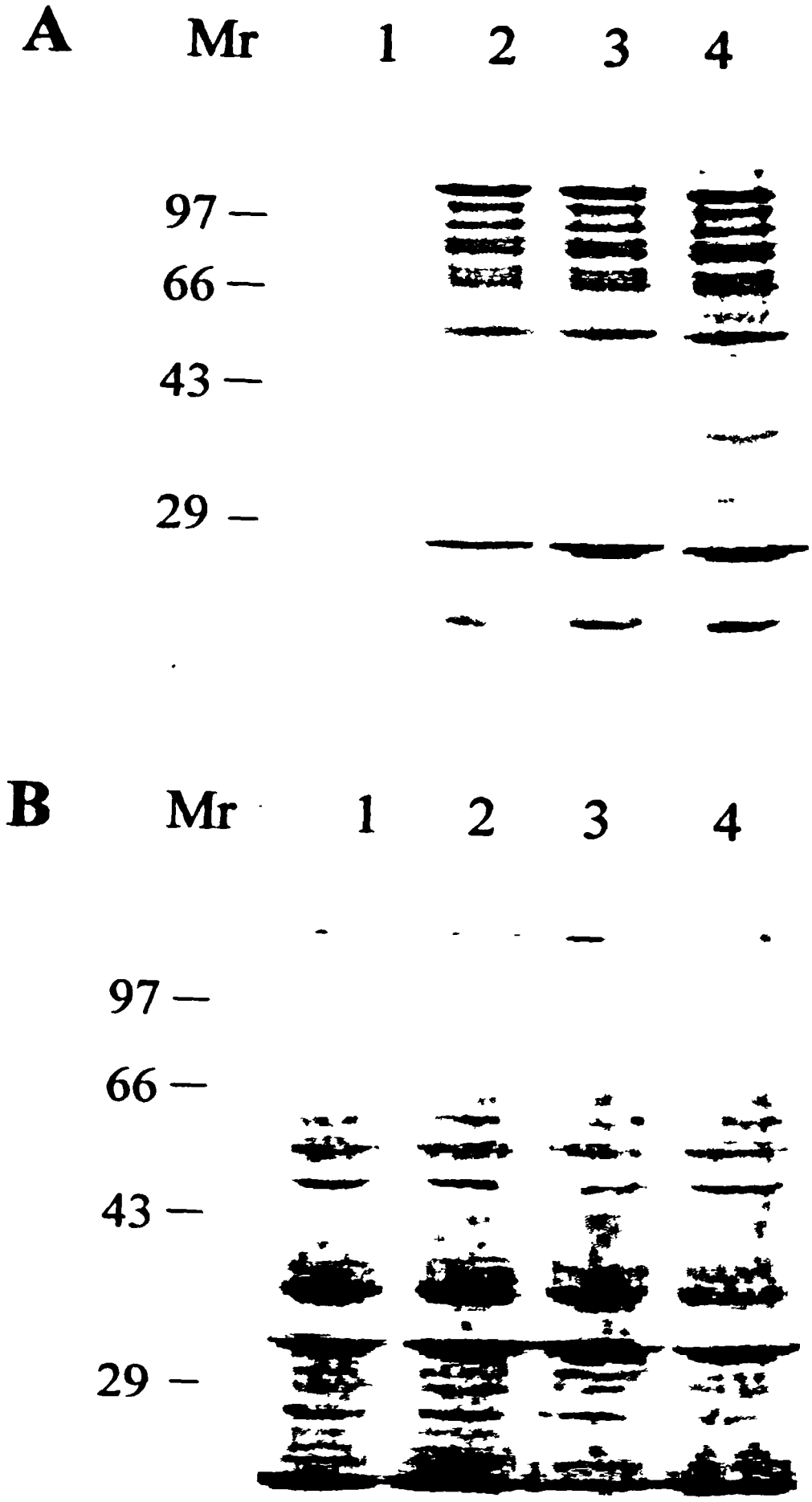
Standard wheat germ lysate protein synthesis assays (25 µl) were carried using BMV RNA (20 µg/ml) at 25°C for 45 minutes in the absence or increasing concentration of caspase-3-specific inhibitor, Ac-DEVD-CHO. The reaction was terminated by addition of 100 ml of 1X sample buffer and the labeled proteins were separated on 10% SDS-PAGE. The gel was coomassie stained, dried and analyzed by phosphorimage.

The figure is a phosphorimage showing [35S] labeled protein products of BMV RNA translated in wheat germ lysate that was treated with or with out caspase inhibitor.

Panel A: Phosphorimage

Panel B: Coomassie stained dried gel

Lanes : Mr, molecular weight marker (kD); 1, WGL; 2, WGL + BMV RNA; 3, WGL + BMV RNA + 50 ng/ml of Ac-DEVD-CHO; 4, WGL + BMV RNA + 200 ng/ml of Ac-DEVD-CHO.


A
Mr
1
2
3
4
97 –
66 –
43 –
29 –
B
Mr
1
2
3
4
97 –
66 –
43 –
29 –

ng/ml of the inhibitor (lanes 3 and 4 vs. 2). This finding suggests that probably caspase-like activity that exists in wheat germ lysates is detrimental to the translation.

3. Discussion

In this study, we observed caspase-like activity in wheat germ lysates that is inhibitory to its translation. Although these observations are preliminary, they can be the basis for purification of caspase-like protein from the wheat germ extract and to identify the substrates for caspase like proteins from plants, particularly the translational factors, in order to determine the mechanism of translational inhibition mediated by caspase activation. Although the caspases are generally in their precursor form and are triggered by different stimuli to become active caspases, we have not yet understood the basis for active caspase like activity associated with the wheat germ extracts that were prepared from wheat embryos. A recent study infact describes that multiple tissues undergo cell death during wheat seed development (Young and Gallie, 2000). This is consistent with our observations in this study. There are no reports to date, however indicating such a caspase-like activity influencing the translational ability or regulating the mRNA translation in plant system.

SUMMARY

The major observations of the present thesis are as follows:

- DTT, GSH and NEM stimulate the phosphorylation of several wheat germ lysate proteins, including WG eIF2α, where as, oxidizing agents such as diamide, GSSG and heat treatment decrease the same.
- Heat treatment causes a profound decrease in the phosphorylation of proteins, inclusion of NEM, DTT or GSH prevents the decrease in phosphorylation. However, these agents do not mitigate the heat-induced translational inhibition. Further, DTT is able to mitigate the translational inhibition caused by oxidizing agents such as diamide
- Heat shock, NEM and diamide decrease the –SH content of wheat germ lysate. NEM treatment results in low –SH content but this is due to alkylation of –SH groups. The decrease in –SH content in diamide and in heat-treated lysates is completely prevented by DTT. But, DTT prevents partially the decrease in phosphorylation, but not translation, that was caused by heat treatment.
    - Overall, these findings suggest that in addition to maintenance of –SH groups, heat-labile proteins may play an important role in translation and phosphorylation abilities of a lysate.
- Heat treatment induces eIF2α phosphorylation at serine 51 residue in reticulocyte lysate, however a similar heat treatment to the wheat germ lysate does not affect the basal phosphorylation of wheat germ eIF2α. This suggests that wheat germ lysates probably do not carry HRI-like protein or an eIF2α kinase that can be activated by heat shock.
- NEM mediated phosphorylation of wheat germ eIF2α occurs at a site other than serine 51. Further, casein kinase II (CKII) mediated phosphorylation of WG eIF2α is enhanced by NEM *in vitro* and in lysates. The phosphopeptide analysis suggests that NEM-induced phosphorylation of WG eIF2α in lysates resembles CKII -mediated phosphorylation. These findings therefore suggest that NEM stimulates CKII-like activity in wheat germ lysates..

- Addition of poly IC to translating wheat germ lysate does not inhibit wheat germ protein synthesis or induce eIF2α phosphorylation.
- Wheat germ translation supported by the addition of BMV RNA is inhibited in PKR-treated wheat germ lysates. The decrease in translation is correlated to increase in WG eIF2α phosphorylation in PKR treated lysates. However, in the present study, we observed that increased PKR levels and thereby increased levels of eIF2α phosphorylation required to inhibit translation of all the four chimaeric RNAs tested. These findings suggest that while wheat germ eIF2α phosphorylation is inhibitory and the translational sensitivity of different RNAs for WG eIF2α phosphorylation is somewhat at variance. Therefore, some mRNAs probably require a higher level of phosphorylation in order to inhibit their translation.
- Phosphomimetic mutant of human eIF2α, S51D, also inhibited wheat germ translation. Further, PKR-mediated translational inhibition is partially mitigated a) in the presence of a recombinant nonphosphorylatable mutant of human eIF2α, S51A, in which 51 serine residue is replaced by alanine and b) also in the presence of K3L protein which is encoded by vaccinia virus and has some homology with the eIF2 to serve as a pseudosubstrate.
    - These findings thus suggest that phosphorylation of serine 51 residue in wheat germ eIF2α is inhibitory to protein synthesis.

