STUDIES ON THE INTERACTION BETWEEN EUKARYOTIC INITIATION FACTOR 2 (eIF2) AND eIF2B PROTEINS: EXPERIMENTS WITH BACULOVIRUS - EXPRESSED RECOMBINANT MUTANTS OF HUMAN eIF2

Thesis submitted to University of Hyderabad for the degree of

Doctor of Philosophy

By

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To my Beloved Mother

Awake, Arise and Rest not Till You Reach The Goal

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Certificate

This is to certify that this thesis entitled **"Studies on the interaction between eukaryotic initiation factor 2 (eIF2) and** eIF2B **proteins: Experiments with baculovirus-expressed recombinant mutants of human** eIF2"comprises the work done by **Akulapalli Sudhahar** for the degree of Doctor of Philosophy under my supervision at the University of Hyderabad, Hyderabad, India. I declare that this work is original and has not been submitted in part or full for any other degree or diploma of any University.

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CONTENTS

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(Sudhakar)

ABBREVIATIONS

A, Ala	: Alanine
AcNPV	: Autographs californica nuclear polyhedrosis virus
AP	: Alkaline phosphatase
APH	: Acetyl phenyl hydrazine
Asp	: Aspartic acid
ATP	: Adenosine 5' triphosphate
BCIP	: 5-bromo-4-chloro-3-indoiyl phosphate
BEVS	: Baculovirus expression vector system
Bis-acrylamide	: N, N'-methylene-bis-acrylamide
Kbp	: Kilo base pair
BSA	: Bovine serum albumin
BV	: Budded virion
Ci	: Curie
CHO	: Chinese hamster ovary cells
CM-S	: Carboxy methyl sephadex
CK II	: Casein kinase II
CP	: Creatine phosaphate
CPK	: Creatine phosphokinase
cpm	: Counts per minute
DAI/dsI/PKR	: double stranded RNA activated inhibitor
dCTP	: Deoxy cytosine triphosphate
DEAE	: Diethyl aminoethyl
DMSO	: Dimethyl sulphoxide
DNA	: Deoxy ribonucleic acid
cDNA	: Complementary DNA

dNTP	: Deoxy nucleotide triphosphate
DTT	: Dithiothreitol
EDTA	: Ethylene diamine tetra acetic acid
eEF	: Eukaryotic elongation factor
EGTA	: Ethylene-bis (β-aminoethyl ether) n, N', N''-tetra aceticacid
eEFs	: eukaryotic elongation factors
eIFs	: Eukaryotic initiation factors
eIF2	: Eukaryotic initiation factor 2
eIF2α	: Alpha subunit of eukaryotic initiation factor 2
eIF2(αP)	: Phosphorylated alpha subunit in eIF2
eIF2B/GEF/RF	:Guaninc nucleotide exchange factor of eIF2/reversing factor
eRF	: Eukaryotic releasing factor or termination factor
EtBr	: Ethidium bromide
FCS	: Fetal calf serum
Fig	: Figure
GDP	: Guanosine 5' diphosphate
GSH	: Reduced gluthathione
GSSG	: Oxidised gluthathionine
GTP	: Guanosine 5' triphosphate
h	: Hours
HS 6B	: Hepharin sepharose 6B
HRI	: Heme regulated inhibitor
HEPES	: N-[2-hydroxyethyl) piperizine-N'-[2-ethane-sulfonic acid]
Hsp	: Heat shock protein

S	: Svedberg
dsRNA	: Double stranded RNA
Met.tRNA,	: Initiator transfer RNA
mRNA	: Messenger RNA
tRNA	: Transfer RNA
RNase	: Ribonuclease
rpm	: Rotations per minute
-SH	: Protein sulfhydryl groups
SDS	: Sodium dodecyl sulphate
Sf9	: Spodoptera frugiperda (fall armyworm) cell line
Sf21	: Spodoptera frugiperda (fall armyworm) cell line
TCA	: Trichloro acetic acid
TEMED	: N', N N, N', N' - tetramethyl ethyl-ethylene diamine
TNM-FH	: Insect cell culture medium
Tris	: Tris (hydroxymethyl) amino methane
μ	: Micro
μl	: Microlitre
μg	: Microgram
ιιM	: Micromolar

CHAPTER I

INTRODUCTION

1. AN OVERVIEW OF PROTEIN BIOSYNTHESIS

1.1. Initiation.

1.2. Elongation.

1.3. Termination.

2. **REGULATION OF INITIATION OF PROTEIN SYNTHESIS**

2.1. eIF2 subunit copmosition, function and regulation.

2.2. Phosphorylation of eIF2 and physiological significance.

2.3. eIF2α kinases, their activation and regulation.

2.4. eIF2B and its regulation.

2.5. eIF2a phosphatases.

2.6. Physiological inhibitors of eIF2α phosphorylation.

3. BACULOVIRUS

3.1. The infection process.

3.2. The advantages of baculovirus expression system.

4.0. **OBJECTIVES**

INTRODUCTION

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

Messenger RNA (mRNA) is monocistronic in eukaryotic cells and regulation of eukaryotic translation might seem unnecessary. Yet, it provides a very rapid way to control gene expression besides the regulation which occurs at other level (promoters, RNA splicing, RNA stability and export of mRNA from

the nucleus to the cytoplasm). Translational control is defined as a change in the efficiency of mRNA translation i.e, in the number of amino acids polymerized per unit time per mRNA molecule. This control may effect a quantitative change in the overall amounts of proteins synthesized, or a qualitative change in the species of proteins produced.

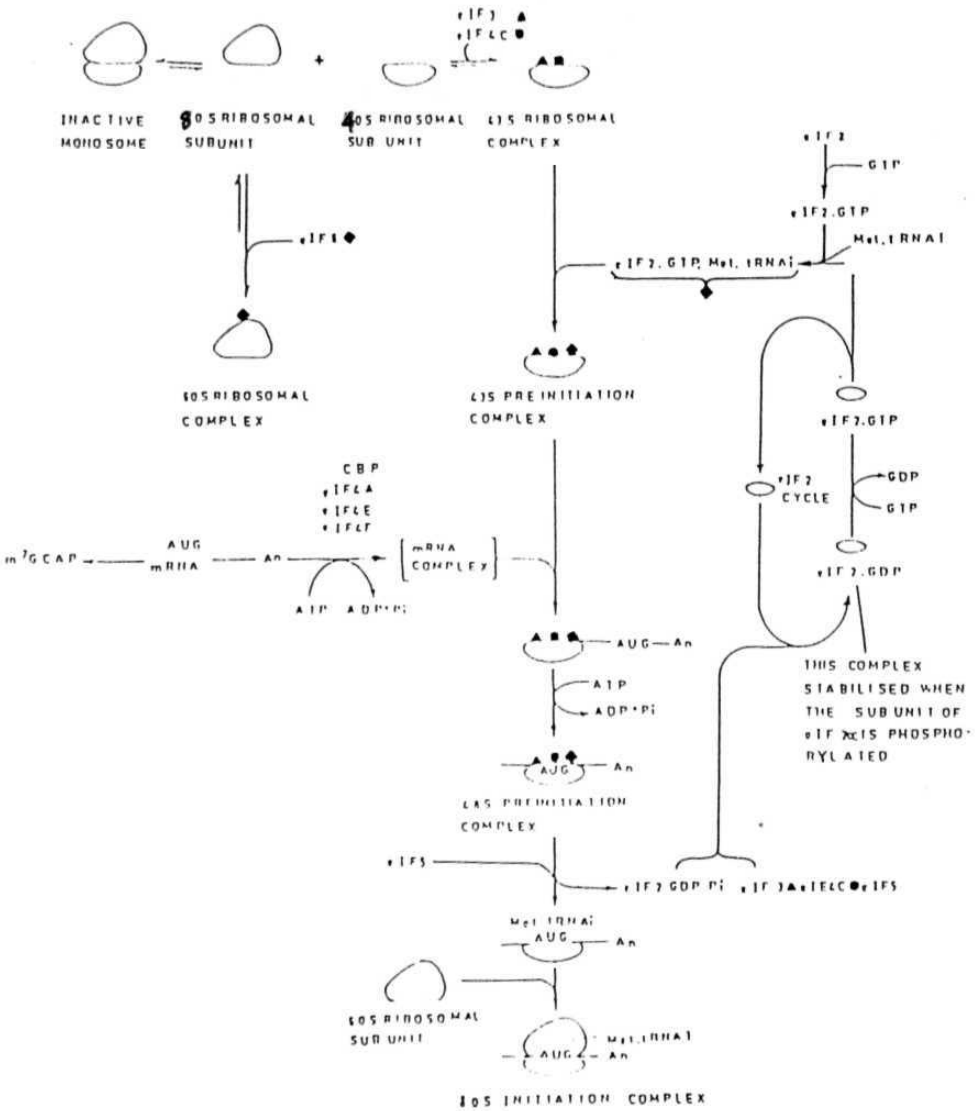
Zamecnick and his colleagues have made rapid strides in protein biosynthesis research since the development of the first cell-free system and way back in the 1950s. A great deal is now known about translation, but perhaps, only a little about what is yet to be learnt. Conventionally, translation is divided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

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

1. AN OVERVIEW OF PROTEIN BIOSYNTHESIS

1.1. Initiation.

This is the most complex of all the three steps, requiring a myriad of initiation factors called as eukaryotic initiation factors (eIFs). Most of the eIFs



Initiation of Protein Synthesis in Eukaryotes
(Ref. Watson et al., 1987)

are multimeric proteins and they are designed to regulate protein synthesis in meaningful ways (Hershey, 1991 and Merrick, 1992).

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

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

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

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

d) This above step involves the recognition of 'start' site on mRNA by Met-tRNA. Prokaryotic mRNAs possess distinct structural features (Shine-Dalgarno sequence) in the mRNA for it to facilitate a distinct hydrogen

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

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

becomes a target for eIF2a kinase (Ramaiah et al., 1992; Pavitt et al., 1998 **and** Mueller et al., 1998).

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

1.2. Elongation.

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

a) eEF-1α catalyses the binding of the aminoacylated tRNA to the 'A' a site of the ribosome, b) The ejection of eEF-1α from ribosome's is accompanied by the GTP hydrolysis. The eEF-1α.GDP thus released is recycled by the eEF-1 p/y. eEF-1 a is comparable to EF-Tu of prokaryotes and the eEF-1 β/ y, which are involved in the exchange of GTP for GDP on eEF-1α.GDP are comparable to EF-Ts of prokaryotes and to the initiation factor eIF2B of eukaryotes. c) The peptide transferase centre, presumably located on the 60S ribosomal subunit, catalyzes formation of peptide bond between the nascent

polypeptide and the incoming aminoacid. d) The elongation factor-2 (EF-2) hydrolyzes GTP and catalyzes the translocation of aminoacyl tRNA from the 'A' site to 'P' site on the ribosome with a concomitant movement of the message (Watson et al., 1987).

1.3. Termination.

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

2. REGULATION OF INITIATION OF PROTEIN SYNTHESIS

Regulation of translation can occur at various stages of protein synthesis. In such a complex sequence of reaction it is natural for the cells to exert control at the first step of initiation of protein synthesis. Two distinct types of translational controls occur. i) General control of overall protein synthesis affecting the bulk of mRNAs of the cell and ii) selective regulation of specific mRNAs or a sub set of mRNAs. The latter may arise due to structural features of specific mRNAs, especially, secondary structures, or, by mRNA binding proteins. These transacting factors may either prevent or facilitate the initiation factor binding to specific mRNAs. Regulation of protein

synthesis by phosphorylation of initiation and elongation factors and also by phosphorylation of ribosomal protein S6 has been well-documented (Hershey, 1991; Merrick, 1992; Proud, 1992 and Jefferies and Thomas, 1996).

In the initiation step of protein synthesis, phosphorylation of eIF2 and eIF4E proteins play a major role in the regulation. There is a strong correlation to enhanced eIF4E phosphorylation, that occurs, in response to growth factors, mitogens, and cytokines, to increased protein synthesis (Morley and Traugh, 1991; Kasper et al., 1990; Fredrickson et al., 1992 and Donaldson et al., 1991). eIF4E is hypo-phosphorylated during mitosis (Boneau and Sonenberg, 1987), following heat shock (Duncan et al., 1989) or infection with several viruses (Huang and Schneider, 1991; Feigenblum and Schneider, 1993) concomitant with a reduction in the translational rates. Interestingly, these conditions which reduce eIF4E phosphorylation enhance eIF2α phosphorylation. However, the connection (if any) between the phosphorylation states of these two proteins is not yet understood. It should be highlighted here that protein synthesis is one of the most complex biochemical process requiring approximately 150 different polypeptides. Seven of these polypeptides (eIF2cx, eIF2Bε, eIF4E, eIF4G, S6, eEF1, and eEF2, have been identified as targets for regulatory pathways to date. Modification of some of these factors affects the overall rate of translation whereas modification of others affects the spectrum of mRNAs translated (Rhoads, 1999). Since the present work deals with the regulation of eIF2 activity and its interaction with eIF2B protein, the introduction here is mainly focused on the current information available on this subject.

2.1. eIF2 subunit composition, function and regulation.

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

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

eIF2 is a heterotrimer composed of three subunits α-(38 kDa/315 aa), p-(50 kDa/333aa) and y-(52 kDa/472aa). All the three subunits of eIF2 from human (Ernst et al.,1987; Pathak et al., 1988 and Gaspar et al.,1994) and yeast (Cigan et al., 1989; Donahue et al., 1988 and Hannig et al., 1993) and also some of the individual subunits such as the (x-subunit from Drosophila (Qu and Cavener, 1994) and bovine (Green et al., 1991) systems and the [5-subunit from wheat germ (Metz and Browning, 1997) and Drosophila (Ye and Cavener, 1994) were cloned and sequenced. Sequencing information of eIF2

reveals that the subunit sequence among different organisms is well conserved to a great extent.

In the presence of physiological concentrations of Mg^{24}, the protein has a 400-fold higher affinity for GDP than for GTP and forms a stable eIF2.GDP binary complex *in vitro* (Panniers and Henshaw, 1983 and Panniers et al., 1988). The joining reaction of eIF2 with the initiator Met-tRNAi requires GTP and is inhibited by GDP (Walton and **Gill,** 1976). Later studies have isolated an heteropentameric initiation factor with guanine nucleotide exchange activity from a variety of mammalian systems and was variously designated as GEF (Panniers and Henshaw, 1983), anti-HRI (Amesz et al., 1979), SP (Siekierka et al., 1981), RF or reversing factor (Siekierka et al, 1981; Matts et al., 1983 and Grace et al., 1982) and or eIF2B (Konieczny and Safer, 1983). It is now referred as eIF2B. The guanine nucleotide exchange activity of eIF2B is estimated from the rate of displacement of labeled [^{3}H or ^{32}P] GDP from the preformed eIF2.GDP binary complex on incubation with labeled GDP or GTP. The eIF2 complex with guanine nucleotides bind to the nitrocellulose filters and is stable in the presence of Mg^{2+}. In the presence of active eIF2B, labeled GDP is exchanged for unlabelled GDP or GTP present in the reaction mixture. Several mechanisms have been proposed to explain the ability of eIF2B to dissociate GDP bound eIF2 (Reviewed in Pain, 1983 and Manchester, 1987). Two of the models were studied in detail which unfortunately do not agree with each other. One group (Rowlands et al., 1 988) proposed an enzyme displacement or substituted mechanism using the nitrocellulose filter binding assay and eIF2B/eIF2 complex as the source of eIF2B. This complex eIF2B is more stable than free eIF2B. According to this mechanism, eIF2.GDP joins the enzyme, eIF2B. This is followed by the dissociation of GDP, binding of GTP and then the release of the enzyme from eIF2.GTP. The reaction thus proceeds by way of eIF2.GDP or eIF2.GTP

binary complexes with an enzyme (eIF2B) intermediate which, free from either substrate, carries the group to be transferred. Another group (Dholakia et al., 1989) used eIF2B free from eIF2 and studied the displacement of labeled GDP and fluorescent GDP derivatives from eIF2 by eIF2B and observed that there was no displacement of labeled GDP unless GTP was present. Hence, they proposed a sequential mechanism which involves a (GTP).eIF2B.(eIF2.GDP) complex. Consistent with their hypothesis, it was observed that the 40 kDa subunit of eIF2B could bind GTP (Dholakia et al., 1989b).