- The binding between wheat germ eIF2 and different IFN-γ mRNA forms are essentially similar, except for d3 mutant and is consistent with what has been observed by Kaempfer's group with reticulocyte eIF2. This suggests that plant and mammalian eIF2 discriminate different mRNA species essentially in the same manner suggesting that plant eIF2 behaves like mammalian eIF2 in its binding to mRNA.

- Wheat germ lysate possess caspase-3 like activity that is inhibited by caspase-3 specific inhibitor Ac-DEVD-CHO. Further, we observed that incubation of rat liver nuclei in wheat germ lysate resulted in specific degradation of DNA in to oligonucleosomal fragments. In addition, inclusion of a caspase inhibitor like Ac-DEVD-CHO improved the traslational ability of the lysate. These results suggest that wheat germ lysate possess potent caspase like activity.

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Translation and Phosphorylation of Wheat Germ Lysate: Phosphorylation of Wheat Germ Initiation Factor 2 by Casein Kinase II and in *N*-Ethylmaleimide-Treated Lysates[1]

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Previously, we observed that *N*-ethylmaleimide (NEM), a thiol-alkylating agent, was found to stimulate the phosphorylation of several proteins in translating wheat germ (WG) lysates, including the phosphorylation of α, the p41–42 doublet subunit, and β, the p36 subunit, of the WG initiation factor 2 (eIF2). We find now that NEM increases phosphorylation of several proteins significantly in lysates which are moderate or low in their translation compared to optimally active lysates. Heat treatment, which stimulates oxidation of protein sulfhydryls, decreases the translation and phosphorylation ability of WG lysates. The decrease in phosphorylation, but not translation, that occurs in heat-treated lysates is prevented very efficiently by NEM and partially by reducing agents such as dithiothreitol (DTT) and GSH. DTT prevents, however, completely the loss of sulfhydryl content of heat-treated WG lysates and does not at all prevent heat-induced inhibition of translation. In contrast, DTT prevents completely the diamide-induced translational inhibition and also the loss of sulfhydryl content. These findings therefore suggest that in addition to the maintenance of sulfhydryl groups, heat-labile proteins and their interactions with other proteins play an important role in overall translation and phosphorylation. It is also observed here that heat treatment stimulates the phosphorylation of rabbit reticulocyte eIF2α but not the α subunit (p41–42 doublet) of WG eIF2. A phosphospecific anti-eIF2α antibody recognizes the WG eIF2α(P) that is phosphorylated by an authentic eIF2α kinase such as double-stranded RNA-dependent protein kinase, but it is unable to recognize the eIF2α that is phosphorylated in NEM-treated lysates. These findings therefore suggest that phosphorylation of WG eIF2α in NEM-treated lysates occurs on a site different from the serine 51 residue that is phosphorylated by authentic eIF2α kinases. In addition, it also suggests that WG eIF2α, unlike reticulocyte eIF2α, is phosphorylated by eIF2α kinases and also by other kinases. Consistent with this idea, it has been observed here that casein kinase II (CKII) phosphorylates WG eIF2α and the phosphorylation is enhanced by NEM *in vitro* and in lysates. The phosphopeptide analysis suggests that WG eIF2α has separate phosphorylation sites for CKII and heme-regulated eIF2α kinase (a well-characterized mammalian eIF2α kinase), and NEM-induced phosphorylation in WG lysates resembles CKII-mediated phosphorylation.
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Protein synthesis in eukaryotes is catalyzed by initiation (eIF),[3] elongation (EF), and termination or release factors (reviewed in 1). Joining of initiator tRNA to the 40S subunit and the formation of the 43S initiation complex require eIF2 and its recycling factor called eIF2B and also eIF3 (reviewed in 2–5). The 5′ cap structure (m7GpppN) of mRNA attracts eIF4F, a heteromultimeric complex, to the mRNA. eIF4F is composed of the cap-binding protein eIF4E, the RNA-de-

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[3] Abbreviations used: eIF2α, the α subunit of eukaryotic initiation factor 2, eIF2α(P), phosphorylated eIF2α; CKII, casein kinase II; HRI, heme-regulated eIF2α kinase, PKR, double-stranded RNA-dependent protein kinase; DTT, dithiothreitol, NEM, *N*-ethylmaleimide; BMV RNA, brome mosaic viral RNA; SV8 protease, protease from *Staphylcoccus aureus;* EF, elongation factor; WG, wheat germ; S6, small ribosomal subunit protein; dsRNA, double-stranded RNA; TCA, trichloroacetic acid; DTNB, 5,5 -dithiobis(2-nitrobenzoic acid .

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pendent ATPase, eIF4A and its stimulatory factor 4B, and eIF4C, the modular factor. The eIF4 factors plus poly(A) binding protein recognize the 5′ terminal cap or 3′ poly(A) tract of mRNA, unwind mRNA structure, and facilitate the joining of the 43S complex to mRNA to form the 48S initiation complex. Identification of the start codon in mRNA by the 40S subunit requires eIF4A, eIF1, and 1A. At this stage, the joining of eIF5 facilitates the release of eIF2 · GDP and joining of the 60S subunit to form the 80S initiation complex (reviewed in 6, 7). The recycling of eIF2 · GDP requires the replacement of GDP by GTP and this guanine nucleotide exchange is catalyzed by eIF2B, a heteropentameric protein (reviewed in 4, 5). Elongation factor eEF1 brings the elongator aminoacyl-tRNA to the A site, whereas eEF2 catalyzes the translocation of the 80S initiation complex on mRNA. Both factors require GTP for their activity (8).

Protein synthesis is regulated through changes in the phosphorylation of eIF2α, eIF2Bϵ, eIF4E, eIF4G, eEF1, and eEF2 (9, 53). The physiological importance of phosphorylation of some of the factors such as the β subunit of eIF2, eIF4B, 4A, and the p107 subunit of WG eIF3 is not clearly established (54, 58). Phosphorylation of the serine 51 residue in the α subunit of trimeric eIF2 (eIF2α) by active eIF2α kinases promotes the formation of a tight complex between eIF2α(P) and eIF2B. This leads to a decrease in the guanine nucleotide exchange activity of eIF2B (10–15), inhibition of the recycling of eIF2 · GDP (16), and impairment of the initiation step of protein synthesis (3, 17, 18). Independent of eIF2α phosphorylation, the activity of eIF2B is inhibited when it is phosphorylated on the ϵ subunit by glycogen synthase kinase-3α and -β (19). Phosphorylation of eIF4E by MAP kinase-interacting kinase-1 and -2 and eIF4G by S6 (small ribosomal subunit protein) kinase up regulates their respective activities and subsequently enhances translation (20–23). The phosphorylation of S6 by S6 kinase is also shown to facilitate the interaction between 40S subunits and mRNA (24). Phosphorylation of eEF1 subunits by multipotential kinases such as casein kinase II (CKII), S6 kinase, and protein kinase C stimulates the factor activity and rate of polypeptide chain elongation (25), whereas phosphorylation of eEF2 by eEF2 kinase inhibits elongation of protein synthesis (26, 27).

The eIF2α phosphorylation pathway is well characterized in yeast, mammalian systems, and other eukaryotes with the major exception of plants. Recent studies in plants suggest that phosphorylation of wheat eIF2α regulates protein synthesis *in vivo* and *in vitro* (28, 29), although the mechanism behind the regulation is not yet understood. In addition, it has been demonstrated that plants encode double-stranded RNA-dependent kinase (pPKR) that is immunologically similar to mammalian PKR. The activation of pPKR requires rather high concentrations of dsRNA suggesting that it is somewhat different from mammalian PKR (30).

Previously, we reported that wheat germ eIF2α (p41–42 doublet) and β (p36) subunits were phosphorylated in *N* ethylmaleimide (NEM) treated translating wheat germ lysates and the phosphorylation did not affect the guanine nucleotide exchange on WG eIF2 (31). WG eIF2 is found to mitigate the inhibition in protein synthesis and eIF2B activity of reticulocyte lysates mediated by eIF2α phosphorylation (32). The present findings suggest that NEM enhances phosphorylation of proteins much more significantly in lysates that are moderate or low in their translation than in optimally active lysates. The phosphorylation of WG eIF2α that occurs in NEM-treated lysates is different from that of PKR or heme-regulated eIF2α kinase (HRI)-mediated phosphorylation and resembles CKII-mediated phosphorylation. Addition of NEM or DTT to the translational mixtures enhances phosphorylation of proteins but does not stimulate the translational ability of the lysates nor does it mitigate the inhibition of protein synthesis caused by heat treatment. Hence the findings also point out that in addition to maintenance of -SH groups, heat-labile proteins and their interactions with other proteins play an important role in phosphorylation and in translation.

MATERIALS AND METHODS

Materials. Wheat germ and reticulocyte eIF2 were purified as described (33, 34). Purified rabbit reticulocyte HRI and double-stranded *RNA-dependent protein kinase were kind gifts received* from Drs. Jane-Jane Chen (MIT, Cambridge, MA) and R. Wek (Indiana University School of Medicine, Indianapolis, IN). Casein kinase II and *Staphylococcus aureus* protease V8-enzyme were purchased from Sigma (St. Louis, MO). Brome mosaic viral RNA was obtained from Promega Corp. (Madison, WI). Wheat germ was obtained locally. Phosphospecific anti-eIF2α antibody was obtained from Research Genetics (Huntsville, AL). [γ-^{32}P]ATP (3000 Ci/mmol was obtained from the Jonaki Centre at CCMB (Hyderabad, India) DTT was obtained from Boehringer Mannheim. All other chemicals were purchased from Sigma.

Protein synthesis in lysates. Gel-filtered wheat germ lysate was prepared as described (31, 35–37). Wheat germ protein synthesis was carried out at 25°C. DTT (1 mM) was added during the preparation of wheat germ lysates but no additional DTT was added to lysates during protein synthesis. The final concentration of DTT in the protein synthesis reaction mixtures was kept at 0.4 mM. Wheat germ lysate protein synthesis was measured by the incorporation of [^{35}S]methionine into TCA-precipitable protein in 5-μl aliquots with time. Other modifications of the standard protocols, if any, are mentioned in the table legends.

Phosphorylation assays. Lysates were pulsed with [γ-^{32}P]ATP (3000 Ci/mmol) for a brief period (5 min) with or without exogenously added eIF2. Phosphorylation assays of wheat germ eIF2 were carried out at 25°C in the presence of 20 mM Tris–HCl buffer (pH 7.5 containing 80 mM KCl, 2.5 mM $Mg(OAc)_2$, and 30 μM unlabeled ATP for 10 min. The samples were separated by 10% sodium dodecyl sulfate–polyacrylamide gels (38). Gels were analyzed by autoradiography. Phosphorylation of eIF2α in the reaction mixtures was also

TABLE I

Protein Synthesis in Wheat Germ Lysates

Sample	Experimental conditions	Protein synthesis ([^{35}S]methionine incorporated, cpm) 15 min	30 min	45 min
Expt 1				
1	WGL 1	14,763	30,435	30,436
2	WGL 2	30,208	55,646	70,182
3	WGL 3	9,766	31,798	45,880
Expt 2				
1	Lysate + 25°C	28,021	33,186	40,219
2	Lysate + NEM 0.5 mM + 25°C	24,175	30,879	37,142
3	Lysate + NEM 1.0 mM + 25°C	25,714	27,802	31,428
4	Lysate + 40°C	23,296	21,868	21,648
5	Lysate + NEM 0.5 mM + 40°C	22,637	20,769	21,538
6	Lysate + NEM 1.0 mM + 40°C	23,186	18,901	18,131
Expt 3				
1	Lysate + 25°C	30,027	36,821	41,755
2	Lysate + DTT 0.5 mM + 25°C	29,479	36,273	39,239
3	Lysate + DTT 1.0 mM + 25°C	28,054	36,164	39,232
4	Lysate + 40°C	23,452	23,342	25,534
5	Lysate + DTT 0.5 mM + 40°C	23,342	22,465	21,917
6	Lysate + DTT 1.0 mM + 40°C	20,273	22,027	21,150
Expt 4				
1	Lysate	18,131	31,758	41,758
2	Lysate + diamide 1 mM	11,208	12,747	14,285
3	Lysate + diamide 1 mM + DTT 1 mM	17,802	32,197	44,725
4	Lysate + DTT 1 mM	16,153	32,637	40,879

Note. Standard lysate protein synthesis was carried out in 25-μl reaction mixtures in the presence of BMV RNA (15 μg/ml) at 25°C for 45 min and was determined by measuring the incorporation of labeled [^{35}S]methionine into acid-precipitable protein in 5 μl of the reaction mixtures with time as described (31, 36). Expt 1, translational ability of three different batches of wheat germ lysate (WGL 1, 2, and 3) in the presence and absence of BMV RNA. Template-dependent translation was determined by subtracting the values obtained in the absence of BMV RNA from the values obtained in the presence of BMV RNA. Expts 2, 3, and 4, effect of SH-reactive agents on translation in control and heat-treated lysates.

analyzed by Western immunoblot analysis using a polyclonal anti-eIF2α antibody that recognizes the phosphorylated form of the protein.

Determination of protein sulfhydryls in translating WG lysates. Protein sulfhydryls were determined in WG lysates obtained from different batches having low to high translational abilities, heat-treated lysates, and lysates treated with different SH reactive agents such as DTT, GSH, GSSG, and diamide using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) as described (39, 40). Total SH content was measured in the presence of 2% sodium dodecyl sulfate and it was omitted for measuring the available protein -SH content. Details of the reaction are mentioned in the legend to Table II.

Phosphopeptide mapping in one dimension. Peptide mapping in one dimension by limited proteolysis in SDS–polyacrylamide gels was done as described (41) for p36 and p41–42 subunits of WG eIF2 that were phosphorylated by CKII and HRI *in vitro* and the p41–42 doublet that was phosphorylated in NEM-treated WG lysates. The bands corresponding to the phosphorylated subunits of wheat germ eIF2 were cut out of the dried gel through the X-ray film. The dried gel pieces containing the phosphorylated subunits were processed for SV8 protease (200 ng/lane) digestion. 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 so that all the residual acetic acid and contaminants like gel drying filter paper were removed. The protease-treated labeled subunits were then separated by 15% SDS–PAGE. To obtain optimal digestion of the protein for a given amount of protease and to ensure proper mixing of the enzyme and substrate, we have used here (a) a 5-cm stacking gel and (b) reversed polarity of current for 5 min just before the dye entered the separating gel. Since there is a distribution of the incorporated radiolabeled phosphate of the subunit into various peptides after digestion, a higher concentration of the substrate (5 μg of eIF2 in 20-μl reaction mixtures) was used in these studies.

RESULTS

Translation and phosphorylation ability of different batches of WG lysates in the presence and absence of NEM. Protein synthesis of three batches of WG lysates with different translational abilities is shown in Table I, Expt 1. Phosphorylation of these control and heat-treated lysates (40°C for 10 min) was studied using labeled ATP in the presence and absence of 1.0 mM NEM (Fig. 1B). NEM stimulated the phosphorylation of several lysate proteins. NEM effect was observed more clearly in lysates that were translationally weak or moderate (as in Lysate III, compare lane 9 vs 11, or Lysate I, compare lane 1 vs 2) than in lysates

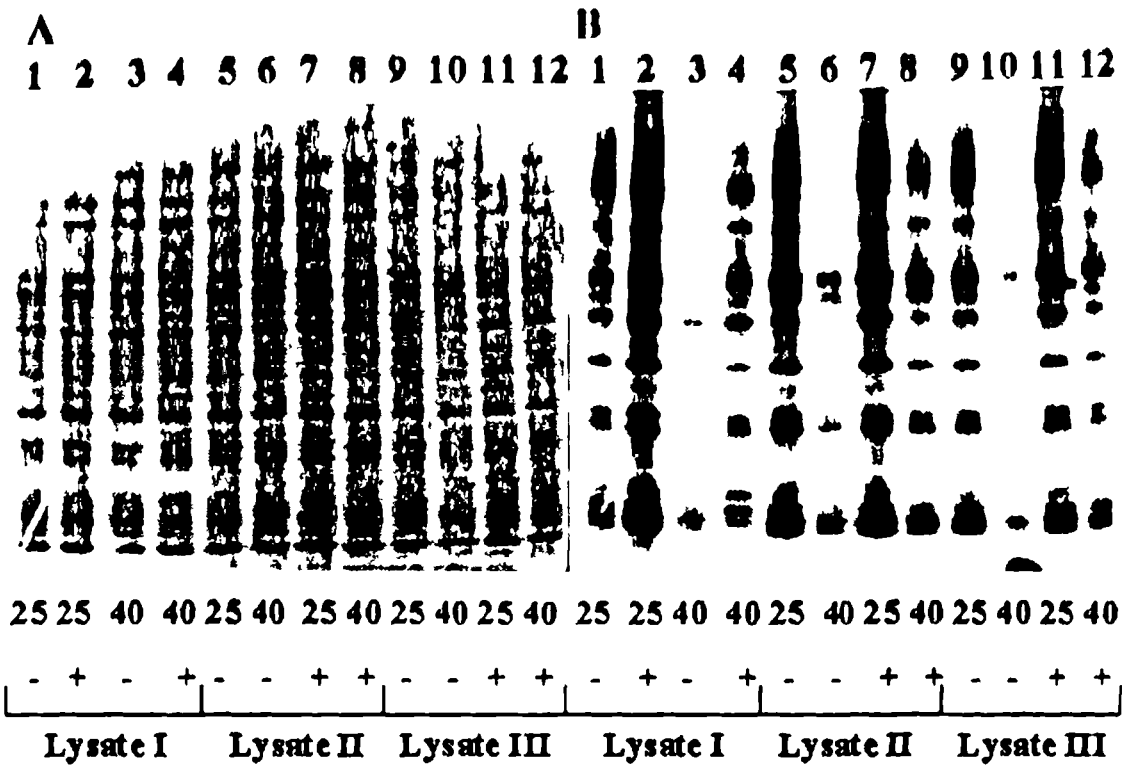


FIG. 1. Phosphoprotein profiles of different lysates in the presence of NEM and or heat treatment. Three batches of wheat germ lysates with different translational abilities as mentioned in Table I, were used for the experiment. Protein synthesis was carried out in standard 25-μl reaction mixtures at 25 and 40°C (heat treatment) for 10 min, with or with out NEM (1 mM) as shown. 10-μl aliquots of protein synthesis reactions were then supplemented with 5 μl of Tris–HCl buffer (20 mM, pH 7.8) containing [γ-^{32}P]ATP (10 μCi). The final reaction mixtures containing 2.5 mM Mg^{2+} were incubated at 25°C for 5 min. Aliquots of 7.5 μl were withdrawn and separated by 10% SDS–PAGE (A) A Coomassie-stained gel and (B) an autoradiogram.