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

Both gamma and beta subunits can be cross linked to guanine nucleotides and to Met-tRNAi (Bommer etal., 1991 and reviewed in Merrick, 1992). The current understanding however is that the N-terminus region of the y-subunit of eIF2, rather than the β-subunit, binds GDP based on the fact that the y-subunit has all the three consensus guanine nucleotide binding domains in

close proximity to each other and mutations in the guanine nucleotide binding domains in human and yeast protein (Naranda etal., 1995 and Erickson et al., 1997) decrease significantly its ability to bind GDP.

The beta subunit has two features which appear to be important in its interaction with nucleic acids and also with other initiation factors such as eIF5 which hydrolyzes eIF2.GTP to eIF2.GDP and or eIF2B, the guanine nucleotide exchange factor. These are, a) three runs of seven lysine residues in the amino-terminal half of the subunit which are conserved in yeast, human and Drosophila (Donahue et al., 1988; Pathak et al., 1988 and Ye and Cavener, 1994) and b) a C2-C2 motif reminiscent of a potential zinc finger structure. However there are no reports that zinc is found on purified eIF2 or required for the eIF2 activity. Mutational analysis of yeast eIF2[5 suggests that the lysine repeats and the C2-C2 motif present in the amino and carboxy-terminal regions of the protein are important for interaction with mRNA. (Laurino et al., 1999). The γ-subunit of yeast eIF2 contains homologous sequences that is found in the elongation factor Tu (EF.Tu) of eubacteria. These sequences have been found important for this factor to bind tRNA (Hannig et al., 1993). Hence it is suggested that it may be involved in initiator tRNA binding. Also, eIF2 containing a suppresser p-subunit binds the Met-tRNAi with lower affinity than the wild type eIF2 complex, thereby suggesting that eIF2fi may be involved in interacting with initiator tRNA (Donahue et al., 1988). Recent information suggests that the binding domain for mammalian and also for yeast eIF5 resides in the N-terminal half of eIF2p and includes the second of the three lysine boxes or all of them respectively (Das et al., 1997 and Asano et al., 1999). In addition, rat liver and yeast eIF2[i-subunit are shown to interact with the 5- and e-subunits of their respective eIF2B proteins (Kimball et al., 1998 and Asano et al, 1999). The interaction requires the N-terminus lysine boxes in the case

of yeast eIF2β whereas, the binding site on rat liver eIF2β has been shown located within approximately 70 amino acids from the C-terminus. In yeast, through mutational analysis, it is suggested that the lysine boxes in eIF2β are required for a tight binding of eIF2B with its substrate eIF2 and the interaction is also dependent on the bipartite motifs rich in aromatic and acidic residues which are conserved at the C-termini of the catalytic subunit of eIF2B (eIF2Bε) (Asano etal., 1999). Based on the unpublished observations that archea lack all the five subunits of eIF2B and archeal eIF2β is devoid of the lysine boxes (Asano etal., 1999), Hinnebusch's laboratory suggested that during evolution, eIF5 and eIF2B acquired domains containing the bipartite motifs, whereas, their common substrate eIF2 acquired polylysine residues.

2.2. Phosphorylation of eIF2 and physiological significance.

Both the α and β-subunits of eIF2 are accessible for phosphorylation by several kinases *in vivo* and vitro. Phosphorylation of the ser^{2} and ser^{67} in the β-subunit occurs by CK-II, where as, ser^{13} and ser^{218} have been shown to undergo phosphorylation in the prevsence of PKC and PKA (Clark et al., 1989 and Welsh et al., 1994). It is not clear if this phosphorylation has any regulatory significance. However, Singh et al (1994) has reported that dephosphorylation of the (i-subunit stimulates the ability of eIF2 to bind GDP. In addition it was shown that phosphorylation of C-terminal portion of this subunit by protein kinase A, increased the guanine nucleotide exchange activity of eIF2B, whereas, the phosphorylation in the N-terminal region by CK-II did not cause a similar effect (Kimball et al., 1998). These studies are consistent with the idea that p-subunit of eIF2 interacts with eIF2B and suggest that probably phosphorylation of this subunit in the C-terminus may also alter this interprotein interaction. The eIF2 protein is partly ribosome

bound. It is isolated from both ribosome and non-ribosome fractions of cytoplasmic extracts.

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

Phosphorylation of the α-subunit in eIF2 occurs in cells or in cell-free translational systems in response to a variety of stimuli such as heme-deficiency (Levin et al., 1976; Kramer et at., 1976; **Farrell** et **at.,** 1977 and Surolia et al., 1991), viral infection or low levels of double-stranded RNA (Farrel et al., 1997 and Levin at al., 1978), amino acid and nutrient starvation (reviewed in Pain, 1994; Dever et al., 1992; Scorsone et al., 1987; Clemens et al., 1987 and Alcazar et al., 1995), purine limitation (Rolfes et al., 1993), serum and growth factor deprivation (Duncan et al., 1985; Montine et al., 1987 and Ito et al., 1994), transient transfection of certain plasmids (Kaufman et al., 1989), during cerebral ischemia (Burda et al., 1998) exercise (Menon et al., 1995), heat shock (Duncan et al., 1984 and De Benedetti et al., 1986), heavy metals (Hurst et al., 1987; Matts et al., 1991 and Alirezaei et al., 1999), release of calcium from the endoplasmic reticulum (ER) or ER-stress (Prostko et al., 1992; Prostko et al., 1993; Prostko et al., 1995; Aktas et al., 1998 and Laitusis et al., 1999), oxidising agents such as oxidised glutathione (Ernst et al., 1979 and Kan et al,, 1988), pyrroloquinoline quinone (Ramaiah et al., 1997), sodium arsenite (Laitusis et al 1999), denatured proteins (Matts et al., 1993) and nitric oxide (Kim et al., 1998) etc.

Phosphoryiation is correlated with a global inhibition of protein synthesis in cell-free translational systems obtained from reticulocyte lysates, or, selective stimulation of certain mRNAs over the others as in the case of yeast subjected to amino acid starvation (reviewed in London et al., 1987; Jackson, 1991 and Hinnebusch, 1993). Increased eIF2a phosphoryiation is correlated to enhanced synthesis of GCN4 in yeast. GCN4 is a transcriptional factor and stimulates the synthesis of several mRNAs that encode proteins which are required in the various amino acid biosynthetic pathways. Recent studies suggest that eIF2a phosphoryiation plays an important role in growth and development and in apoptosis (Donze et al., 1995; Qu et al., 1997; Der et al., 1997; Srivastava et al., 1998 and Alcazar et al., 1995). Abrogation of eIF2 phosphoryiation by expressing non-phosphorylatable mutant eIF2a or a mutant eIF2a kinase like PKR can lead to malignancy (Donze et al., 1995; Koromilas et al., 1992). Also, a recent study reported that eIF2α was cleaved in apoptotic Saos-2 cells on treatment with poly(I).poly(C) or tumor necrosis factor α and in the presence of caspase-3 *in vitro.* By site directed mutagenesis, the cleavage site was mapped to an Ala-Glu-Val-Asp$^{300}\downarrow$ Gly^{301} sequence located in the C-terminal protein of eIF2α. (Satoh et al., 1999). Recent studies demonstrated the presence of eIF2 in the nucleus, interaction of eIF2 with DNA-dependent protein kinase (DNA-PK) and the phosphoryiation of the β-subunit of eIF2 by DNA-PK (Ting et al., 1998). In addition, eIF2α and eIF4E expression is increased in reponse to growth induction by c-myc (Rosenwald et al., 1993). These findings suggest that probably the role of eIF2 is not limited to translational initiation but it may as well be associated with DNA repair, apoptosis and in malignancy.

2.3. eIF2(x kinases, their activation and regulation.

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

From a variety of studies such as amino acid sequencing (Colthrust et al., 1987), site specific mutagenesis (Pathak et al., 1988), genetic studies (Vazquez de Aldana et al., 1993) and through the over expression of mutants of eIF2(x (Kaufman et al., 1989; Choi et al., 1992 and Murtha-Riel et al., 1993) which can overcome the inhibitory effects caused by endogenous

eIF2α phosphorylation, it is well established now that serine 51 residue is the only phosphorylation site in mammalian eIF2α. However, in some systems like in yeast, the eIF2α contains three more additional sites for phosphorylation in the highly acidic C-terminal region which can be phosphorylated by CK-II (van den Heuvel et al., 1995). Similarly eIF2α, the p41-42 doublet, in wheat germ is accessible for phosphorylation both by reticulocyte eIF2α kinases and CK-II as has been reported by us earlier (Janaki et al., 1995). In addition, the eIF2α from sea urchin, Brine shrimp and Artemia embryos is phosphorylated by CK-II suggesting the presence of a second site of phosphorylation in the alpha-subunit (Dholakia et al., 1990 and Mehta et al., 1986). The physiological significance of these additional phosphorylation sites in eIF2a is however not clear. Purified mammalian eIF2 alpha kinases from one source can phosphorylate eIF2 from other sources as well (Dever et al., 1993 and Janaki et al., 1995).

2.4. eIF2B and its regulation.

Phosphorylation of a small portion of eIF2α subunit (20-30%) in the trimeric eIF2 decreases protein synthesis globally in reticulocytes and their lysates during heme-deficiency and double-stranded viral infection or selectively in yeast during amino acid deprivation (reviewed in London et al., 1987; Jackson, 1991 and Hinnebusch, 1994). Since phosphorylation of only a portion of eIF2α suffices to inhibit protein synthesis completely (Leroux and London, 1982), it is suggested that there must be yet another rate-limiting initiation factor which must be regulating eIF2 activity. Subsequent studies lead to the identification of eIF2B, a rate-limiting factor that regulates eIF2 recycling when the α-subunit in eIF2 is phophorylated. This is because i) addition of purified eIF2B restores the inhibition of protein synthesis caused by eIF2α phosphorylation in cell-free translational systems (Amesz et al,

1979; Grace et al.,1982 and Matts et al., 1983) and ii) phosphorylation of eIF2a is associated with the inhibition in the guanine nucleotide exchange (GNE) activity of eIF2B *in vitro* (Clemens et al., 1982 and Matts et al., 1983) and in translating extracts (Matts et al., 1984 and Rowlands et al., 1988). Later studies have shown that eIF2B activity, measured in small amounts of translating lysates, decreases specifically whenever eIF2a is phosphorylated but is not affected by general inhibition in protein synthesis caused by inhibitors such as cycloheximide, puromycin and pactamycin that do not affect eIF2 phosphorylation (Babu and Ramaiah, 1996). In reticulocyte lysates, eIF2B pool is estimated to be 1 5% on a molar basis of the eIF2 pool. Hence low levels (20-25%) of eIF2cx phosphorylation in reticulocyte lysates completely inhibit the eIF2B activity and leads to the shut down in protein synthesis. In contrast, a lower ratio of eIF2 to 2B, as has been observed in Ehrlich ascites cells, requires a higher level of eIF2a phosphorylation (Rowlands et al., 1988).

Several investigations were carried out to understand the mechanism by which phosphorylated eIF2α inhibits the GNE activity of eIF2B. It was shown that phosphorylation of eIF2α sequesters eIF2B activity into a 1 5S complex, eIF2α(P).eIF2B, in which eIF2B becomes inactive (Thomas et al., 1985). Rowlands et al (1988b) explained the apparent stoichiometric sequestration of eIF2B by eIF2α(P) as has been demonstrated by Thomas et al., is due to the differences in affinities of eIF2B for eIF2α(P).GDP and eIF2.GDP. This is because eIF2α(P).GDP is not a substrate but is a competitive inhibitor of eIF2B competing with eIF2.GDP for binding to eIF2B and that eIF2B has higher affinity for the inhibitor eIF2α(P) than for the substrate, eIF2.GDP (Goss et al., 1984 and Rowlands et al., 1988). The difference in the dissociation constants, K_D of the clF2B.eIF2 and eIF2B.eIF2α(P) estimated by fluorescence anisotropy was only two fold in

the presence of GDP (Goss et al., 1984) rather than the 150-fold estimated by kinetic measurements (Rowlands et al., 1988b). Recent studies using polyhistidine tagged yeast eIF2, it has been further demonstrated that binding of all the five subunits of over expressed yeast eIF2B to eIF2a(P) was two fold higher than to unphosphorylated eIF2 and ten fold higher than the background level of binding observed in the presence of eIF2 (Pavitt et al., 1998). In mammalian systems, it has been hypothesized that serine 48 residue is required for the interaction to occur between phosphorylated eIF2a(P) and eIF2B (Ramaiah et al., 1994) and is consistent with the observations that over expression of this 48A mutant eIF2a (in which 48 serine residue is replaced by alanine and the mutant can still be phosphorylated on its 51 serine residue) escapes the inhibition of protein synthesis and eIF2B activity caused by phosphorylation of endogenous eIF2α (Choi et al., 1992; Murtha-Riel et al., 1993 and Ramaiah et al., 1994).

In related studies, point mutations in the a, (3- and δ–subunits of the enzyme eIF2B resulted in a phenotype in yeast that is insensitive to eIF2α phosphorylation (Vazquez de Aldana and Hinnebusch, 1994). These above studies suggest that probably eIF2α subunit may be involved in binding one or more of the above subunits of eIF2B. However, results of a recent study (Kimball et al., 1998a) indicate that eIF2B is shown to bind only to the p-subunit of eIF2 by far-western analysis. Since phosphorylation of the a subunit in the trimeric eIF2 complex increases its interaction with eIF2B, it is suggested that phosphorylation of eIF2α results in a conformational change in the eIF2 holoprotein that alters the affinity of eIF2B for eIF2[3 (Kimball et al., 1998a).