hat were active optimally (Lysate II, compare lane 5 vs 7). Further, heat treatment reduces the overall phosphorylation of all the lysate proteins (Fig. 1B, anes 3, 6, and 10) compared to their controls incubated at 25°C (lanes 1, 5, and 9). This is not due to any differences in the amounts of protein loaded in the gels Fig. 1A). Addition of NEM to such heat-treated lysates enhances significantly the phosphorylation of most of the proteins compared to their controls in which NEM s not added (compare lanes 3 vs 4, 6 vs 8, 10 vs 12). NEM enhances significantly phosphorylation of non-heat-shocked lysates that are low or moderate in their translational activity (lanes 9 vs 11 or 1 vs 2), whereas the increase in phosphorylation of proteins in the presence of NEM is not so much evident in batch II lysates that are optimally active in their translation (lanes 5 vs 7). These findings suggest that translationally active lysates may have most of their sulfhydryl groups unoxidized and hence one can see a higher level of phosphorylation of these proteins. Addition of NEM under those conditions does not cause any significant difference. This is substantiated by heat or heat and NEM treatment here.

Translation in WG lysates: Effect of heat shock and SH-reactive agents. To understand the importance of -SH groups in WG translation, the lysate translation was studied in the presence of NEM or DTT and during heat treatment. While addition of DTT does not stimulate translation any further, NEM is found to be inhibitory (Table I, Expts 2 and 3). Addition of NEM o DTT does not prevent the translational block caused b heat treatment (Table I, Expts 2 and 3). In contrast DTT is able to mitigate the translational inhibitio caused by oxidizing agents such as diamide (Table I Expt 4) and GSSG (data not shown). These finding therefore suggest that the oxidation of protein sulfhy dryls and also the denaturation of heat-labile protein may contribute to the poor performance of certai batches of lysates and to the translational inhibitio observed in heat-treated lysates.

Phosphorylation of WG eIF2 in NEM-, NEM an CKII-, and heat-treated lysates. Earlier, we reporte that NEM-treated WG lysates showed increased phos phorylation of the p41–42 doublet that is considered t be the α subunit. However, it did not inhibit the ex change of guanine nucleotides on WG eIF2 (31). Also, i is known that NEM or heat treatment enhances eIF2 phosphorylation in reticulocyte lysates (42, 43, 55, 56 Hence, we studied here the phosphorylation of W eIF2α in NEM- and heat-treated lysates using a phos phospecific anti-eIF2α antibody. Heat shock facilitate the phosphorylation of reticulocyte eIF2α in hemi supplemented reticulocyte lysates that is recognize here by the above phosphospecific anti-eIF2α antibod The latter recognizes specifically eIF2α that is pho phorylated on its serine 51 residue by eIF2α kinase (Fig. 2, compare lane 2 vs 3). In contrast, a similar he

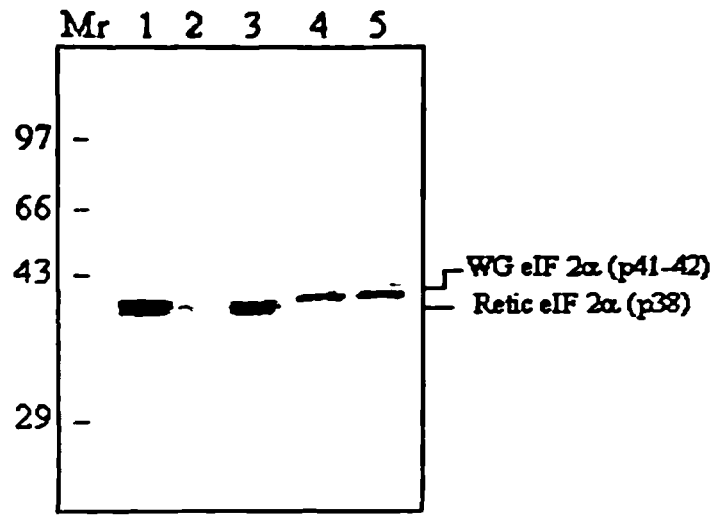


FIG. 2. eIF2α phosphorylation in translating heat-treated reticulocyte and wheat germ lysates. Protein synthesis was carried out in 25-μl reaction mixtures in heme-sensitive reticulocyte lysate (with or without added hemin) as described (34) and in wheat germ extract (25 μl) at 30 or 25°C, respectively, for 15 min as mentioned above. Heat treatment was given to samples at 40°C for 15 min. The reactions were then terminated and subjected to pH 5.0 precipitation as described (32). The samples were separated by 10% SDS–PAGE. A Western blot probed with a phosphospecific anti-eIF2α antibody is shown. Lanes 1, without heme; 2, with heme; 3, with heme + heat treatment at 40°C; 4, with wheat germ extract; 5, with wheat germ extract + heat treatment at 40°C.

treatment does not affect the basal phosphorylation status of wheat germ eIF2α (Fig. 2, lane 4 vs 5).

NEM-induced WG eIF2α phosphorylation in lysates is not recognized by the phosphospecific antibody in the Western blot (Fig. 3C, lane 1 vs 2) but can be detected with [γ-^{32}P]ATP (Fig. 3A, lanes 1 vs 2). These findings therefore suggest that WG eIF2α is phosphorylated by more than one kinase. Hence, the phosphorylation of WG eIF2α in lysates treated with CKII or CKII and NEM (Fig. 3) was also assessed. It was observed that CKII phosphorylates WG eIF2α (Figs. 3A and 3B, lanes 1 vs 3) and it is enhanced by the addition of NEM (lane 3 vs 4). NEM induces a 6-fold increase in the phosphorylation of proteins in lysates as judged from the quantification (lane 1 vs 2 and also the corresponding bar diagram in Fig. 3B). Also NEM increases the phosphorylation of lysate proteins by added CKII at least by 1 fold (lanes 3 and 4 and the corresponding bar diagram in 3B). The CKII-mediated (Fig. 3, lane 3) and/or the NEM-induced WG eIF2 phosphorylation in lysates (Fig. 3, lanes 2 and 4) is not recognized by the phosphospecific antibody. These findings therefore suggest that NEM- or CKII- or NEM and CKII-mediated phosphorylation of WG eIF2α occurs at a site different from the serine 51 residue. While the above studies were done in lysates, we have also carried out similar studies *in vitro* with the purified components to determine if CKII-mediated WG eIF2 phosphorylation is enhanced by NEM (Fig. 4). Our findings indicate that purified WG eIF2 is not phosphorylated in the absence of any added CKII (Figs. 4A and 4B, lane 1). Addition of NEM, however, increases the phosphorylation of this band by twofold (Figs. 4A and 4B, lane 1 vs 3), suggesting that the WG eIF2 preparation contains some CKII-like activity. Addition of CKII causes 3.5-fold increase in WG eIF2α phosphorylation (lane 1 vs 2), whereas addition of NEM and CKII causes an approximately 16-fold increase in its phosphorylation (lane 1 vs 4). However, there is no such increase in the phosphorylation status of WG eIF2α when the Western blots are probed with phosphospecific antibody (Fig. 4C). Thus both *in situ* and *in vitro* studies indicate that NEM stimulates WG eIF2 phosphorylation that is mediated by CKII.