Unlike other guanine-nucleotide exchange factors which are generally monomers, eIF2B involved in the initiation of protein synthesis is a

heteropentameric protein consisting of α, p, y, 8, and e and the yeast equivalents are designated as GCN3, GCD7, GCD1, GCD2, and GCD6. In order to understand the subunit structure, function and interaction with other subunits of the protein and also with other proteins, the yeast and mammalian eIF2B subunits are cloned (Hannig and Hinnebusch, 1988; Price et al., 1994 & 96a and b; Asuru et al., 1996; Flowers et al., 1995 and 1996) and the coding regions of these subunits in both systems show considerable homology (Bushman et al., 1993; Price et al., 1996a & b and Pavitt et al., 1997). Interestingly, a (GCN3), β (GCD7), and the carboxy-terminal half of δ (GCD2) subunits share sequence similarity (Paddon et al., 1989 and Bushman et al., 1993a), form a stable complex *in vivo* and the over expressed subunits can partially suppress the toxic effects of eIF2α(P) in yeast cells (Yang and Hinnebusch,1996). This suggests that the homologous regions may be devoted to this regulatory function i.e in recognizing the phosphorylation status of eIF2α. Also, the y, and E- subunits share sequence similarity and can form a complex together (Pavitt et al., 1998). This second subcomplex however is unable to overcome the growth inhibitory effects caused by eIF2α phosphorylation in yeast cells (Yang and Hinnebusch, 1996). Further, the first subcomplex, that is formed by a, β and 5 subunits, does not carry any guanine nucleotide exchange activity and it binds eIF2α(P) at least 10-fold above back ground and three fold higher than eIF2 as has been demonstrated (Pavitt et al., 1998) in yeast in a pull-down assay with extracts over expressing this subcomplex and purified his-tagged eIF2. In contrast, the second sub complex formed by the y- and c-subunits has higher GNE activity than wild type 5-subunit eIF2B of this subcomplex (y and ε– subunits). The GNE activity or its interaction with eIF2 is not affected by eIF2α phosphorylation. The *in vitro* biochemical assays suggest that the ε-subunit of yeast and mammalian eIF2B actually carries the catalytic or GNE activity (Fabian et al., 1997 and Pavitt et al., 1998) and is enhanced by the

presence of the γ-subunit of eIF2B (Pavitt et al., 1998). Further, inhibition of eIF2B activity that occurs due to eIF2α phosphorylation under amino acid starvation conditions (Pavitt et al., 1998; Dever et al., 1992) stimulates GCN4 translation (reviewed in Hinnebusch,1997). As has been mentioned earlier, the deletion of the a- subunit of eIF2B or point mutations in this subunit inhibits the induction of GCN4 mRNA translation under amino acid starvation conditions (Hinnebusch and Fink, 1983; Hannig and Hinnebusch, 1988 and Pavitt et al., 1997). Moreover, deletion of eIF2Bα reduces the growth inhibitory effect of high level eIF2α phosphorylation induced by over expression of the human double-stranded RNA dependent eIF2a kinase (Dever et al., 1993). Consistent with these findings, biochemical studies *in vitro* indicate that both human and yeast eIF2B devoid of the a-subunit are found tobe insensitive to eIF2α phosphorylation (Pavitt et al., 1998; Fabian et al., 1997 and Kimball et al., 1998). However, eIF2B lacking the a-subunit has showed a higher affinity binding of eIF2a(P) versus eIF2 characteristic of wild-type eIF2B (Pavitt et al., 1998 and Kimball et al., 1998). This information suggests that the mutant eIF2B devoid of the α-subunit can accept eIF2α(P).GDP as a substrate and the mutation or deletion of this subunit does not affect the GNE activity of eIF2B (Fabian et al., 1997; Pavitt et al., 1998 and Kimball et al., 1998) or simply reduce the binding affinity between eIF2B for the inhibitor eIF2a(P) versus the substrate as has been hypothesized earlier (Hinnebusch, 1994). These findings summarize that the alpha-subunit of eIF2B is not required for the catalytic activity but is needed for inhibition by eIF2a(P) (Pavitt et al., 1998 and Fabian et al., 1997). Interaction of the regulatory subcomplex but not the catalytic complex, with eIF2 is significantly increased when the 51 serine residue in eIF2a is phosphorylated as has been demonstrated (Pavitt et al., 1998). These observations also correlate with the ability of the regulatory complex to overcome partially the growth inhibitory effects in yeast caused by eIF2a

activity (and indirectly eIF2 acivity as well) is modulated by signalling pathways (reviewed in Rhoads,1999). eIF2B is reported to be associated with NADPH (Dholakia et al., 1986) and its activity is decreased when there is an increase in NADP/NADPH ratio (Proud et al., 1992; Dholakia et al., 1986 and Akkaraju et al., 1991). Also redox factors like pyrroloquinoline quinone (Ramaiah et al., 1997), oxidised glutathione and low concentrations of sugar phosphates (Kan et al., 1988) can alter eIF2B activity. Reducing conditions stimulate the GNE activity of eIF2B while oxidising conditions are found to inhibit its activity. In addition, higher concentrations of sugar phosphates and nucleotides like ATP and inositol phosphate are shown to modulate eIF2B activity by allosteric means (Singh and Wahba, 1995 and Kimball and Jefferson, 1995).

Previous studies have shown that expression of eIF2α mutants in which the putative phosphorylation sites, that is, the serine residues at 48 and 51 were changed to alanine, can relieve the inhibition of protein synthesis (Choi et al., 1992 and Murtha-Riel et al., 1993) and mitigate the inhibition in the GNE activity of eIF2B (Ramaiah et al., 1994) that is caused by eIF2α phosphorylation in cultured mammalian cells. While the 51A mutant is not phosphorylated, the 48A mutant is found to be substrate for phosphorylation in these studies. In contrast, expression of 51 aspartic acid mutant (51D) causes inhibition of protein synthesis. These eIF2α mutants are useful in resolving the phosphorylation sites in mammalian eIF2α (Pathak et al., 1988 and Choi et al., 1992), in localizing protein synthesis defects that occur due to eIF2a phosphorylation in such cases as heat shock and calcium sequestration (Murtha-Riel et al., 1993 and Srivastava et al., 1995), in determining the importance of eIF2α phosphorylation in growth and development and in apoptosis (Donze et al., 1995 and Srivastava et al.,

1998), and also in the expression of eIF2α kinases that are inhibitory for protein synthesis (Chefalo et ai., 1994).

2.5. eIF2a Phosphatases.

So far, no protein phosphatase has been shown to rescue protein synthesis inhibition caused by eIF2α phosphorylation, although both type1 and 2 protein phosphatases are found to dephosphorylate eIF2a(P) *in vitro* (Pato et al., 1983a and b and Redpath and Proud, 1990). There was no such indication however that such preparations could restore protein synthesis inhibition or eIF2B activity caused by eIF2a phosphorylation in translating lysates. With the help of inhibitors of type1 and 2 phosphatases, this laboratory suggested that probably a type1 phosphatase may be involved in the dephosphorylation eIF2α(P) and restoration of eIF2B activity in heme-deficient inhibited lysates rescued by the delayed addition of hemin. These observations are consistent with the earlier observations that addition of an inhibitor of type1 phosphatase stimulates eIF2α phosphorylation and inhibits protein synthesis in hemin-supplemented reticulocyte lysates (Ernst et al., 1982) and involvement of a type1 phosphatase in controlling the extent of eIF2α phosphorylation in yeast (Wek et al., 1992). Also, a type1 protein phosphatase is shown to form a complex with the viral gene product, $\gamma_1$34.5 protein in herpes simplex virus-infected cells and blocks the shutoff of host protein synthesis that occurs due to the activation of double stranded RNA dependent eIF2α kinase by directing the protein phosphatase 1a to dephosphorylate eIF2a (He et al., 1997).

2.6. Physiological Inhibitors of eIF2α Phosphorylation.

The rate of protein synthesis is tightly correlated with the growth state of the cell. Since small changes in eIF2a phosphorylation influences dramatically the rate of initiation of protein synthesis, cells have evolved different strategies to regulate their eIF2α kinases and the level of eIF2a phosphorylation. Heme, an iron protoporphyrin compound is known to inhibit the activation of HRI kinase by promoting disulfide bonds in the protein (Chen et al., 1989). However HRI kinase as mentioned above appears to be present mainly in erythroid tissues and is activated during heme-deficiency (Crossby et al., 1994). Unlike HRI, PKR is ubiquitously found in most of the cells. This protein kinase activation occurs not only in the presence low concentrations of double stranded viral RNA or by viral infection, but also by various stress conditions such as heat shock, growth factor deprivation, treatment with tumor necrosis factor α, and release of calcium ions from the endoplasmic reticulum. In addition, other regulatory molecules such as polyanions like heparin and dextran sulfate also cause activation (reviewed in Clemens and Elia 1997). In view of this wide ranging conditions that stimulate PKR activation, cells and viruses have evolved various mechanisms to check or stimulate the activation and activity of PKR. Several cellular and viral proteins and also the viral RNAs as mentioned below are known to regulate PKR activation and eIF2α phosphorylation These products are produced as part of cellular or viral defense mechanisms. These regulatory molecules inhibit or activate eIF2α phosphorylation through multiple mechanisms as mentioned in here. 1) A block in eIF2α phosphorylation by active HRI and PKR occurs as in the case of a cellular glycosylated protein like p67 (Chakraborty et al., 1994). 2) Inhibition of the activation of PKR enzyme occurs by RNA transcripts produced by the interferon-resistant viruses such as adenovirus small RNA VA1, EBER-1 of Epstein-Barr Virus RNA, the TAR-RNA of HIV-1 and the Hepatitis C Virus envelope protein E2, through competition and induction of a conformational change in PKR protein

that disrupts the association of PKR with ribosome at all the sites (reviewed in Clemens and Elia, 1997 and Taylor et al., 1999). 3) DsRNA molecules are sequestered by viral proteins like E3L and sigma 3 proteins coded by Vaccinia and Reovirus so that PKR activation does not take place (Chang et al., 1992 and Giantini et al., 1989). 4) The viral proteins serve as pseudo substrates or mimick eIF2cx as in the case of K3L and PK2 proteins produced by Vaccinia and Baculovirus (Davies et al., 1993 and Dever et al., 1998). 5) Herpes Simplex Virus products, the $\gamma_1$34.5 gene product which is homologous to the mammalian protein known as GAAD34 (growth arrest and DNA damage protein 34) and a ribosome-associated RNA-binding protein, (Us11) blocks the inhibition of protein synthesis caused by PKR activation by directing the protein phosphatase 1a with which it is associated to dephosphorylate eIF2α(P) as in the case of $\gamma_1$34.5 gene product (He et al., 1997, 1998 and Mulvey et al., 1 999) or, by blocking PKR activation as in the case of Us11 as has been postulated Mulvey et al., (1999). 6) Activation of a cellular inhibitor, p58, as identified originally in cells infected with influenza virus inhibits the dimerization of PKR (Lee et al., 1994). 7) L18, one of the proteins of the 60S subunits competes with dsRNA for binding to PKR and prevents PKR activation by dsRNA while PKR is associated with the ribosome (Kumar et al., 1999). In addition to these inhibitors, there are other cellular inhibitors of PKR kinase and eIF2a phosphorylation whose mechanism of action is not yet well characterized. These include a) TAR RNA binding protein of Human immunodeficiency virus whose over expression induces a transformed phenotype in NIH3T3 cells (Park et al., 1994; Benkirane et al., 1997 and Cosentino et al., 1995) b) a 100 kDa cellular protein, induced in NIH3T3 cells in response to the expression of the transforming Harvey ras oncogene (Mundschau and Faller 1992) c) a 15 kDa protein, produced in Murine 3T3F442α cells that are induced to differentiate into adipocytes (Judaware and Petryshyn 1992) d) the La antigen which probably inhibits

PKR by sequestering and unwinding dsRNA (Xiao et al., 1994) e) inactivation of PKR by Alu RNA (Chu et al., 1998) and f) Simian virus 40 large-T antigen rescues translational inhibition with out affecting PKR activation and eIF2a phosphorylation probably at a step down stream of eIF2α phosphorylation (Swaminathan et al., 1996).

3. BACULOVIRUS

Baculoviruses or Nuclear polyhedrosis viruses are a family of insect viruses. They infect mostly Lepidopterans (butter flies and moths), Hymenoptera (sawflies) and Coleoptera (butterflies). They are also found to infect Crustaceans. The baculo portion of the name refers to the rod shaped capsids of the virus particles. Within the capsid, the DNA is condensed into a nucleoprotein structure known as the core. The DNA of baculoviruses is double stranded, covalently closed circular structure and is 130-200 kb in length. In nature, baculoviruses occur as virions that are occluded within the proteinaceous crystals known as polyhedra on plant leaves, plant debris and soil. Viral occlusion bodies are formed in the nucleus. Polyhedral occlusion bodies of nuclear polyhedrosis viruses (NPV) are known as polyhedra, occluded viruses (OV), or polyhedral inclusion bodies. The word nuclear in the NPV is used to distinguish it from the nuclear polyhedrin protein from the matrix polyhedrin protein characteristic of cytoplasmic polyhedrosis viruses which are members of reoviridae. Among the various viruses of baculoviridae, Autographa californica nuclear polyhedrosis virus and Bombyx mori nuclear polyhedrosis virus are the most well characterised. Recently, the genome of another baculovirus, Lymantria dispar nuclear polyhedrosis virus has been sequenced and is composed of 161 kb (Kuzio et al, 1999).

Baculoviruses have proven to be the most powerful and versatile eukaryotic expression vectors available. Although several mammalian viruses like SV40, adenoviruses, herpes simplex, vaccinia virus, cytomegalovirus and mammary tumour virus etc., have been used to express foreign proteins very sucessfully, most of these viruses have their own limitations in terms of safety, biological containment, level of expression, proper post translational modifications and proper processing. The baculovirus expression system bypasses most of these limitations. In addition, they have been recognised as eco-friendly and possessing the ability to develop into potential biopesticides. Baculoviruses have recently been found to infect mammalian cells, but they fail to replicate, thereby contributing to the additional application of baculoviruses as vectors for the delivery of foreign genes in mammalian systems (Hofman et al., 1995; Boyce and Bucher 1996 and Sandig et al., 1996). The p29 and p10 promoters of baculovirus system are simple in architecture but they are powerful in driving foreign gene expression to spectacular levels (400 μg/ml). However, several factors influence the level of expression (Hasnain et al., 1994 and Ranjan and Hasnain, 1995a and b).

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

The most extensively studied baculovirus strain is Autographa californica nuclear polyhedrosis virus(AcNPV). During recent years its entire genome is mapped and sequenced (O'Reilly et al., 1992 and Ayres et al., 1994). Although, AcNPV was first isolated from the alfalfa looper (Autographa californica), it multiplies readily in cell lines derived from the fall armyworm

(Spodoptera frugiperda) and the cabbage looper (Trichoplucia ni). Though most of the expression vectors harness AcNPV infection of Spodoptera frugipedra cells, heterologous proteins also produced in silkworm larvae (Bombyx mori) by infecting them with recombinant BmNPV (Maeda, 1989). Recombinant virus construction is based on the homologous recombination between the plasmid carrying the foreign gene and viral genome.

3.1. The infection process.

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

The infection cycle is divided into three phases: early, late and very **late.** During the early phase, there is transcription of genes, whose product is essential for viral DNA replication, and these genes are transcribed by **the** RNA polymerase encoded by the host (Grula et al., 1981) This phase continues up to 5 to 6h post- infection (h p.i). Between 5 and 18h p.i, late phase genes are transcribed which encode structural proteins and budding of nucleocapsid. The very late phase starts from around 20h p.i and is characterised by the transcription of occlusion-specific genes (polyhedrin gene, p10 gene) involved in viral occlusion process. The promoters of these genes are so strong that these genes continue to be overexposed such **that** 50-75% of the total protein in an infected cell is polyhedrin protein. The late

phase and very late phase genes are transcribed by virus-encoded or virus-modified host RNA polymerase (Huh et al., 1990). The Autographa californica nuclear polyhedrosis virus polyhedrin gene is transcribed at high levels very late in the lifecycle and involves a virus-specific or a virus modified host RNA polymerase. Reversible phosphorylation is an important posttranscriptinal modification that can modulate the functions of many cellular enzymes and transcription factors, control protein synthesis and macromolecular assembly, and regulate cell cycle propagation and signal transduction pathways (reviewed in references Boulikas,1995; Edelman et al., 1987; Hunter, 1995 and Hunter et al., 1992). The activation of many viral proteins also can be regulated by phosphorylation, and this can be an important factor in viral replication (reviewed in references (Leader, 1988 and Prives, 1990).

AcNPV infection produces two types of viral progeny which are different structurally and functionally. The occluded or polyhedra-derived viral forms (PDV) are responsible for primary infection and embedded within a matrix of proteinaceous structures called occlusion bodies (OBs). During natural infection, the larvae ingest PDV containing OBs that contaminate their food. Then in the midgut of the larvae, the polyhedra are dissolved due to the presence of an alkaline environment. This process releases the embedded virions. The liberated PDV infect the midgut columnar epithelial cells by receptor mediated membrane fusion (Horton and Burand, 1993). The first viral occlusion bodies of wt AcNPV develop 2 days p.i, but continue to accumulate and reach a maximum between 5-6 days p.i. Occlusion bodies are visible under light microscope as dark, polygonal shaped bodies filling the entire nucleus of the infected cell These infected cells produce the BV or extracellular viruses. The budded virus form (BV) transmits infection from cell to cell during secondary infection. ODV infects gut cells by fusion of the viral

envelope with the columnar cell microvillar membranes, whereas BV entry of other cells occurs by Adsorptive endocytosis (Volkman and Goldsmith, 1985). Once the viral DNA is released the host nucleus becomes enlarged and forms distinct electron dense granular structure, called the virogenic stroma (Fraser 1986). Around 1 2h, progeny BVs are formed and are released to the extracellular compartment. Polyhedra soon begin to be formed thereafter and mature PDVs surrounded by envelopes become occluded. PDV and BV differ significantly in their ability to infect the insect through the gut and, viral envelopes and nucleocapsids of the two viral forms contain different proteins, or proteins that are processed differently (Braunagel et al., 1988). The most distinctive difference observed to date is the presence of viral encoded glycoprotein, gp64, which is found in BV but not in PDV. During secondary infection, gp64 is intimately involved in virus entry into the cells via the process of Adsorptive endocytosis. Other differences include the presence of an O-glycosylated protein, gp41 and the protein p74 in PDV, but not in BV.

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

3.2. The advantages of baculovirus expression system.

Two important features of the baculovirus account for the success of this virus as an expression vector. First, the virus contains at least two non-essential regions that can be replaced by foreign genes. Second, many of these genes especially the late genes are under the control of very strong promoters that allow hyper expression of the foreign genes. Most of these transfer vectors make use of p29 and p10 promoters along with

neighbouring sequences to allow homologous recombination. Both p29 and p10 are nonessential genes and deletion of these genes does not affect replication of virus in cell culture (Smith et al, 1983 and Were et al, 1989).

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

Since the baculovirus gene is too large to manipulate *in vitro* to produce recombinant virus, transfer vector or transplacement vectors are widely used (Luckow and Summer, 1989). Although, there are several reports describing direct insertion of foreign genes into the genome via enzymatic ligation (Peakman et al., 1992), through the use of large bacterial plasmids and transposon element (Luckow et al., 1993) or inserting a yeast replicon element (Patel et al., 1992). However, these methods are cumbersome. The transfer vectors contain a bacterial plasmid, a portion of the baculoviral genome encompassing a gene promoter and a transcriptional terminator. Two regions of AcNPV have been used to construct transfer vectors. These are 7.3 Kb EcoRI fragment containing the polyhedrin gene and 2.0 Kb EcoRI-P fragment containing the p10 gene. Earlier transfer vectors used to have too long flanking sequences but now the size of these vectors have been significantly reduced to accommodate larger insert. A number of improved

methods are now available to simplify the tedious process of identifying the polyhedra negative recombinant viruses. These include methods wherein linearized baculovirus DNA (Kitts et al., 1990; Kitts and Possee 1993) were used to increase the number of recombinant viruses obtained after cotransfection. An engineered baculovirus was constructed such that two sites for Bsu36l were introduced in the flanking sequences upstream to the promoter and in the downstream orf129 encoding an essential gene, the viral replicase. This linearized modified viral genome is cotransfected with the plasmid carrying a gene of interest under the polyhedrin promoter and deleted portion of the viral genome. Bsu361 digested viral genome lacking the portion of the essential downstream gene, even after *in vivo* repair and recircularization is unable to produce viable viruses. Only when it recombines with the transfer vector carrying the missing segment along with the gene of interest it can form viable progeny virus. This approach results in assured recombination and results in >90% recombinants. Clontech markets this system under the trade name of BacPAK and is marketed by Pharmingen as 'baculogold'.

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

4. OBJECTIVES

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

The availability of site-specific mutants of eIF2a like the 48A or 51A in which the serine residues in the respective positions of eIF2α have been replaced by alanine, has advanced our understanding in identifying that, a) serine 51 residue in eIF2α is the only site for phosphorylation in mammalian eIF2u (Pathak et al., 1988), b) the translational block caused by adenoviral mRNAs, plasmid derived mRNAs, heat shock or calcium release from the endoplasmic reticulum is due to increased eIF2α phosphorylation (Kaufman et al., 1 989; Choi et al., 1 992; Murtha-Riel et al., 1 993 and Srivastava et al., 1995) or in localising the translational inhibition caused by eIF2a phosphorylation, and c) phosphorylation of eIF2α plays critical role in cell proliferation and development (Donze et al., 1995 and Qu et al., 1997) . In addition, the coexpression of a mutant eIF2α (51 A) which cannot be phosphorylated has facilitated the expression of mammalian eIF2α kinases like the heme-regulated kinase in insect colls (Chefalo et al., 1994).

In order to obtain a good amount of purified wt and mutants of eIF2a for a variety of biochemical characterizations to determine protein-protein interaction and eventually express the other subunits of initiation factor and other initiation factors, it is felt that we should have a system that is relatively easy to handle and maintain. In this context we chose to express eIF2α subunit and mutants of eIF2a in Spodoptera frugiperda insect cells using baculovirus expression system. The following site-specific mutations of eIF2α were cloned into baculovirus vector and expressed.

The mammalian cells were unable to express 51D mutant (aspartic acid mutant) of eIF2a efficiently and the cells expressing this mutant were killed. This is probably because the mutation created to mimic the charge of a phosphorylated serine and the mutant behaves like phosphorylated species. One of the objectives of this thesis is also to see if it is possible to express 51D mutant protein in insect cells using baculovirus expression system.

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

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

c) 48A is another mutant in which 48 **serine** residue is replaced by an alanine. This mutant is phosphorylated on its 51-serine residue. However

over expression of this mutant escapes the inhibition in protein synthesis and mitigates the inhibition in eIF2B activity caused by endogenous eIF2a phosphorylation (Kaufman et al., 1989; Choi et al., 1992., Murtha Riel et al., 1993 and Ramaiah et al., 1994).

CHAPTER II

MATERIALS AND METHODS

1.0. CELL FREE TRANSLATION SYSTEM

1.1. Preparation of heme sensitive rabbit reticulocyte lysates.

1.2 .Measuring reticulocyte lysate protein synthesis.

2.0. **RABBIT RETICULOCYTE eIF2 AND eIF2B PURIFICATION**

2.1. Purification of rabbit reticulocyte eIF2 from post ribosomal salt wash.

2.2. Purification of eIF2B from post ribosomal salt wash.

3.0. **GUANINE NUCLEOTIDE EXCHANGE ACTIVITY OF eIF2B**

3.1 . Preparation of eIF2.[^{3}H]GDP binary complex, the substrate.

3.2. Assay for endogenous eIF2B activity in protein synthesising lysates and insect cell extracts.

4.0. **PHOSPHORYLATION ASSAYS**

4.1. *In situ* phosphorylation.

4.2. *In vitro* phosphorylation.

5.0. **ANALYSIS OF eIF2α(P).eIF2B COMPLEX IN RETICULOCYTE LYSATES**

6.0. INSECT CELL TISSUE CULTURE

6.1. Cell lines and virus.

6.2. Preparation of TNM-FH medium.

6.3. Freezing and revising of Sf9 cells.

7.0. MOLECULAR CLONING OF eIF2α cDNA INTO BACULOVIRUS TRANSFER VECTOR pBacPAK8

7.1. Transformation, amplification, purification and excision of human eIF2a cDNA (wild type and mutants).

7.2. Isolation and restriction digestion of plasmid DNA.

7.3. Ligation of 1.6 Kb eIF2α cDNA into pBacPAK8 transfer vector.

7.4. Colony hybridization and probe preparation.

7.5. Southern Hybridization.

8.0. CO-TRANSFECTION AND IDENTIFICATION OF RECOMBINANT BACULOVIRUSES

8.1. Plaque Assay.

8.2. Amplification of the recombinant viral stock.

9.0. ANALYSIS OF THE BACULOVIRUS EXPRESSED RECOMBINANT eIF2a PROTEIN

9.1. Dot – Blot hybridization.

9.2. Determination of eIF2α expression and preparation of cell extract.

9.3. Sodium dodecyl sulphate – polyacrylamide gel electrophoresis.

9.4. Western immunoblot analysis.

10.0. PURIFICATION OF BACULOVIRUS EXPRESSED RECOMBINANT eIF2α

MATERIALS

The various fine chemicals, enzymes, isotopes and antibodies were supplied by various companies or received as gifts from other laboratories as mentioned below.

Amersham-Pharmacia Biotech, UK: I) Multiprime DNA labelling kit, Hybond N' membranes., 0.45 μM Nitrocellulose Membranes, X-Ray films, Restriction enzymes,T4 DNA ligase, L-[U-^{14}C]leucine (11.2 GBq/mmol, 303 mCi/mmol, 50 mCi/ml) and [8-^{3}H]GDP (429 GBq/mmol, 11.6 Ci/mmol,1.0 mCi/ml) and Protein molecular weight markers.

Biorad, USA: Protein molecular weight markers, Biorad protein estimation reagent, Acrylamide and Bis-acrylamide.

Biological E. Ltd, India: Heparin and Newzealand White Male Rabbits.

Boehringer and Mannheim GmbH, Germany: Creatine phosphokinase, Creatine phosphate, GDP, GTP, DTT, Mlu I, Sca I, EcoR I and T_4 DNA ligase.

Calbiochem, USA: Poly (IC).

Clontech, USA: Clontech kit for expression in Sf9 cells using the BacPAK vectors.

BRIT and **JONAKI,** India: [γ-^{32}P] ATP (3000 Ci/mmol) and [α-^{32}P]dCTP (4000 Ci/mmol).

DIFCO, USA: Bactoagar, Bacto-tryptone and Yeastolate.

Dupont, NEN, USA: [^{14}C]Leucine (330 mCi/mmol,100 mCi/ml) and [8-^{3}H]GDP (2 mM, 9 Ci/mmol).

Flow laboratories, Scotland: Neutral red staining solution.

GIBCO BRL, USA: Graces' insect cell culture medium with or without methionine, Fetal calf serum, 1 kb DNA ladder and Competent DH5u cells.

Indu India: Developer and Fixer.

Loba-Chemie, India: TEMED, β- mercaptoethanol.

Merck, India: Glycine.

Millipore, USA: 0.22 and 0.45 μM Nitro-cellulose filter discs.

Sartorius, Germany: Filter units, 0.45 μM and 0.22 μM filter discs.

NESTLES, India: Non- fat dry milk.

New England Biolab, USA: DNA polymerase I, Klenow fragment, Restriction enzymes and Protein molecular weight markers.

Pharmacia, Sweden: S-300, CM- Sephadex and Heparin Sepharose 6B.

Whatman, UK: Whatman 1 and 3 filter papers, DEAE-cellulose, Phosphocellulose and Nitro-cellulose membranes.

Promega Corporation Inc, USA: Restriction enzymes, Anti-mouse IgG raised in rabbit-AP conjugate, NBT and BCIP.

Research Genetics, Inc. USA: Anti-eIF2α antibody (A polyclonal phosphospecific, anti-eIF2α antibody).

Qiagen, **USA:** Qiagen kit for DNA purification.

Sigma, St. Louis, USA: Acetyl phenylhydrazine, ATP, GDP, HEPES, Magnesium acetate, Sucrose, TNM-FH medium, Bromophenol blue, Coomassie R250, BSA, Antimycotic-antibiotic solutions, Ficoll, Low gelling temperature agarose, Agarose, Sodium bicarbonate, PMSF, PVP, Sonicated salmon sperm DNA, Trypan blue, L- Amino acids, Tissue culture flasks (T-25 and T-75), Calcium chloride, Triton-X 100, Twin 20, and Potassium acetate.

Qualigens, India: KCl, NaCl, Glucose, HCl, H_2SO_4, HNO_3, Magnesium chloride, Acetone, Toluene, Glycerol, Ammonium sulphate, Disodium hydrogen phosphate, Sodium fluoride, Acetic acid, Ammonium acetate, Ammonium carbonate, Silver nitrate, Methanol, Ammonium persulphate, Sodium fluoride, and EDTA.

SISCO, India: Phenol.

Spectrochem, India: Tris-HCl, Glycine, POP and POPOP.

Sarabhai chemicals, India: Hydrogen peroxide.

Gifts: Monoclonal antibodies for eIF2α raised in Dr. Henshaw's laboratory, eIF2Bε produced in Dr. Christopher Proud's laboratory and HRI produced in Dr. London's laboratory were kindly provided by Dr. Jane-Jane Chen in MIT, Cambridge. eIF2u wild type, 48A, 51A and 51D mutant cDNA's were generously provided by Prof. Randall J. Kaufman, University of Michigan Medical Center, Ann Arbor, Michigan.

METHODS

1.0. CELL FREE TRANSLATION SYSTEM

1.1. Preparation of heme sensitive rabbit reticulocyte lysates.

White male Newzealand rabbits were used for the preparation of heme-deficient rabbit reticulocyte lysates. The lysates were prepared as described (Hunt et al., 1972 and Ernst et al., 1978). Each of several rabbits was injected with 1% acetyl phenyl hydrazine 2.5 ml (APH) daily for 4 days. After five days the rabbits were bled through the ear vein or through the optic vein. The blood was collected into 30 ml pre-cooled corex tubes containing heparin (300 IU for 30-50 ml of blood). The blood was collected separately and processed at 4 °C. The RBC cells were isolated by centrifuging the blood at 3,000 rpm for 5 minutes at 4 °C in a Remi high speed centrifuge. The supernatant was carefully removed with a pasture pipette and the cell pellets were suspended in buffered saline (5 mM Hepes pH 7.2, 130 mM NaCl, 5 mM KCl, 5 mM $Mg(OAc)_2$ and 5 mM Glucose). The RBC cell pellet was briefly suspended in buffered saline and centrifuged at 4 °C for 5 min at 3000 rpm. This step was carried out at least three times, each time the buffy coat (that was present on the top of the cell pellet) was removed carefully without touching the cell pellet. The buffy coat was removed carefully and the cells were lysed in equal volume of ice cold sterile water. The cells were lysed on ice for about 2-5 min and then were spun at 4 °C for 20 min at 10,000 rpm. The 10 K supernatant was collected and was stored in 250 µl aliquots in liquid nitrogen. Bulk preparation of lysate was also carried out for preparation of initiation factor 2 and 2B.

1.2 Measuring reticulocyte lysate protein synthesis.

The reticulocyte lysate protein synthesis was performed in **a** 25 µl reaction volume. The reaction mixture contained the following ingredients: 60% reticulocyte lysate, 10 mM Tris-HCl, pH 7.7, 80 mM KOAc, 1 mM Mg $(OAc)_2$, 40 µM amino acid mix, 200 µM GTP, 5 mM CP, 120 µg **CPK and** 33 µM [^{14}C] leucine (8 µci/ml) (Ernst et al., 1980).

25 µl reaction mixtures were incubated at 30 ^{0}C with or without the addition of 20 µM hemin to study the incorporation of [^{14}C] labeled leucine into protein. At different times, 5 µl of protein synthesizing lysates were spotted on Whatman No. 1 filter paper. The filters were dried and immersed in 10% cold TCA (trichloroacetic acid) for 20 min to precipitate **the** protein. The filters were then transferred to 5% boiling TCA for 5 min and subsequently were washed with 5% TCA at room temperature to remove non-specific radioactivity. The filters were then washed with crude ethanol and acetone and dried. Afterwards, the filters were soaked in chilled (1:1) diluted H_2O_2 for 5 min to bleach the colour. Once again the filters were washed with ethanol and acetone and dried. Dried filters were counted for measuring the incorporation of labeled amino acids using toluene based scintillation fluids in a Beckman liquid scintillation counter.