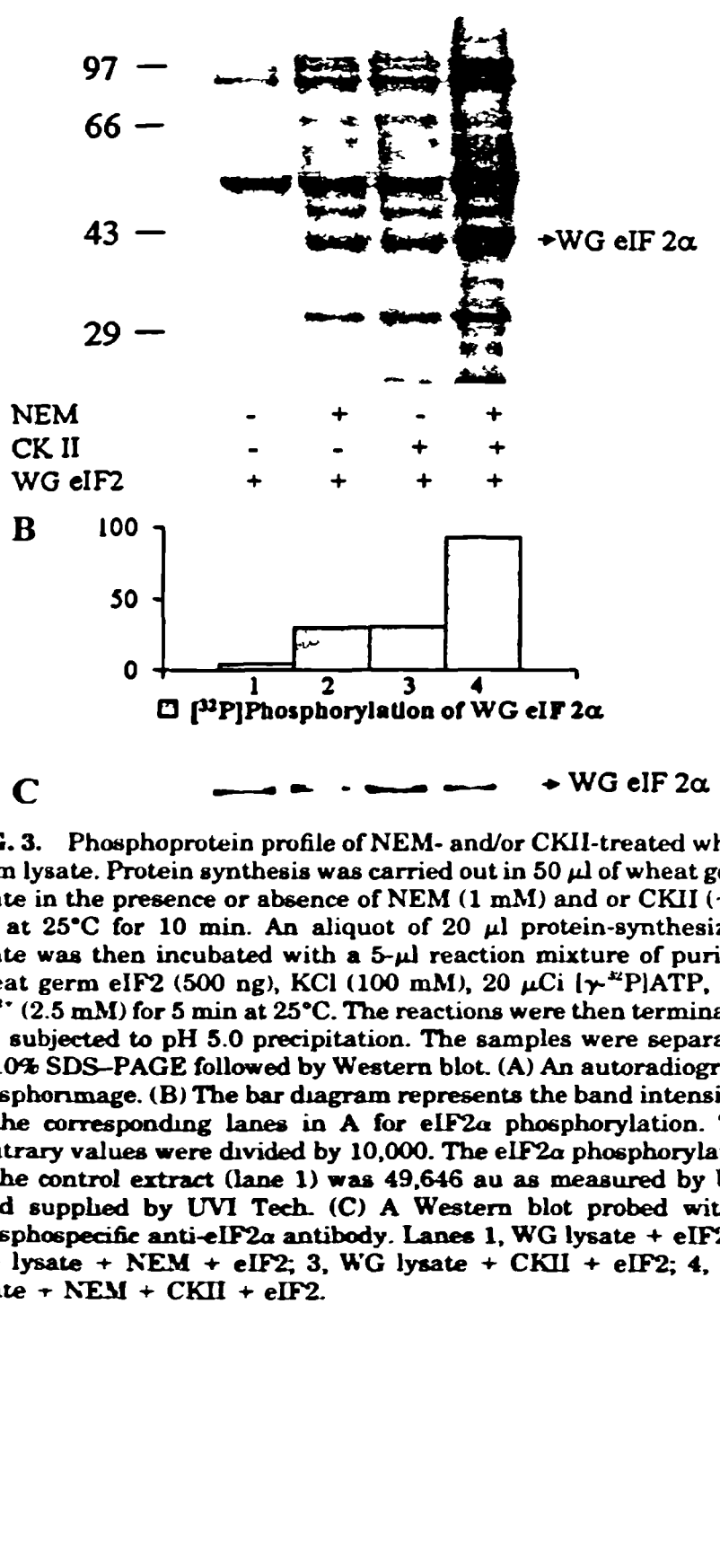


FIG. 3. Phosphoprotein profile of NEM- and/or CKII-treated wheat germ lysate. Protein synthesis was carried out in 50 μl of wheat germ lysate in the presence or absence of NEM (1 mM) and or CKII (~40 ng) at 25°C for 10 min. An aliquot of 20 μl protein-synthesizing lysate was then incubated with a 5-μl reaction mixture of purified wheat germ eIF2 (500 ng), KCl (100 mM), 20 μCi [γ-^{32}P]ATP, and Mg^{2+} (2.5 mM) for 5 min at 25°C. The reactions were then terminated and subjected to pH 5.0 precipitation. The samples were separated by 10% SDS–PAGE followed by Western blot. (A) An autoradiogram/phosphorimage. (B) The bar diagram represents the band intensities of the corresponding lanes in A for eIF2α phosphorylation. The arbitrary values were divided by 10,000. The eIF2α phosphorylation in the control extract (lane 1) was 49,646 au as measured by UVI band supplied by UVI Tech. (C) A Western blot probed with a phosphospecific anti-eIF2α antibody. Lanes 1, WG lysate + eIF2; 2, WG lysate + NEM + eIF2; 3, WG lysate + CKII + eIF2; 4, WG lysate + NEM + CKII + eIF2.

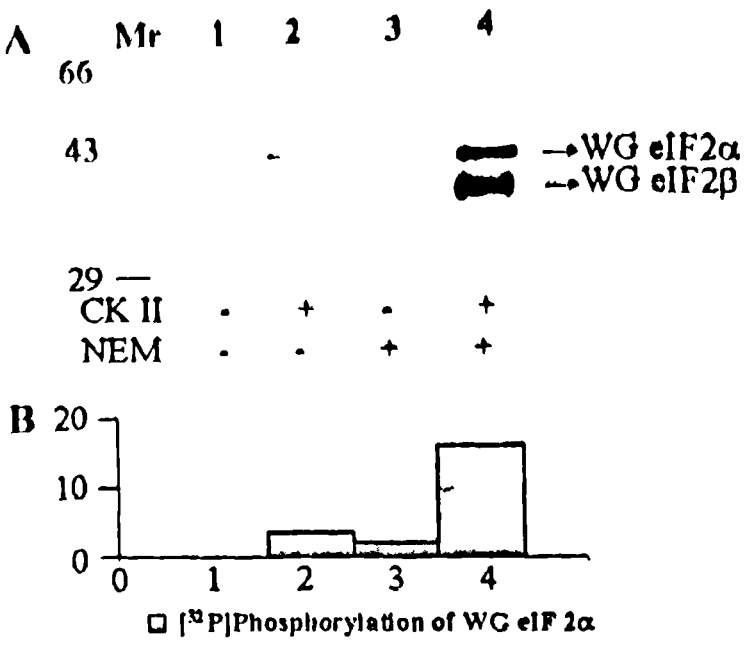


FIG. 4. Phosphorylation of WG eIF2 by NEM and/or CKII *in vitro*. Phosphorylation reactions were carried out in standard 20-μl reaction mixtures containing 20 mM Tris–HCl, pH 7.8, 2.5 mM $Mg(OAc)_2$, 30 μM unlabeled ATP and 10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol), and purified WG eIF2 (500 ng) with or without pure CKII (40 ng) and NEM (1.0 mM). Reaction mixtures were incubated at 25°C for 10 min and then separated by 10% SDS–PAGE. The gel was then transferred to a nitrocellulose membrane. (A) An autoradiogram showing labeled protein bands. (B) The bar diagram represents the band intensities of the corresponding lanes in A for eIF2α phosphorylation. The arbitrary values were divided by 10,000. The eIF2α phosphorylation in the control extract (lane 1) was nil as measured by UVI band supplied by UVI Tech. (C) A Western blot probed with a phosphospecific anti-eIF2α antibody. Lanes 1, WG eIF2; 2, WG eIF2 + CKII; 3, WG eIF2 + NEM; 4, WG eIF2 + CKII + NEM.

Further, we have shown that CKII-mediated phosphorylation is different from the phosphorylation caused by known eIF2α kinases such as PKR (a double-stranded RNA-dependent kinase). Both PKR and CKII enzymes are able to phosphorylate the p41–42 subunit of WG eIF2 as is shown in the autoradiogram (Fig. 5A, lanes 1 and 3). However, the PKR-mediated WG eIF2α phosphorylation is different from CKII-mediated phosphorylation as is evident from the fact that the phosphospecific antibody recognizes the PKR-mediated eIF2α phosphorylation but not CKII-mediated phosphorylation (Fig. 5B).

Effect of SH-reactive agents on WG eIF2α phosphorylation in control and heat-treated lysates and estimation of protein SH content. To determine the importance of protein sulfhydryls in the phosphorylation of WG eIF2α in lysates, we have studied the phosphorylation of lysate proteins in the presence of NEM, DTT, GSH, GSSG, and diamide and with heat treatment (40°C for 10 min) (Fig. 6A). While DTT, GSH, and NEM stimulated the phosphorylation of several lysate proteins, including WG eIF2α (Fig. 6A, lanes 2, 3, and 4 vs lane 1, respectively), oxidizing agents such as diamide (lane 6), GSSG (to a lesser extent, lane 5), and heat treatment (lane 7) decreased the same. The increase in phosphorylation was higher in NEM treated lysates and was followed by DTT and GSH. However, there was no increase in eIF2α phosphorylation as judged by the phophospecific antibody (data not shown). This finding therefore suggests that none of these reagents activate eIF2α kinase-like activity in WG lysates and is thus different from what has been reported earlier in reticulocyte lysates (17, 18).

To determine the importance of -SH groups in phosphorylation and in translation, the phosphorylation of WG lysate proteins was studied in heat-treated lysates in the presence of SH-reactive agents as mentioned above. While heat treatment causes a profound decrease in the phosphorylation of proteins (Fig. 6B, lane 1 vs 2) as mentioned earlier, inclusion of NEM, DTT, or GSH (Fig. 6B, lanes 3, 4, and 5) prevents the decrease in phosphorylation. However, NEM prevents almost completely, but DTT and GSH prevent partially, the heat-induced inhibition in phosphorylation. These agents, however, do not mitigate the heat-induced translational inhibition as shown in Table I.