2.0. RABBIT RETICULOCYTE eIF2 AND eIF2B PURIFICATION.

2.1. Purification of rabbit reticulocyte eIF2 from post ribosomal salt wash.

eIF2 was purified from ribosomal salt wash (Andrews et al., 1985; Babu and Ramaiah, 1996). 1:1 diluted reticulocyte lysate was layered over a glycerol cushion [50% glycerol, 10 mM Tris- HCl pH 7.7, 5 mM NaCl, 25 mM KCl and 2 mM $Mg(OAc)_2$] and centrifuged at 45,000 rpm for 4 h at 4 °C in Ti 70 rotor in Beckman Ultracentrifuge. The ribosomal pellet obtained was suspended in TDEG buffer [20 mM Tris- HCl pH 7.8, 2 mM $Mg(OAc)_2$, 80

mM KCl, 10% glycerol and 100 μM EDTA], treated with KCl (0.5 M final concentration) and centrifuged at 55,000 rpm for 3 h at 4 °C in a Ti 80 rotor in Beckman Ultracentrifuge. The supernatant (ribosomal salt wash) was concentrated (0-80% ammonium sulphate) and dialysed against buffer containing 80 mM KCl. The dialyzed ribosomal salt wash was passed through different ion-exchange chromatography columns as has been shown in the purification profile (Fig. 3). eIF2 has also been purified from the post ribosomal supernatant.

2.3. Purification of eIF2B from post ribosomal salt wash.

The flow chart for eIF2B preparation is shown in Fig. 5. eIF2B was purified from post ribosomal salt wash with the help of ion-exchange chromatography. The various fractions obtained during the purification of eIF2B were separated on a 10% SDS-PAGE to check the purity of eIF2B. The various fractions were probed with an eIF2B polyclonal antibody to determine the presence of eIF2B in the various fractions.

3.0. GUANINE NUCLEOTIDE EXCHANGE ACTIVITY OF eIF2B

3.1. Preparation of eIF2.[^{3}H]GDP binary complex, the substrate.

The ability of eIF2 to bind GDP in the presence of 1 mM Mg(0Ac)?is used as an assay for the presence and activity of eIF2. Purified eIF2 from ribosomal salt wash was incubated with labeled GDP (2 μM) in a 20 μl reaction mixture containing 20 mM Tris-HCl pH 7.8, 80 mM KCl, 100 μg/ml CPK and 1 mM DTT at 30 °C for 10 min followed by another 10 min on ice. The binary complex was stabilized by the addition of 1 mM Mg^{2+} and incubated on ice for 10 more min. The amount of labeled binary complex [eIF2.(^{3}H)GDP] formed was assessed by terminating the reaction with 3 ml cold wash buffer

(20 mM Tris-HCl pH 7.8, 80 mM KCl, and 1 mM Mg^{2+}). The reactions were then filtered through 0.45 μM nitrocellulose filter membrane and the filters were there after washed with another 6 ml of ice cold wash buffer. The filters were dried and the amount of labeled GDP that bound to eIF2 and retained on the Millipore filter was determined by a liquid scintillation counter.

3.2. Assay for endogenous eIF2B activity in protein synthesising lysates and insect cell extract.

The guanine nucleotide exchange activity of eIF2B in translating lysates and in the insect cell extracts was estimated from the dissociation of labeled GDP from the preformed reticulocyte eIF2.GDP binary complex as described earlier (Matts et al., 1984, Babu and Ramaiah, 1996 and Sudhakar et al., 1999). Lysate protein synthesis reactions (20-25 μl) were carried out at 30 °C as described above except that in the place of labeled leucine, unlabeled leucine was used. Protein synthesis was carried out with reticulocyte lysates 10-15 min prior to the addition of preformed-labeled binary complex (20 μl), prepared as described above. Reactions were stopped at specified time points by the addition of cold wash buffer, filtered and dried. The pmol of eIF2.[^{3}H]GDP binary complex retained on the filter were measured. The difference between the pmol of labeled binary complex added initially to the lysates and that retained on the filters after the exchange assay is the eIF2.[^{3}H]GDP dissociated and reflects the eIF2B activity present in the lysates. The assay system is very specific and sensitive as pointed out earlier (Babu and Ramaiah, 1996).

Dissociation of preformed binary complex in reticulocyte lysates was measured in heme-deficiency, during poly (IC)-treatment and in hemin-

supplemented lysates. In addition, the eIF2B activity in inhibited hemin and poly (IC)-treated translating reticulocyte lysates was also studied in the presence of insect cell extracts expressing recombinant eIF2a or partially purified recombinant eIF2a. For analyzing eIF2B activity in insect cell extracts alone, the extracts were incubated with preformed reticulocyte eIF2.GDP binary complex at 30 °C for 10 min. Reactions were stopped at specified time points by the addition of cold wash buffer, filtered and dried. The GNE activity of insect cell extracts is then estimated from the radioactive counts as described above.

4.0. PHOSPHORYLATION ASSAYS

4.1. *In situ* phosphorylation.

Heme-sensitive rabbit reticulocyte lysates (15 μl) were pulsed with [γ-^{32}P] ATP for 7 min during protein synthesis reactions. Protein synthesis was carryout at 30 °C with cold leucine. The reactions were terminated 7 min after the addition of [γ–^{32}P] ATP. 12 μl of the reaction mixture was then taken out and terminated by the addition of 800 μl of ice cold pH 5.0 solution (50 mM NaF, 5 mM EDTA and 12 μl of 0.5 M glacial acetic acid). The pH 5.0 solution contains NaF and EDTA which inhibit the phosphatase activity. The pH 5.0 precipitate collected at 1 2 K was dissolved in 20 μl of 1X SDS sample buffer and heated for about 2 min in boiling water bath. The proteins were separated on 10% SDS-PAGE gel and the gel was dried and the eIF2a phosphoproteins was estimated by autoradiography or by western analysis immunoblot using a phosphospecific anti-eIF2α antibody..

4.2. *In vitro* phosphorylation.

In vitro phosphorylation assays were carried by incubating purified eIF2 with HRI and [γ-^{32}P]ATP (3,000 Ci/mmol) in a 20 μl cocktail containing 20 mM Tris-HCl pH 7.8, 2 mM Mg^{2+}, 80 mM KCl and 30 μM unlabeled ATP. The protein kinase assays were terminated by the addition of 4X SDS sample buffer (Tris-HCl pH 6.8, 10% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.1% bromophenol blue). The samples were boiled for about 2-min in boiling water bath, then run on the 10% SDS-PAGE. The gel was dried and analyzed by autoradiography by phosphospecific anti-eIF2a antibody.

5.0. ANALYSIS OF eIF2α(P).eIF2B COMPLEX IN RETICULOCYTE LYSATES

Protein synthesis reactions (100 μl) were carried out at 30 °C for 15 minutes in heme-deficient, hemin-supplemented (20 μM heme) or heme and poly (IC)-treated (20 μM and 300 ng/ml) reticulocyte lysates in the presence of non-recombinant or recombinant viruses infected cell extracts/partially purified recombinant eIF2α protein as described in the legends to the figures. At the end of the protein synthesis reaction, the lysates were diluted with equal volume of chilled TKM buffer consisting of 20 mM Tris-HCl pH 7.6, 100 mM KCl and 2 mM $Mg(OAc)_2$ to terminate the reaction. Samples were layered on a 4.5 ml exponential sucrose gradients (10-30%) which were prepared with the above dilution buffer. Samples were run at 40,000 rpm for 6 hours at 4 ^{0}C in a SW 50.1 rotor to separate free eIF2 from [eIF2α(P).eIF2B] complex as described (Krishna et al., 1997). Fractions (400 μl) were collected by upward displacement of the gradients with the help of an ISCO gradient fractionator. Fractions were concentrated by pH 5.0 precipitation in the presence of 50 mM NaF, 5 mM EDTA and 12 μl of 0.5-M glacial acetic acid (for every 800 μl of pH 5.0 reaction mixture) to prevent the dephosphorylation of eIF2α. Samples were incubated on ice for about 60 min and centrifuged at 12,000 rpm for about 20 min, then the pellets were suspended in 1X SDS sample

buffer, briefly boiled and separated on 10% SDS-PAGE gel. Proteins were transferred to nitrocellulose membranes and eIF2α and eIF2Bε of the various gradient fractions were detected by using an anti-human eIF2α/eIF2Bε monoclonal antibody as described (Krishna et al., 1997).

6.0. INSECT CELL TISSUE CULTURE

6.1. Cell lines and virus.

Sf9 cell lines (Vaughn et al., 1977), which serve as hosts for AcNPV was used for the expression study. Sf9 cells were maintained in complete medium (TNM-FH from Sigma) supplemented with 10% FCS and 100 μg/ml antibiotic and antimycotic solution as described by Summer and Smith, 1987.

6.2. Preparation of TNM-FH medium.

TNM-FH medium (HINK, 1970) is Grace's insect cell culture medium (Grace, 1962) which is supplemented with lactalbumin hydrolysate and yeastolate. The medium is enriched in all the basic nutrients for the growth of insect cells and it is buffered with sodium phosphate. To make 1 liter of TNM-FH medium, 51.2 gm of Grace's medium was dissolved in 700 ml of distilled water, 350 mg of $NaHCO_3$ was also added and the medium was adjusted to pH 6.2 with autoclaved 0.5 M KOH. Then the volume was adjusted to 900 ml with autoclaved double distilled water. The medium was filter sterilized by filtering through 0.22 μM filter using sterile filter unit in the hood. The filtered medium was kept at room temperature for about 48 hours to check the contamination. After 48 hours, 10% fetal calf serum and 100 μg/ml antibiotic and antimycotic solution were added to make the complete medium.

Sf9 insect cells were maintained at 27 °C in complete medium and grown as monolayer and in suspension culture. Sf9 insect cells double every 24 hours at 27 °C. Sf9 cells were maintained in T-25 cm^2 or in T-75 cm^2 tissue culture flasks or in spinner flasks for obtaining monolayer and or suspension cultures respectively. Cells were dislodged by washing the surface by gentle pipetting (O'Reilly et al., 1992). For each subculture, 1-3 million cells were seeded depending on the flask size. Before every splitting or subjecting the cells to infection, the viability of the cells were checked by staining with 10% v/v trypan blue (dead cells stain blue). Only cells with greater than 90% viability were used for the expression, freezing and splitting.

6.3. Freezing and revising of Sf9 cells.

Sf9 cell line stocks were prepared from >90% healthy log-phase cultures. The cells were dislodged from the confluent flask and harvested and the pellet was suspended in complete medium containing 10% DMSO. The final cell density was maintained approximately at 4-millions/ml of cell suspension. The cell suspension was aliquot in to a screw capped eppendorfs (1ml) and frozen slowly. The cells were initially placed at 4 ^{0}C and then kept at -20 ^{0}C for 1 hour before transferring them to -70 °C. After keeping them overnight at -70 °C, the cells were transferred to vapour phases of liquid nitrogen.

The above stocks were taken out from liquid nitrogen when required and thawed by gentle agitation in a 37 °C water bath. Once the stocks were thawed, the vials were surface sterilized with 70% ethanol before taking in to the hood. The cells were transferred to a centrifuge tube and spun at 4,000 rpm for about 5 min at 27 ^{0}C. The cell pellet was suspended initially in 1 ml of complete medium and seeded in a T-75 cm^2 flask. Then, 9 ml of

complete medium was added to the flask. Once the cells were seeded, then the medium was replaced by the fresh medium.

7.0. MOLECULAR CLONING OF eIF2α cDNA INTO BACULOVIRUS TRANSFER VECTOR pBacPAK8

7.1. Transformation, amplification, purification and excision of human eIF2a cDNA (wild type and mutants).

Competent DH5α cells (100 μl) were transformed with 50-100 ng of plasmid DNA by incubating the cells for 30 min on ice (Sambrook et al., 1989). The cells were heat shocked for about 60 seconds at 42 °C. 500 μl of LB (for 1 lit of LB: 10 gm bacto-tryptone, 5 gm yeast extract, 10 gm NaCl, pH 7.5 with NaOH) was added and incubated for 30 min at 37 °C. The transformed cells were plated on LB agar (LB + 15% bactoagar) plates containing ampicillin (100 μg/ml). The plates were incubated for 1 2 to 14h at 37 °C to allow the growth of transformed ampicillin resistant bacterial colonies.

Larger cultures were incubated with a single isolated colony with the help of an autoclaved toothpick. The cultures were left at 37 °C **for** 18-20 hours.

7.2. Isolation and restriction digestion of plasmid DNA.

The plasmid DNA was isolated from small cultures by alkaline-SDS lysis protocol (Sambrook et al., 1989). Plasmid DNA from large cultures was isolated using Qiagen columns and the DNA from the agarose gel was isolated using polyethylene glycol.

All the manipulations of DNA preparations were carried **out** according to Sambrook et al. (1989). 1 μg of plasmid DNA was incubated at 37 °C for 1 hour with 2-10 units of restriction endonuclease and suitable buffer.

Isolated plasmid DNA, as well as DNA treated with various restriction enzymes were separated on 1 % agarose gel in TAE buffer (0.04 M Tris-acetate, 1 mM EDTA pH 8.0 and 250 μg/ml of ethidium bromide). A gel loading dye (0.025% bromophenol blue and 0.025% xylene cyanol) was added to DNA samples. Electrophoresis was carried out at 100 volts in TAE buffer. The DNA bands were viewed under UV light.

7.3. Ligation of 1.6 Kb eIF2α cDNA into pBacPAK8 transfer vector.

All ligation reactions were carried out using T_4 DNA ligase in 1X ligation buffer. The ligation reaction was carried out at 16 °C **for** 16 h or 4 °C for overnight or at room temperature for about 1-2 hours in a total volume of 10 μl. The ligation mix was used for transforming competent DH5α cells. The positive clones carrying the insert were used for transforming competent DH5(x cells. The positive colonies carrying the insert were identified by colony hybridization.

7.4. Colony hybridization and probe preparation.

Colony hybridization was done as described (Anjali, 1996). The bacterial colonies obtained after transformation with the ligation mix were transferred on to a nylon membrane (nylon N^+, Amersham Biotech, UK) and grown **over** night at 37 °C. After 10-12 hours, the membrane was placed on polythene sheet, colonies facing up and treated as follows: DNA denatured twice for 3 min each with 0.5 N NaOH. The alkali was neutralized with 1 M Tris-HCl pH

7.5 and 1.5 M NaCl. The membrane was air-dried and the DNA was immobilized by baking at 80 °C for 2h. The membrane was briefly soaked in 4X SSC buffer and was cleaned gently with a cotton swab. The membrane was pre-hybridized with a buffer containing 6X SSC buffer pH 7.0, 5X Denhardt's solution, 20 μg/ml sonicated Salmon sperm DNA and 0.5% SDS at 55 °C for 5 hours in a rotary shaker as described (Sambrook et al., 1989). After pre-hybridization, the blot was hybridized with radioactive eIF2α cDNA probe at 55 °C for 16h. The membrane was washed as described. In 2X SSC, twice for 10 min each at room temperature. In 0.2X SSC with 0.1% SDS, twice for 10 min each at 55 °C. In 0.1XSSC with 0.1% SDS, 10 min at 65 °C. The membrane was then dried, covered with saranwrap and subjected to autoradiography to identify the positive colonies.

To prepare a radioactive DNA probe, 75-100 ng of DNA fragment was labeled by random priming using a multiprime DNA labeling kit as described (Anjali, 1996). DNA was heated at 90 °C for 5 min for denaturation to occur and chilled on ice for 10 min. Random hexanucleotide primer, dNTPs (-dCTP), buffer (Tris-HCl pH 7.8, $MgCl_2$, and β-mercaptoethenol), [α-^{32}P]dCTP (30 μCi) and klenow fragment of E.coli DNA polymerase I (2 units for reaction) were added and the reaction volume was made up with water to 50 μl and incubated at 37 °C for 30 min. The temperature was then raised further to 95 ^{0}C for 10 min for denaturation of DNA and inactivation of the enzyme to occur. The radioactive probe was then added to the prehybridization solution containing the membrane in the previous step.

7.5. Southern Hybridization.

After electrophoresis, the gel was transferred to a glass-baking dish (Borosil India). The gel was soaked for 30 min in a denaturation solution (1.5 M NaCl

and 0.5 M NaOH) with constant gentle agitation to denature the DNA. The gel was briefly rinsed with deionised water and then neutralized by soaking for 30 min in several volumes of 0.5 M Tris-HCl pH 7.4 and 1.5 M NaCl at room temperature. The gel was again rinsed in deionised water and blotted on to a nylon membrane (Hybond N+, Amersham Biotech, UK) using 20X SSC for 16 hours (Southern, 1975). The membrane was then neutralized in 6X SSC for 2-3 min, air dried and baked at 80 ^{0}C for 2 hours. This was followed by prehybridization and hybridization with labeled probe [α-^{32}P] dCTP (30 μCi) for 16 hours at 55 °C. The blot was washed as described earlier and exposed over night to hyper film, MP (Amersham Biotech, UK) at -70 ^{0}C.

8.0. CO-TRANSFECTION AND IDENTIFICATION OF RECOMBINANT BACULOVIRUSES

Sf9 cells were co-transfected with pBacPAK8 viral transfer vector carrying cDNA of interest and with Bsu36l digested BacPAK6 viral DNA to obtain an infectious complete viral genome. Sf9 cells (>95 % viable) were seeded in 35 mm tissue culture dishes (2.2×10^{6} cells) just before co-transfection. Old medium was replaced by 2 ml of incomplete medium and incubated for 60 min (serum proteins inhibits the co-transfection). Meanwhile the DNA-lipofectin complex was prepared as per the manufacturer's instructions in polystyrene tubes in 100 μl volume containing 25 μl of recombinant plasmid DNA (20 ng/ml), 5 |il of BacPAK6 viral DNA, 66 μl of sterile double distilled water and 4 μl of Bacfectin solution was added, gently mixed and incubated at room temperature for 30 min to allow the bacfectin to form complex with the DNA.

Meanwhile, the medium was removed from the cell monolayer. 1.5 ml of incomplete medium was then added and incubated for 30 min. Afterwards the medium was removed and bacfectin-DNA complex was added drop wise to the cells with gentle swirling. The mixture was incubated for 5 h at 27 °C. The medium containing bacfectin-DNA was removed from the culture plate with out disturbing the cell monolayer. This medium contains most often a few recombinant viruses and the reaction mixture. This can be saved at 4 ^{0}C and can be reused if the cells are not infected with the virus. Now the monolayer cells in the dish were washed once with 1.5 ml of complete medium and then incubated 72 hours at 27 °C with 1.5 ml of complete medium without a moist towel. The recombinant viral supernatant was later used in the plaque assay. A negative control, without BacPAK6 viral DNA and mock without any DNA was also maintained.

8.1. Plaque Assay.

This step was carried out to obtain active recombinant virus from transfected cells. The procedure is as follows:

1. $1.8x10^{6}$ cells were seeded in 35 mm tissue culture dishes. Several dilution's of recombinant viruses from the lysates of the transfected cells ranging from 10^{-1} to 10^{-7} in 1 00 µl complete medium were made.

2. The medium from the cells was removed and 100 µl of viral dilution was added drop by drop to each petridish. The petridishes were incubated at 27 °C temperature for 1 hour with gentle rocking for every 1 5 min.
3. The viral inoculum was removed and 2 ml of LGT agarose overlay was added to the monolayer cells in the petridish. The petridishes were then taken out from the laminar hood to allow the agarose to gel. Then 1 ml of

complete medium was added to each dish and then these were incubated at 27 °C for about 3-5 days with a few layers of soaked paper towels around the petidishes. Later, the liquid medium was removed from the top of the agarose overlay and the petridishes were dried by placing them in an inverted position on a paper towel. Then 2 ml of neutral red staining solution (made by adding 1-2 ml of neutral red in 20 ml of plaque assay buffer: 0.82 gm of NaCl, 0.2 gm of KCl, 0.1 14 gm of Na_2HPO4 and 0.02 gm of KH_2PO_4 in 100 ml of H_2O, pH 7.3) was added to each dish.

4. The petridishes were incubated for about 1-2 hours at room temperature. The stain was then drained and the petridish was kept inverted for a few hours to over night for the formation of clear plaques.
5. The recombinant plaques were scored on the basis of differential refraction by placing it on the top of an illuminated (white light) box.
6. The plaques were observed under the light microscope. The clear plaques appear with some cell debris. These plaques were counted in each case and viral titer (Plaques forming units per ml, pfu/ml) was calculated by using the following formula: pfu/ml = Average No. of plaques X 1/ml of inoculum per plate X 1/dilution factor.
7. The plaques were picked up and released into an eppendorfs containing 100 µl of complete medium. The eppendorfs were left at 4 °C for the virues to be released into the medium. This virus was used for the infection studies.

8.2. Amplification of the recombinant viral stock.

A stepwise amplification of the recombinant viral titer (determined by dot-blot) was done from a 96 well plate to a 35 mm dish through a 24 well and 6 well plates and finally to a T-25 cm^2 flask.

96 well plate: 0.1 $\times 10^6$ cells in 100 µl of medium were seeded in each well of **a** 96 well plate. After 2 hours, the medium was carefully removed without disturbing the monolayer. 50 µl of viral stock was added to each well and left for 1 hour with gentle rocking for every 1 5-min. 50 µl of complete medium was added to each well and the plate was wrapped with parafilm and left at 27 °C in an incubator with a moist paper towel in a box for 48-60 hours. Later the viral supernatant was removed and stored at 4 ^{0}C for further amplification.

24 and 6 well plate: The viral stocks obtained from the 96 well plate was used for infecting the cells for further amplification of the virus. **The final** volumes were 500 µl and 2 ml in each well of a 24 well and 6 well plates respectively. The plates were incubated at 27 °C for about 5 days and the viral supernatant was collected for further amplification.

35-mm tissue-culture dishes: 2 x **1** 0^6 cells in 2 ml of complete medium were seeded in 35-mm tissue-culture dish. After 1-hour, medium was aspirated carefully and 1 50 µl of recombinant virus obtained from the 6 well plate was added to each dish and processed as mentioned above. At the end of 1 hour the supernatant was removed and stored at 4 °C. 2 **ml** of complete medium was carefully added and the dishes were incubated at 27 °C for **about** 5 days. Later the supernatant was collected and used for further amplification.

T-25 cm^2 flask: The procedure was same as mentioned above but here, 4 x 10^6 cells were seeded and 150 µl of recombinant virus obtained from 35 mm dish and 4 ml of complete medium was used. The flasks were left for one week until all the cells become well infected.

The mock as well as wild type AcNPV infected control was also used in all the above amplification steps. The cells were observed under the light microscope to monitor the course of infection. A plaque assay was done to

determine the viral titer. High viral titer stocks were aliquoted and stored at -70 °C for long term storage.

9.0. ANALYSIS OF THE BACULOVIRUS EXPRESSED RECOMBINANT eIF2α PROTEIN

9.1. Dot - Blot hybridization.

To check whether the recombinant vector harboring the 1.6 Kb insert cDNA has been incorporated into the viral genome, dot-blot hybridization has been performed. A radioactive probe against the insert cDNA was prepared as mentioned previously and was used to detect the insert in the recombinant infectious virus and thereby identify the positive plaques. 10^5 Sf9 cells in 100 µl of medium were seeded in each of the 96 well plate. One negative control with wild type virus (non-recombinant) and a positive control with the insert (that was the template for the probe) were also used. 50 µl of the virus (virus released from the plaques obtained with different dilution's of the virus stock) was added to each well after the medium was carefully removed. The plates were left for 1h at 27 ^{0}C and 50 µl of complete medium was added and the plates were again incubated in above containing moist paper.

After the cells were infected, the supernatant was removed and stored at 4 °C. To each well, 200 µl of 0.5 N NaOH and 50 µl of 4 M ammonium acetate was added. Samples were transferred using the dot blot apparatus onto a nitrocellulose membrane soaked in warm water (55 °C-65 ^{0}C) and in dot blot solution (0.2 N NaOH, 1 M ammonium acetate). After the transfer, the membrane was baked for 2h, treated with pre-hybridization and hybridization solution as mentioned before and then exposed to X- ray film to identify the positive plaques.

9.2. Determination of eIF2a expression **and preparation of cell extract.**

Recombinant virus from the positive plaques were amplified and later used to infect cells to identify and analyze the recombinant protein.

v

$4x10^6$ cells were seeded in T-25 tissue-culture flask. One flask was used for one time point for each virus (recombinant viruses or nonrecombinant virus) as well as one flask for Sf9 (with out virus) as control. Cells were infected as described earlier. At each time point, cells were suspended in the medium and the cell suspension was centrifuged at 3,000 rpm for 5 min to harvest the cells. The supernatant was discarded and the cells were washed twice in ice cold PBS (pH 6.2) The cells were lysed in 100 µl of lysis buffer (20 mM Tris-HCl pH 7.8, 1 mM Mg^{2+}, 1 mM DDT, 1X pepstatin A (1 mg/ml in methanol, 1000X), 1X leupeptin (1 mg/ml in sterile water, 1000X), and 1X aprotinin (1 mg/ml in sterile water, 1000X)) and centrifuged at 10,000 rpm for 10 min. To the lysate supernatant, PMSF (1 mM prepared in isopropanol,) and KCl (80 mM) were added and immediately aliquot and stored under liquid nitrogen.

25 µg of extract protein was separated on 10% SDS-PAGE and transferred to nitrocellulose membrane to determine the expression of eIF2a based on its migration on SDS-PAGE and also its ability to interact with a monoclonal eIF2a antibody (Krishna et al., 1997 and Rowlands et al., 1988).

Protein estimation was carried out **in** all the cell extracts using the Biorad protein assay kit.

9.3. Sodium dodecyl sulphate -- polyacrylamide gel electrophoresis (SDS – **PAGE).**

Proteins were separated on a modified Laemmli method (1970). The 10% separation gel mix, 30 ml, contained 7.5 ml of 1.5 M Tris-HCl pH 8.8, 10 ml of 30:0.8 acrylamide: Bis-acrylamide mixture, 0.3 ml of 10% SDS, 0.1 ml of 10% ammonium per sulphate, 7.5 µl TEMED and 12.1 ml water. The 4.5% stacking gel mix in a total volume of 6 ml contained 0.9 ml of 30:0.8 acrylamide:Bis-acrylamide mixture, 1.5 ml of 0.5 M Tris-HCl pH 6.8, 0.1 ml of 10% SDS, 0.06 ml of 10% APS, 3.6 ml of water and 6 µl of TEMED. Protein samples were prepared in sample buffer containing Tris-HCl pH 6.8, glycerol, SDS, β-mercaptoethanol and bromophenol blue. Vertical slab gel electrophoresis was carried out at 120 volts with Tris-SDS-Glycine buffer until the dye front ran into the lower buffer. The gel was stained either by coomassie or by silver nitrate.

9.4. Western immunoblot analysis.

The proteins separated on SDS-PAGE were transferred onto a nitrocellulose membrane electrophoretically at 90 volts at 4 °C cabinet. The transfer was done for 3h in transfer buffer (25 mM Tris, 195 mM Glycine in 20% methanol). After the transfer, the membrane was stained with ponceau S solution to check that the transfer had occurred and also to mark the molecular weight marker proteins. The stain was removed by rinsing the membrane with 1X TBS and water. The membrane was thereafter soaked in blocking solution (3% blot grade BSA in TBS-10 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM Sodium azide). After 1h, the blocking solution was replaced with TBS containing the primary antibody in the required dilution for for overnight at 4 °C. The membrane was later washed thrice with 1X TBST for10 min each time to remove the non-specifically bound antibody. Later the membrane was incubated in TBS containing the appropriate anti-IgG-AP

conjugate for 60 min. The membrane was again washed thrice with TBST, 10 min each time. Then the membrane was developed with a colour development solution with NBT (66 μl) and BCIP (33 |il) as substrates in 10 ml of AP buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$). Washing the membranes in distilled water arrested the colour development. The membrane was air-dried and stored between filter papers and kept away from light.

10.0. PURIFICATION OF BACULOVIRUS EXPRESSED RECOMBINANT eIF2α

The Sf9 cells infected with the recombinant eIF2α wt and mutants of 48A, 51A and 51D were lysed and the extracts were treated with 0.5 M KCl. Cell extracts were then incubated on ice for about 20 min and spun at 12,000 rpm/20 min. The supernatant was concentrated by 0-80% ammonium sulfate and centrifuged at 12,000 rpm for about 20 min. The pellet was resuspended in buffer A [20 mM Tris-HCl pH 7.8, 2 mM $Mg(OAc)_2$, 50 mM KCl, 5% glycerol and 100 μM EDTA] and then directly loaded on S-300 (Pharmacia) gel filtration column which was pre-equilibrated with buffer A/50 mM KCl. The elution was done with buffer A and 800 μl fractions were collected (100 fractions). Then 100 μl from each fraction was TCA precipitated (10%). Samples were resuspended in 10 μl of 1 M Tris buffer (pH 8.8 to bring the acidic pH of the pellet to 6.8) + 10 μl of 2X SDS sample buffer and boiled briefly before loading on a 10% SDS-PAGE and transferred to a nitrocellulose membrane. The peak fractions, recognized by eIF2cx antibody. The peak fractions were pooled and precipitated with the addition of 2.5 volumes of saturated ammonium sulphate and then spun about 12,000 rpm for about 60 min. The pellet obtained was resuspended in 100 μl of buffer A/50 mM KCl and dialyzed against the same buffer.

The dialyzed S300 protein was loaded on 2 ml DEAE-52 column which is preequilibrated with buffer A/50 mM KCl. The proteins were eluted with buffer A/150 mM KCl and 250 mM KCl. 250 µl of fractions were collected and 20 µl of the fractions were directly loaded on 10% SDS-PAGE. The proteins in the gel were transferred and the eIF2α in the fractions was identified with the help of a monoclonal antibody. eIF2a was mostly eluted in the 250 mM KCl fractions. The peak fractions were pooled, concentrated (0-80% ammonium sulphate fractionation) and dialyzed against buffer A/50 mM KCl.

The various fractions obtained during purification of recombinant eIF2α were separated by 10% SDS-PAGE to show the various stages during purification.