The lysate -SH content was also measured using DTNB (Table II), in order to relate the changes in phosphorylation and translation to -SH content of the

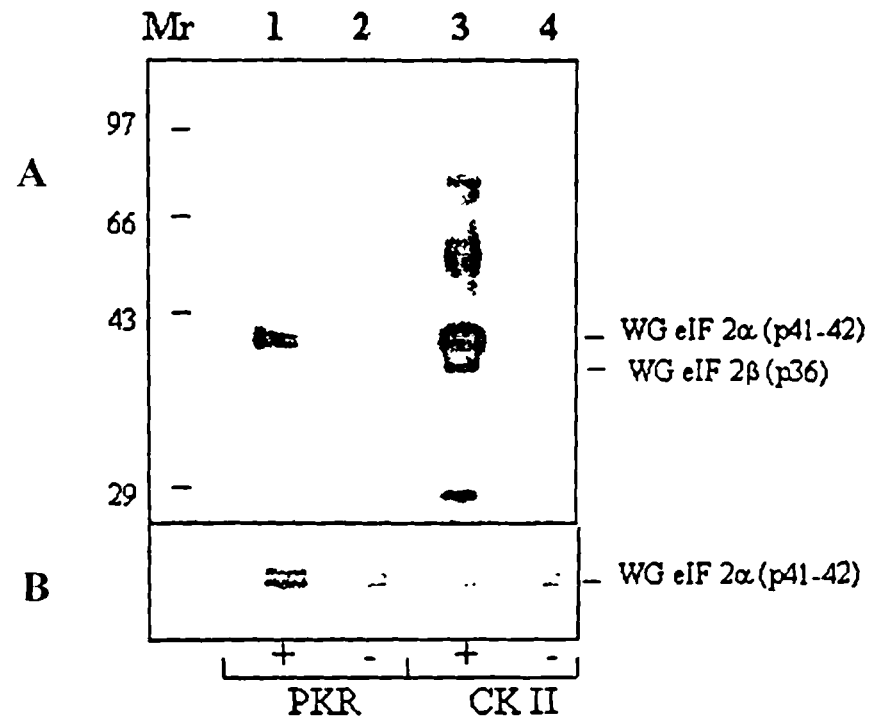


FIG. 5. Phosphorylation of wheat germ eIF-2 *in vitro* by PKR or CKII. Phosphorylation reactions were carried out in standard 20-μl reaction mixtures containing 20 mM Tris–HCl, pH 7.8, 2.5 mM $Mg(OAc)_2$, 30 μM unlabeled ATP, 10 μCi [γ-^{32}P]ATP (3000 Ci/mmol), and purified wheat germ eIF2 (~500 ng) with or without CKII (~40 ng) or PKR (~40 ng). Reaction mixtures were incubated at 25°C for 10 min, separated by 10% SDS–PAGE, and then transferred to a nitrocellulose membrane. The membrane was analyzed by autoradiography (A) and was also probed with a phosphospecific anti-eIF2α antibody (B). Lanes 1, WG eIF2 + PKR; 2, WG eIF2 (substrate control); 3, WG eIF2 + CKII; 4, WG eIF2 (substrate control).

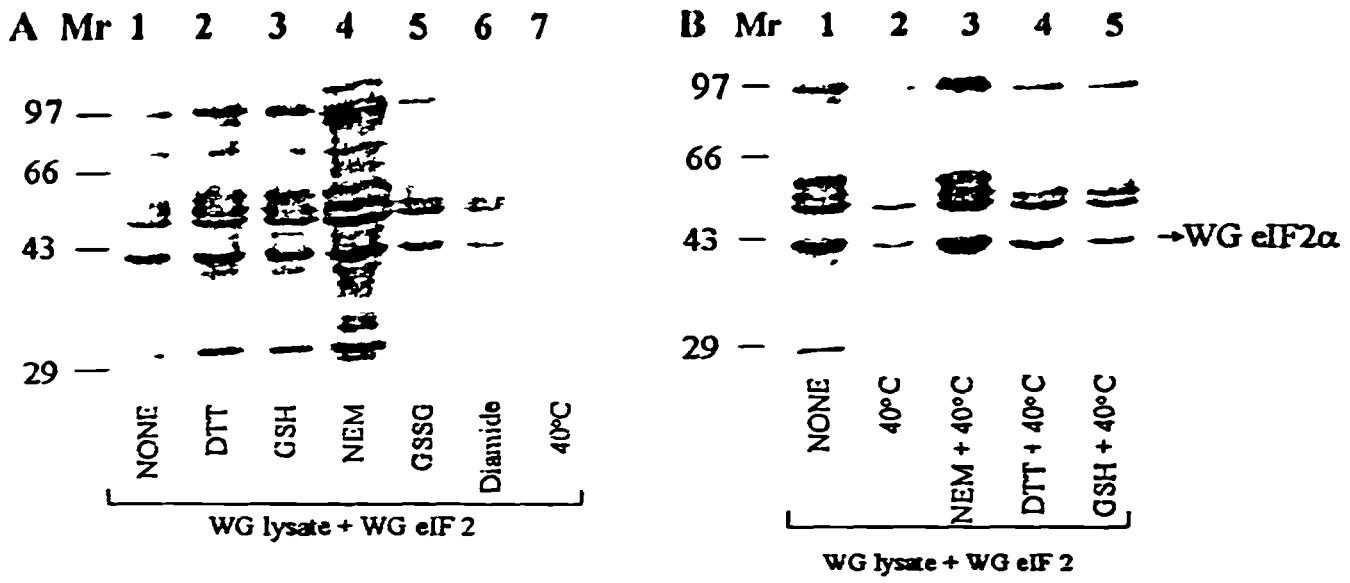


FIG. 6. WG lysate phosphorylation in the presence of SH-reactive agents and during heat treatment. (A) Phosphorylation of the lysate proteins was carried out and the samples were processed as described in the legend to Fig. 3. The lysates were treated with DTT, NEM, GSH, or diamide (1.0 mM each) or exposed to heat treatment for 10 min at 40°C. An autoradiogram/phosphorimage is shown. Lanes 1, WG lysate + eIF2; 2, WG lysate + eIF2 + DTT; 3, WG lysate + eIF2 + GSH; 4, WG lysate + eIF2 + NEM; 5, WG lysate + eIF2 + GSSG; 6, WG lysate + eIF2 + diamide; 7, WG lysate + eIF2 + heat treatment at 40°C. (B) Effect of SH-reactive agents on the phosphorylation of proteins in heat-treated lysates. WG lysates were subjected to heat treatment at 40°C for 10 min in the presence and absence of NEM, DTT, and GSH. An autoradiogram is shown. Lanes 1, WG lysate + eIF2; 2, WG lysate + eIF2 + 40°C; 3, WG lysate + eIF2 + 40°C + NEM; 4, WG lysate + eIF2 + 40°C + DTT; 5, WG lysate + eIF2 + 40°C + GSH.

lysate. Total -SH content measured in the presence of SDS was found to be 47–50% more than available -SH (fifth column vs third column in Table II). The latter was measured in the absence of SDS. Lysates with high translational ability showed more -SH than lysates with low translational ability (see No. 12 vs 13 and 14). Heat shock decreased the -SH content (No. 8 vs 1). NEM presence also showed low -SH content (No. 4 vs 1) but this was due to alkylation of -SH groups. Alkylated -SH groups are not accessible for DTNB reaction. Alkylation, however, causes stabilization of -SH groups and protects them from further oxidation. An oxidizing agent like diamide (No. 6) caused more decrease in the lysate -SH than GSSG (No. 5). The decrease in -SH content in diamide-treated lysates and in heat-treated lysates was completely prevented by DTT (No. 6 vs 1 with 7 vs 1; 8 vs 1 with 9 vs 2, respectively). However, DTT prevented partially the decrease in phosphorylation (Fig. 6B), but not translation, that was caused by heat treatment (Table I, Expt 3). Also, the presence of DTT did not improve the translational performance of the moderate lysate in the absence of heat treatment as has been shown here (Table I, Expt 3). The addition of reducing agents like GSH and DTT to a lysate did not give an additive effect (Table II). The total amount of -SH decreased significantly in DTT-supplemented lysates compared to GSH-supplemented lysates, comparing their independent values (that is lysate alone or DTT or GSH alone). This suggests that the reducing ability of these agents is utilized by the system and DTT is used up perhaps more efficiently than GSH.

Overall, these findings suggest that in addition to maintenance of -SH groups, heat-labile proteins may play an important role in translation and phosphorylation abilities of a lysate.

Phosphopeptide analysis of WG eIF2. Purified reticulocyte eIF2α kinases such as HRI or PKR were unable to phosphorylate p36, the small subunit (equivalent to the β subunit of mammalian eIF2) of wheat germ eIF2. We reported earlier that this subunit is phosphorylated by CKII (31). The phosphopeptides generated by SV8 digestion of this p36 subunit that is phosphorylated by CKII are shown in Fig. 7 (lane 2). As we are interested in the p41–42 doublet subunit, the phosphopeptides of HRI- or CKII-phosphorylated p41–42 subunit are further analyzed here (Fig. 7). SV8 protease digestion of the WG eIF2, which is phosphorylated by CKII or HRI, yielded three strongly labeled species, but of different molecular weights (Fig. 7, lanes 1 and 3). This finding indicates that HRI and CKII phosphorylate WG eIF2 at different sites and the p41–42 subunit of WG eIF2 appears to have more than one phosphorylation site.

To determine if there are any overlapping sites of phosphorylation in the p41–42 subunit, phosphorylation was carried out in the presence of CKII, HRI, or a combination of both enzymes added together at the beginning of the reaction or at 7 min. In the latter case, WG eIF2 was phosphorylated in the presence of CKII and unlabeled ATP for 7 min before the addition of reticulocyte HRI and labeled ATP (Fig. 8A). The results indicate the following: (a) Phosphorylation of this sub-

TABLE II

Sulfhydryl Content of Wheat Germ Lysate Measured as Described (39, 40)

Sample No	Reaction sample	Total SH (mM)	±SD	Available SH (mM)	±SD
1	WG lysate (WGL)	1.09	0.06	0.58	0.03
2	WGL + DTT	2.55	0.12	1.17	0.06
3	WGL + GSH	1.94	0.10	1.23	0.06
4	WGL + NEM	0.48	0.07	0.35	0.01
5	WGL + GSSG	0.94	0.05	0.51	0.03
6	WGL + diamide	0.56	0.04	0.40	0.02
7	WGL + diamide + DTT	1.10	0.07	0.57	0.04
8	WGL + 40°C	0.81	0.03	0.42	0.05
9	WGL + 40°C + DTT	2.76	0.12	1.15	0.08
10	DTT	2.33	0.12	2.33	0.08
11	GSH	0.88	0.04	0.87	0.02
12	WGL strong	1.20	0.06	0.64	0.01
13	WGL moderate	1.09	0.06	0.58	0.02
14	WGL weak	0.89	0.04	0.46	0.01
15	Diamide + DTT	0.0		0.0	

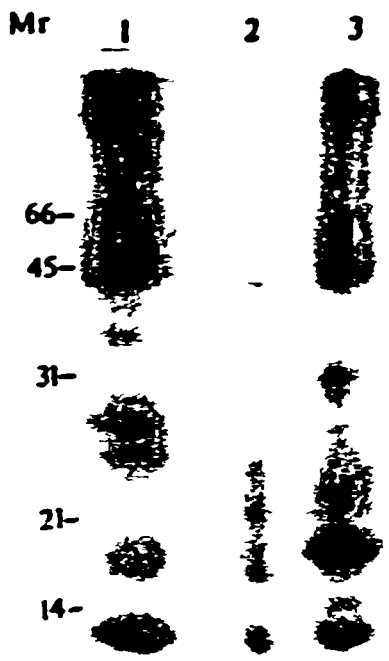
Note. The lysate was treated with and without the various redox agents (1.0 mM) as indicated. The -SH content was estimated in a total reaction mixture of 50 μl containing 45 μl of WGL (3 mg/ml) and 5 μl of the respective -SH reagent as indicated to give a final concentration of 1.0 mM. In control reactions, lysate is omitted (Nos. 10, 11, and 15). The mixtures were all incubated for 20 min at 25°C or at 40°C where indicated. To this reaction mixture, 33 μl of DTNB solution (40 mg DTNB in 10 ml of 0.1 M sodium phosphate buffer, pH 8.0) and 1.0 ml solution containing 2% SDS, 0.08 M sodium phosphate buffer, pH 8.0, and 0.5 mg/ml EDTA were added. The color developed for 15 min and was measured at 410 nm against the lysate mixture in SDS to give apparent absorbance. A reagent blank was subtracted from he apparent absorbance to give the net absorbance. For calculation of sulfhydryl content, the net absorbance was employed with a molar absorptivity value of 13,600 M^{-1} cm^{-1}. Based on [^{35}S]methionine incorporation in 5 μl of translational lysate, the lysates were characterized as strong, moderate, and weak. Strong lysates showed an incorporation of 65,000–70,000 cpm, whereas the moderate and weak lysates showed an incorporation of 35,000–45,000 or 20,000–30,000 cpm, respectively, in 5-μl aliquots after 45 min of a translational assay. Available protein -SH content was measured in the same manner in the absence of SDS and the absorbance was monitored at 410 nm.

unit does not occur without added kinase (lane 1), suggesting that there is little or no kinase contamination with the preparation of eIF2. (b) Phosphopeptides generated by SV8 digestion of CKII- and HRI-phosphorylated p41–42 subunit are different, as has been shown here (Fig. 8A, lanes 2 vs 3), and resemble the data presented in Fig. 7. A similar pattern of phosphopeptides was observed here when the substrate was treated with unlabeled ATP without the addition of kinase and then phosphorylated in the presence of the respective kinase and labeled ATP (Fig. 8A, lanes 5 and 6 match Fig. 7, lanes 2 and 3, respectively). (c) Consistent with these findings, phosphorylation of the p41–42 doublet by unlabeled ATP and CKII and then by labeled ATP and HRI yields a phosphopeptide map that is similar to that obtained by HRI alone (lane 7 vs lanes 3 and 6). Finally, (d) the phosphopeptides, however, generated by SV8 digestion of the p41–42 subunit that is phosphorylated by the addition of both HRI and CKII resemble the pattern that is produced in the presence of HRI alone (lane 4 vs lane 3). It may be because of a higher affinity of WG eIF2 for HRI than for CKII or, alternatively, the phosphorylation of eIF2α by HRI causes a conformational change that causes the CKII sites to become hidden. These possibilities have to be further tested.

In addition, phosphopeptide maps obtained from WG eIF2α phosphorylated *in vitro* by CKII and from NEM-treated lysates resemble each other (Fig. 8B, lane 1 vs lane 2, respectively). This further confirms that NEM stimulates CKII activity in WG lysate.

DISCUSSION

Phosphorylation of the serine 51 residue in eIF2α inhibits the guanine nucleotide exchange activity of eIF2B (3, 11–15, 45). This in turn inhibits the recycling of eIF2 · GDP to eIF2 · GTP and protein synthesis (16). This is a major regulatory mechanism in gene expression at the translation level in many eukaryotes studied to date. Previously, we have shown that the p41–42 doublet subunit and the p36 subunit of WG eIF2 are phosphorylated in NEM-treated lysates and also by CKII (31). This phosphorylation did not affect the exchange of guanine nucleotides on eIF2. Subsequent studies have shown that the p41–42 doublet subunit of WG eIF2 is equivalent to mammalian eIF2α (30) and the p36 is equivalent to mammalian eIF2β (44). Addition of higher concentrations of poly(IC) stimulated probably plant PKR-like activity in WG lysates and facilitated phosphorylation of the p41–42 subunit of WG eIF2. Phosphorylation of the p41–42 doublet sub-



[G. 7. Cleveland partial peptide digestion of phosphorylated heat germ eIF2 subunits. The phosphorylated bands of wheat germ F2 subunits were obtained from a 10% SDS–PAGE. The bands ere identified by superimposing the developed X-ray film on the ied gel and were then cut out through the X-ray film. The dried gel eces containing the phosphorylated subunits were equilibrated and ade ready for SV8 protease as described under Materials and ethods. The protease-treated labeled subunit was then separated a 15% SDS–PAGE. An autoradiogram is shown. All lanes repre- nt the SV8 protease-digested radio labeled products. Lanes 1, SV8 gest of p41–42 subunit of wheat germ eIF2 phosphorylated by KII; 2, SV8 digest of p36 subunit of wheat germ eIF2 phosphory- ed by CKII; 3, SV8 digest of p41–42 subunit of wheat germ eIF2 osphorylated by HRI.

nit under those conditions is found associated with hibition of protein synthesis in translating wheat rm lysates, although the mechanism underlying this hibition of protein synthesis is not yet understood 9).

In the present article, we have further studied the EM-mediated phosphorylation of WG eIF2α. We ob- rved that NEM stimulates phosphorylation of sev- al lysate proteins, including WG eIF2α (Figs. 1 and . However, the ability of NEM to stimulate the phos- orylation was found to be dependent on the transla- nal ability of the lysates. NEM stimulates efficiently e phosphorylation of lysates that are relatively low in eir translational ability compared to optimally active sates Table I, Expt 1, and Fig. 2 . The active lysates ay have their protein sulfhydryl groups well main- ined, and addition of NEM alkylates the -SH groups and thereby prevents the formation of disulfide bonds. Hence, no significant difference in phosphorylation is observed under those conditions. In contrast, addition of NEM to weak or moderate lysates enhances the phosphorylation of proteins much more significantly.