CHAPTER

RESULTS AND DISCUSSION

1. PROTEIN SYNTHESIS IN RETICULOCYTE LYSATES.

2. PURIFICATION OF eIF2 AND eIF2B.

3. CLONING OF 51A AND 51D HUMAN eIF2a IN BACULOVIRUS.

4. EXPRESSION. PARTIAL PURIFICATION, FUNCTIONAL CHARACTERIZATION OF RECOMBINANT eIF2a Wt AND MUTANT PROTEINS AND INTERACTION BETWEEN eIF2 AND eIF2B.
   - 4.1. Introduction.
   - 4.2. Expression, immunoreactivity and partial purification of recombinant 51A and 51D mutants of eIF2α.
   - 4.3. Human recombinant eIF2α protein is less efficiently phosphorylated than the eIF2α present in the purified trimeric reticulocyte eIF2.
   - 4.4. Recombinant wt and 48A mutant eIF2a decreases the phosphorylation of reticulocyte eIF2α in the trimeric preparation *in vitro.*
   - 4.5. Phosphorylation of reticulocyte and recombinant eIF2α wt, 48A, 51A and 51D proteins *in vitro* by HRI kinase and γ-[^{32}P]ATP.
   - 4.6. Kinetics of reticulocyte eIF2α phosphorylation in hemin and poly(IC)- treated lysates in the presence and absence of partially purified recombinant eIF2α wt and mutants.

4.7. Reticulocyte eIF2α phosphorylation by HRI is decreased in the presence of AcNPV- infected cell extracts (using γ-[^{32}P]ATP).

4.8. Uninfected Sf9 cell extracts show higher cellular eIF2α phosphorylation and can phosphorylate efficiently purified reticulocyte eIF2 compared to virus-infected cell extracts (using phospospecific-anti eIF2a antibody).

4.9. Insect cell eIF2α phosphorylation is decreased more efficiently in the early stages of virus infection than in the later stages.

4.10. 48A mutant decreases the inhibition of eIF2B activity in poly (IC)-treated reticulocyte lysate.

4.11. eIF2.[^{3}H]GDP dissociation in hemin or hemin and poly (IC)-treated reticulocyte lysates in the presence of partially purified recombinant eIF2α wt and the three mutants.

4.12. Gradient analysis of eIF2α(P).eIF2B Complex.

DISCUSSION

RESULTS

1. PROTEIN SYNTHESIS IN RETICULOCYTE LYSATES

Several batches of lysates from rabbit reticulocytes were prepared as mentioned in the Methods in order to carry out the studies on eIF2 phosphorylation and the complex formation between eIF2α(P) and 2B in translating reticulocyte lysates under quasiphysiological conditions in the presence and absence of recombinant eIF2α wt and its mutants. Of the several lysates that were prepared, some of them were found hemin-insensitve or displayed a weak response to added hemin. These lysates, however were found otherwise translationally active and were able to incorporate [^{14}C] leucine into the acid precipitable protein as shown in Fig. 1. Some of them were unable to support protein synthesis (data not shown). The translational efficiency varies from batch to batch. If the internal or endogenous heme is not completely depleted, the lysates would not be able to show a good response upon added hemin. Infact, most of the commercially available lysates do not show a heme-sensitivity. Sometimes this could be because these lysates were pretreated with hemin. Such hemin-insensitive lysates were also used here sometimes to activate double stranded RNA-dependent protein kinase (dsl or PKR) and to stimulate eIF2α phosphorylation. PKR in the lysates can be activated in the presence of double stranded viral RNA or synthetic poly (IC). Commercially available lysates are expensive and are difficult to be imported since they have to be shipped in dryice. Hence, we solely relied on the lysates that are prepared in this laboratory.

Some of the lysates prepared had shown a typical response to added hemin. In the absence of hemin, protein synthesis was linear for 10-15 min. and

Fig. 1. Protein synthesis in different batches of heme-insensitive rabbit reticulocyte lysates.

Protein synthesis was carried out in different batches of heme-insensitive rabbit reticulocyte lysates in 25 μl volume at 30 °C under two different conditions: a) - heme and b) + heme, 20 μM, as described in 'Materials and Methods'. Protein synthesis was measured by the incorporation of [^{14}C] leucine into TCA precipitable protein in 5 μl aliquots at 15, 30, 45 and 60 min.

Protein synthesis in **heme** insensitive reticulocyte lysates

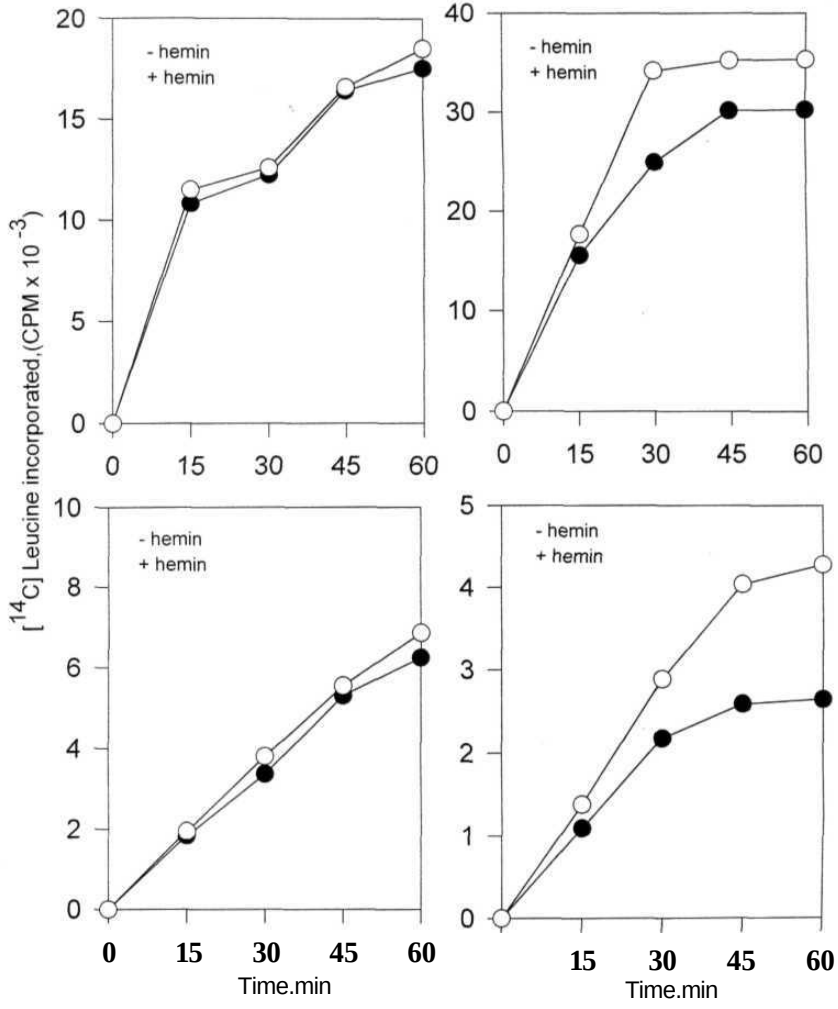
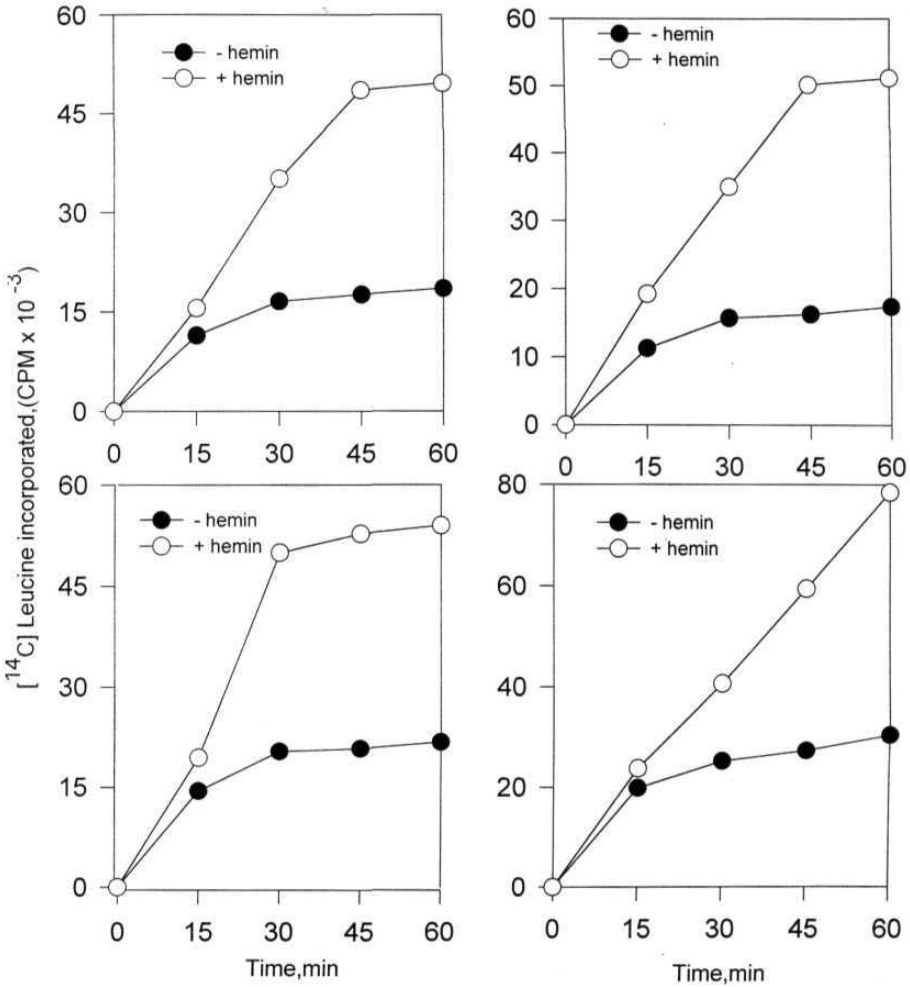


Fig. 2. Protein synthesis in different batches of heme-sensitive rabbit reticulocyte lysates.

Protein synthesis was carried out in different batches of heme-sensitive rabbit reticulocyte lysates in 25 μl volume at 30 °C under two different conditions: a) - heme and b) + heme, 20 μM, as described in 'Materials and Methods'. Protein synthesis was measured by the incorporation of [^{14}C] leucine into TCA precipitable protein in 5 μl aliquots at 1 5, 30, 45 and 60 min.

Protein synthesis in **heme** sensitive reticulocyte lysates



SCHEME FOR PURIFICATION OF RETICULOCYTE eIF2

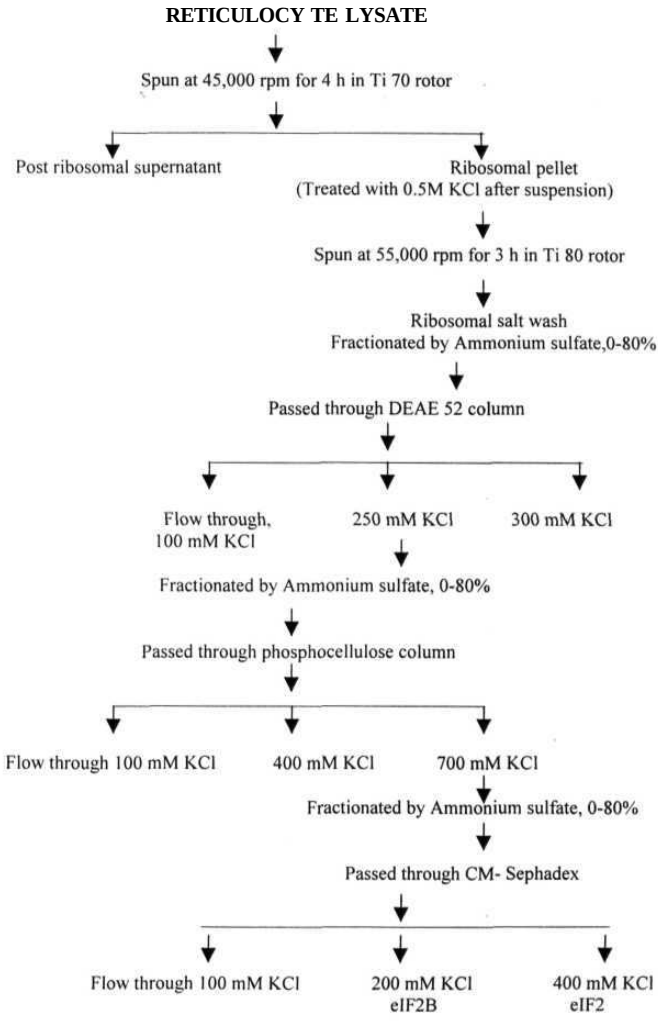


Fig. 3. Scheme for rabbit reticulocyte eIF2 purification

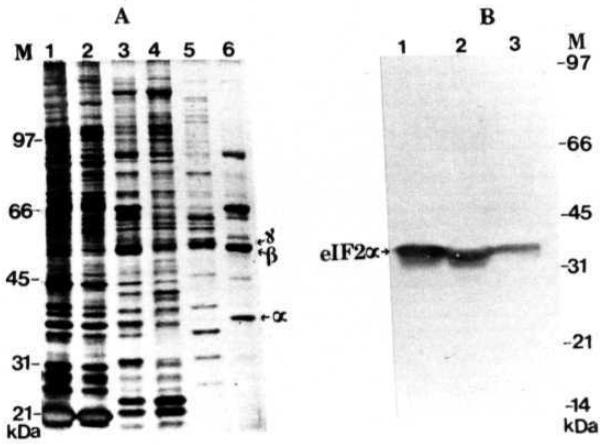
eIF2 was purified from ribosomal salt wash as well as from postribosomal supernatant by ion-exchange chromatography as described in 'Materials and Methods'

Fig. 3a. Purification of eIF2 from ribosomal salt wash

Panel A is a silver-stained 10% SDS-PAGE showing the purity of eIF2 at different steps of purification. Different lanes are as follows: lanes 1- ribosomal salt wash, 2- DEAE-52 0.2 M KCl fraction; 3- Phosphocellulose 0.4 M KCl fraction; 4- Phosphocellulose 0.7 M KCl fraction; 5- CM Sephadex 0.2 M KCl fraction; 6- CM Sephadex 0.4 M KCl fraction.

Panel B is an immunoblot of the gel showing eIF2 in peak fractions of the DEAE, P11 and CM-Sephadex columns. Lanes 1-DEAE 0.2 M KCl fraction; 2- Phosphocellulose 0.7 M KCl fraction; 3- CM Sephadex 0.4 M KCl fraction. After the proteins were separated on a 10% SDS-PAGE gel, they were transferred to the nitrocellulose membrane and immunoblotted with an eIF2α monoclonal antibody to identify the α subunit (38 kDa) of eIF2.

eIF2 PURIFICATION PROFILE



SCHEME FOR PURIFICATION OF RETICULOCYTE eIF2B

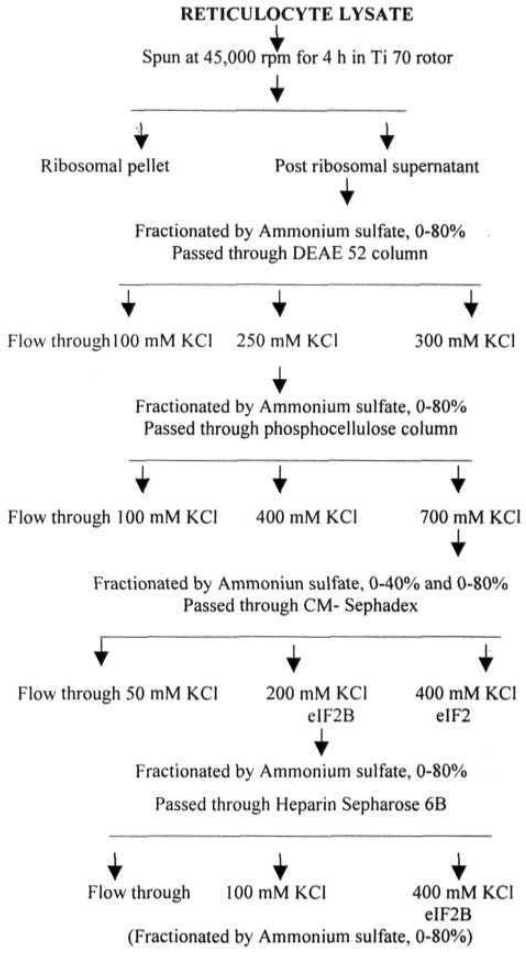


Fig.4 Scheme for rabbit reticulocyte eIF2B purification.