The findings that diamide treatment causes reduced phosphorylation (Fig. 6A) and the heat-induced decrease in phosphorylation is prevented significantly by the addition of NEM, DTT, or GSH (Figs. 1 and 6) support the hypothesis that oxidation of protein -SH groups can lead to a decrease in phosphorylation. However, NEM decreases the lysate sulfhydryl content (either by alkylation of protein -SH or lysate GSH) (Table II) and inhibits translation (Table I, Expt 2), whereas diamide causes oxidation of protein -SH groups. In contrast, DTT and GSH maintain protein -SH groups, do not inhibit protein synthesis, but stimulate phosphorylation, albeit to a lesser extent than NEM. Neither DTT nor NEM can prevent heat-induced inhibition of protein synthesis (Tables I and II and Fig. 6). On the contrary, DTT prevents diamide- (Table I) and GSSG-induced (data not shown) inhibition of protein synthesis. These findings therefore suggest that in addition to the maintenance of sulfhydryl groups, heat-labile proteins or denatured proteins and their interactions with other proteins play an important role in overall translation and phosphorylation. This conclusion is supported by other studies wherein heat shock protein 70 has been shown to restore the inhibition of protein synthesis that occurs due to the activation of HRI in heme-deficient reticulocyte lysates (55). It is suggested that this is a general mechanism for translational control in response to cellular stress wherein the denatured proteins formed in response to heat or other stress may bind to heat shock proteins in competition with HRI, thus leading to the activation of HRI (56, 57). In addition, heat shock protein 72 has been implicated in preventing the activation of kinases such as Jun N-terminal kinase and p38 through the maintenance of a cellular phosphatase activity during heat shock, ethanol or oxidative stress, and other stress conditions that cause protein damage in lymphoid tumor cell lines (59, 60).

Further, our findings here indicate that heat treatment stimulates the phosphorylation of reticulocyte eIF2α, but not the wheat germ eIF2α, in their respective translational lysates. This finding suggests that wheat germ lysates probably do not carry HRI-like protein. The eIF2α phosphospecific antibody, as mentioned above, has been used to distinguish the phosphorylation of the p41–42 doublet of WG eIF2 caused by eIF2α kinases and a multipotential serine/threonine kinase like CKII. The findings presented in Fig. 5 suggest that these enzymes phosphorylate WG eIF2α at different sites. Also we observed that NEM stimulates CKII-mediated phosphorylation of WG eIF2α

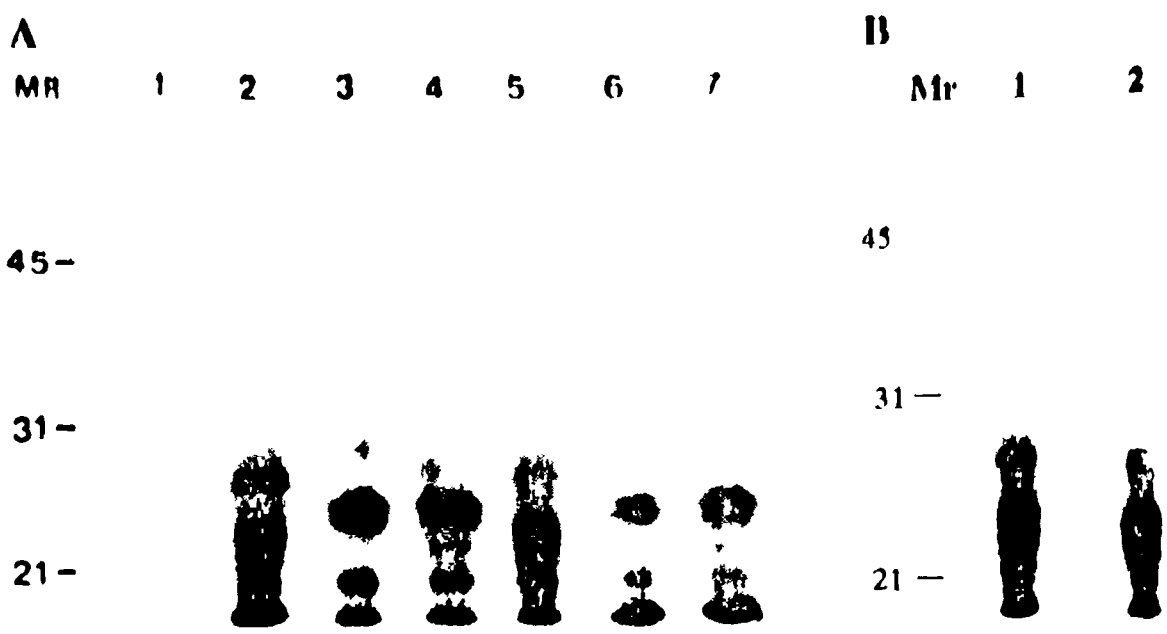


FIG. 8. SV8 protease digestion products of p41–42 subunit of phosphorylated wheat germ eIF2 *in vitro* (A) and in lysates (B). (A) To determine if there are any overlapping sites of phosphorylation in the p41–42 subunit of wheat germ eIF2, phosphorylation of the substrate (4 μg of eIF2) was carried out with [γ-^{32}P]ATP in the presence of CKII, HRI, or a combination of both enzymes. Where both enzymes (CKII and HRI) were added, they were added together at 0 min or added at different time intervals (0 and 7 min) as described below. Also, to determine the presence of any endogenous kinase-like activity associated with purified wheat germ eIF2, phosphorylation of the latter was carried out in the absence of any added kinase. Phosphorylated products were separated by 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. An autoradiogram is shown. Lanes 1, no kinase added; 2, with CKII; 3, with HRI; 4, with CKII + HRI (added together at 0 min); 5, no kinase + unlabeled ATP (0–7 min); with CKII + labeled ATP (7–14 min); 6, no kinase + unlabeled ATP (0–7 min), + HRI + labeled ATP (7–14 min); 7, with CKII + unlabeled ATP (0–7 min), + HRI + labeled ATP (7–14 min). (B) Phosphorylation of the p41–42 subunit of WG eIF2 in NEM-treated lysates (50 μl lysate) and by CKII *in vitro* was carried out. The protease digestion of the phosphorylated product was carried out as described for A. The protease-treated labeled subunit was then separated by 12.5% SDS–PAGE. An autoradiogram is shown. Lanes 1, WG eIF2 + CKII; 2, WG eIF2 + NEM-treated (1.0 mM) lysate.

both in lysates (Fig. 3) and in an *in vitro* system (Fig. 4). These studies suggest that NEM stimulates CKII-like activity in wheat germ lysates. This is further supported by the evidence that phosphopeptides of WG eIF2α in NEM-treated lysates resemble the phosphopeptide map of WG eIF2α that is phosphorylated by CKII *in vitro* (Fig. 8B). CKII is a multipotential kinase with several substrates. Consistent with this idea, addition of NEM stimulates phosphorylation of several lysate proteins. While it is known that reticulocyte eIF2α is phosphorylated so far exclusively by eIF2α kinases, WG eIF2α is phosphorylated by eIF2α kinases and CKII.

The physiological significance of CKII-mediated WG eIF2α phosphorylation or the mechanism of activation of CKII in the presence of NEM is not yet understood. Although mammalian eIF2α may not contain any phosphorylation site other than the serine 51 residue that is phosphorylated by eIF2α kinases, yeast eIF2α has been found phosphorylated by both eIF2α kinases and CKII. The C-terminus of yeast eIF2α contains three additional phosphorylation sites that are constitutively phosphorylated *in vitro* and *in vivo* by CKII. Mutations in these CKII phosphorylation sites of yeast eIF2α are found to affect the GDP/GTP exchange on eIF2, presumably by affecting the eIF2B activity (46). Recent studies by Le *et al.* (47) have shown that the phosphorylation status of eIF2α varies considerably during wheat seed development and germination. However, the authors find it difficult to explain how phosphorylation at each site of wheat eIF2α affects its activity. This is because plant eIF2α has more than one phosphorylation site and that is consistent with our observations here.

Phosphorylation of eIF4 factors (4E, 4A, 4B, 4G) is also known to alter translational rates (58). Earlier studies have shown that mammalian eIF4B is phosphorylated *in vitro* by several kinases, including CKI and CKII (51, 52), but the physiological importance of this phosphorylation is not yet understood. However, in plants, eIF4B is found dephosphorylated in wheat embryos, but it is well phosphorylated in leaves, and is also found dephosphorylated during heat shock (50). Thus eIF4B phosphorylation correlates with increased rates of protein synthesis. Based on several results it is suggested that eIF4E phosphorylation in animal systems enhances its affinity for mRNA (22) but the role of this phosphorylated factor in plant systems is not clear. Phosphorylation of other eIF4 factors like eIF4A has also been shown to occur during a variety of environmental stress conditions and is known to decrease and also increase protein synthesis both in plant and in mammalian systems, thereby suggesting that phosphorylation occurs at different residues on these pro-

teins, some of which are inhibitory and some of which are stimulatory (58). In addition, the interaction of heat shock proteins with the kinases that phosphorylate some of these substrates may also modulate the activity of these initiation factors, as has been illustrated for HRI kinase activity (56, 57). Our observations suggest that there is a general increase in phosphorylation of several proteins including eIF2α in an active wheat germ cell-free translational system and this appears to be because of the presence of an active CKII in the system. The mechanism through which CKII is activated and how the CKII-mediated eIF2 phosphorylation or the phosphorylation of other initiation factors improves the translational performance of lysates have to be established by future studies.

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