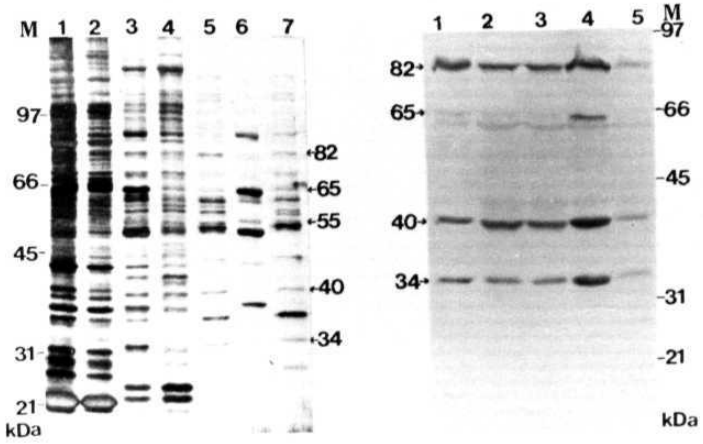
eIF2B was purified from post ribosomal supernatant by ion-exchange chromatography as described in 'Materials and Methods'.

Fig. 4a. Purification of eIF2B from postribosomal **supernatant (PRS).**

Panel A is a silver stained 10% SDS-PAGE. The purity of eIF2B at different steps of purification. Lanes 1-PRS; 2- 0.2 M KCl fraction of DEAE-52; 3- Phosphocellulose; 4- 0.4 M KCl fraction of Phosphocellulose 0.7 M KCl fraction; 5- 0.2 M KCl fraction of CM Sephadex; 6- 0.4 M KCl fraction of CM Sephadex, 7- 0.4 M KCl fraction of Heparin Sepharose **6B.**

Panel B is an immunoblot of the gel showing, Lanes 1-DEAE 0.2 M KCl fraction; 2- Phosphocellulose 0.4 M KCl fraction; 3- CM Sephadex 0.2 M KCl fraction; 4- CM Sephadex 0.4 M KCl fraction; 5- Heparin Sepharose 6B 0.4 M KCl fraction. After the proteins were separated on a 10% SDS-PAGE gel, they were transferred to a nitrocellulose membrane and immunoblotted with an eIF2B polyclonal antibody to identify the different subunits of eIF2B.

eIF2B PURIFICATION **PROFILE**



then it was shut down (Fig. 2). In the absence of hemin, a kinase called heme-regulated kinase (HRI) is activated which phosphorylates eIF2cx and inhibits the guanine nucleotide exchange activity of eIF2B and thereby protein synthesis (Babu and Ramaiah, 1996 and Ramaiah, et al., 1997). Addition of hemin inhibits the HRI kinase activity probably by promoting disulfide bond formation in the protein (Chen et al., 1989). HRI kinase is different from PKR although both of them, when activated, can stimulate eIF2α phosphorylation and inhibit protein synthesis in reticulocye lysates.

2. PURIFICATION OF eIF2 AND eIF2B.

The flow chart describing the purification of eIF2 from the ribosomal salt wash is shown in Fig. 3. Various fractions of eIF2 are shown in Fig. 3a. Purified protein contains three subunits α (38 kDa), β) and y . The β and y subunits (51 and 52 kDa) migrate close to each other on a 10% SDS-PAGE. eIF2 in the fraction is recognised by using a monoclonal antibody that is raised against the alpha-subunit. In addition to these three bands, the CM-Sephadex purified eIF2 contains few other contaminant bands. Previously, we characterized such eIF2 preparations for their ability to bind GDP and to serve as a substrate for eIF2α kinases such as HRI and PKR (Sepuri V. N. Babu Ph.D. Thesis). Here we have used the CM purified trimeric eIF2 for various studies in which the eIF2 binding to GDP and phosphorylation were monitored.

eIF2B protein was purified from the postribosomal supernatant as described in the flow chart (Fig. 4). The various fractions containing eIF2B were separated on 10% SDS-PAGE (Fig. 4a) and analysed by their cross reactivity with a polyclonal anti eIF2B antibody as shown in Fig. 4a. eIF2B contains five subunits a (82kDa), β (65 kDa), y (55 kDa), S (40 kDa) and **e** (34 kDa).

Fig. 4b. Restoration of protein synthesis inhibition in heme-deficient lysates by eIF2B fractions.

Protein synthesis was carried out in heme-sensitive rabbit reticulocyte lysates at 30 °C under different conditions: 0 + heme (20 μM hemin); •- heme; 0 - heme + PC eIF2B; A - heme + CM eIF2B and • - heme + HS6B eIF2B as described in 'Materials and Methods'. Protein synthesis was measured by the incorporation of [^{14}C] leucine into TCA precipitable protein in 5 μl aliquots at 15, 30, 45 and 60 min.

Reticulocyte lysate protein synthesis

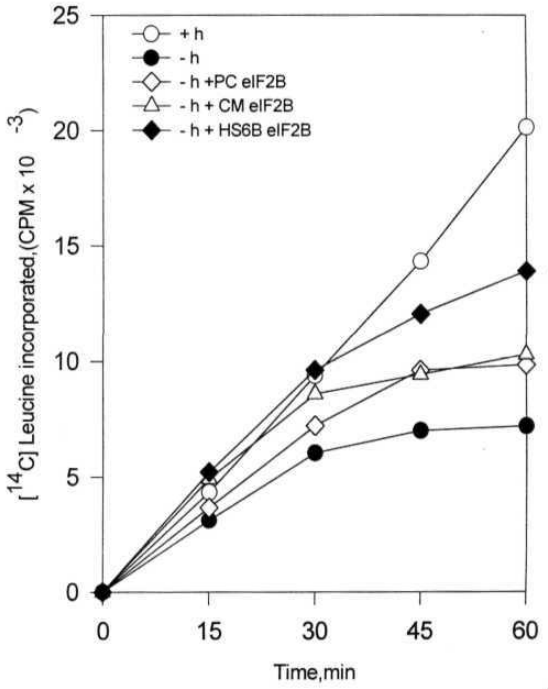
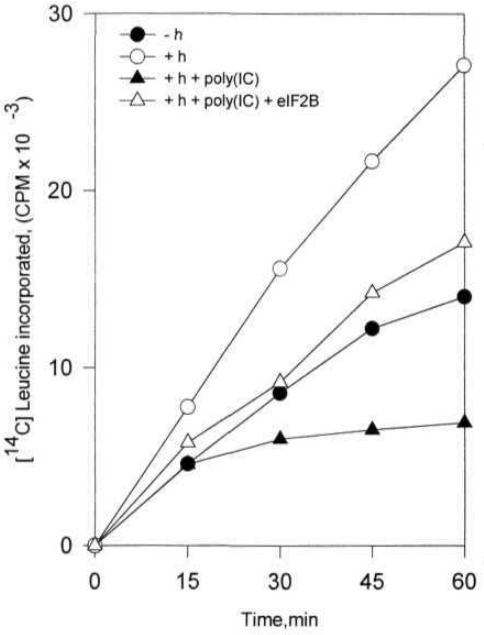


Fig. 4C. Restoration of protein synthesis inhibition in hemin and poly (IC)-treated reticulocyte lysates by purified Heparin-Sepharose eIF2B.

Protein synthesis was carried out in heme-sensitive rabbit reticulocyte lysates at 30 °C under different conditions: 0 + heme (20 μM); • - heme; • + heme + Poly (IC) (300 ng/ml) and A + heme + poly (IC) + eIF2B as described in 'Materials and Methods'. Protein synthesis was measured by the incorporation of [^{14}C] leucine into TCA precipitable protein in 5 μl aliquots at 15, 30, 45 and 60 min.

Reticulocyte lysate protein synthesis



CLONING EXPRESION AND PURIFICATION OF HUMAN WT, 48A, 51A AND 51D eIF2α IN INSECT CELLS USING BACULOVIRUS EXPRESSION SYSTEM

A FLOW CHART

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Transformation, Amplification, purification and Excision of human eIF2α cDNA (51A and 51D) from the parent vector, pETFVA

↓

Ligation of cDNA insert into baculovirus transfer vector, pBacPAK8.

↓

Transformation of DH5α cells by recombinant vector pBacPAK8 and purification.

↓

Preparation of radioactive probe of the insert to identify colonies is containing recombinant vector using colony hybridization technique.

↓

Double restriction digestion of the recombinant vector construct pBacPAK8 to determine the orientation of the insert.

↓

Maintenance of Sf9 cells and transfection of these cells with the recombinant vector, pBacPAK8 and also with Bsu 361 digested pBacPAK6 viral DNA using lipofectin. This step is carried out to obtain homologous recombination between viral vectors and to obtain a recombinant infectious genome with eIF2α cDNA insert.

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Plaque assay, identification of positive plaques by Dot-blot hybridization

↓

Amplification of recombinant viruses (positive plaques)

↓

Identification of positive plaques by Dot- blot hybridization

↓

Infection of positive viruses to Sf9 cells for analysis of the recombinant protein by SDS-PAGE, immunoblot analysis and functional characterization by purification and phosphorylation assays.

Fig. 5. This is flow chart showing the various steps involved in cloning and expression of human eIF2α 51A and 51D mutants in *spodoptera frugiperda* (Sf9) insect cells using the baculovirus expression system.

Fig. 5a. Agarose gel showing the pETFVA vector carrying 1.6 Kb fragment of eIF2α cDNA (51D and 51A).

DH5α cells were transformed with pETFVA vector harboring the eIF2α cDNA and ampicillin resistant gene. Bacterial cells were cultured at 37 °C. Plasmid DNA was isolated from the transformed DH5a cells and the DNA was treated with and without EcoR I at 37 °C in 10 μl reaction mixture for 60 min. The samples were then treated with 6x DNA loading buffer and loaded on 1 % agarose gel containing ethidium bromide (0.5 μg/ml). The DNA bands are visualised under UV light. The various lanes are as follows:

Lane 1, 1 Kb ladder DNA as marker; lanes 2 and 4, pETFVA vector carrying 51D and 51A eIF2α cDNA without EcoR I digestion, lanes 3 and 5, pETFVA vector carrying 51D and 51A eIF2α cDNA with EcoR I digestion showing the presence of 1.6 Kb fragment on the gel.

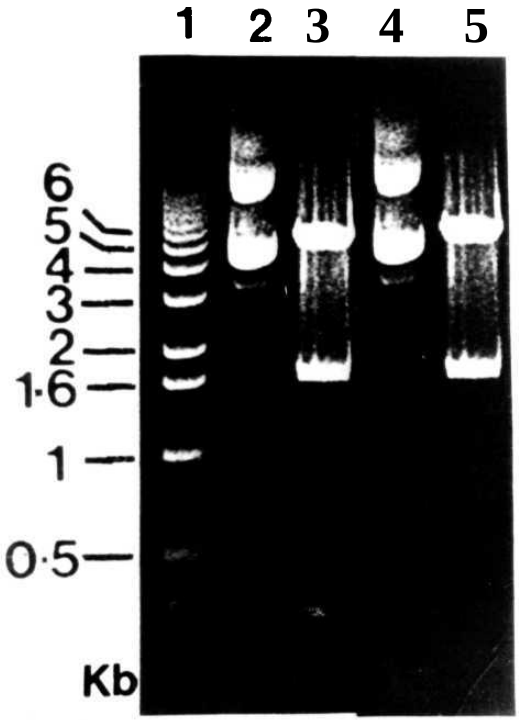

1
2
3
4
5
6
5
4
3
2
1·6
1
0·5
Kb

Fig. 5b. Agarose gel showing the linearised pBacPAC8 vector DNA and 1.6 Kb fragment of eIF2α cDNA (51A and 51D mutants).

Baculovirus transfer vector, pBacPAC8, eIF2α cDNAs, 51D and 51A from the parent vector pETFVA, were bulk digested with EcoR I. These were separated on 1% agarose gel, eluted and concentrated by using polyethylene glycol. A small amount of concentrated DNA was separated by 1% agarose gel and viewed under UV light. The various lanes in the gel are as follows:

Lane 1, linearised pBacPAK8 viral DNA; lane 2, marker DNA (1 Kb ladder); lanes 3 and 4 are 1.6 Kb fragments of eIF2a cDNA 51A and 51D respectively.

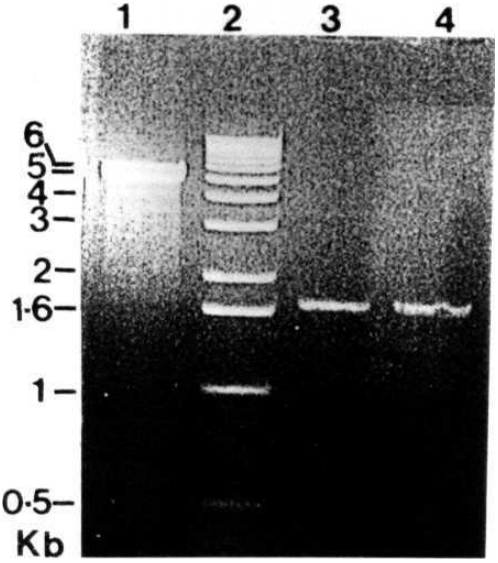
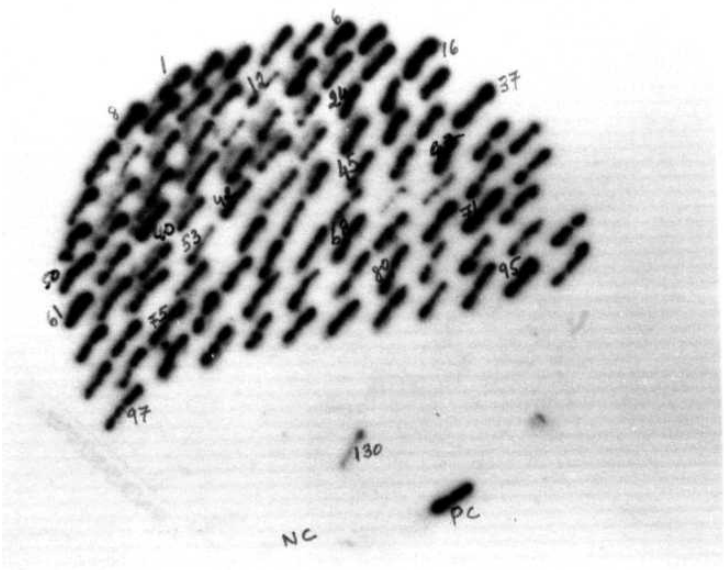

1
2
3
4
6
5
4
3
2
1·6
1
0·5
Kb

Fig. 5c. Colony hybridization to identify the recombinant pBacPAK8 with the mutant eIF2a.

A total of 150 (for 51D, upper panel of the figure) and 180 (for 51A, bottom panel of the figure) transformed colonies were screened using a radioactive eIF2a cDNA probe as mentioned in 'Materials and Methods'. The positive colonies which have taken up the recombinant pBacPAK8 transfer vector **and** harboring 51D and 51A eIF2α DNA were identified by autoradiography. The film was exposed to a nitrocellulose membrane for 2 hours at -70 °C.


1
8
12
16
37
40
53
61
97
130
NC
PC

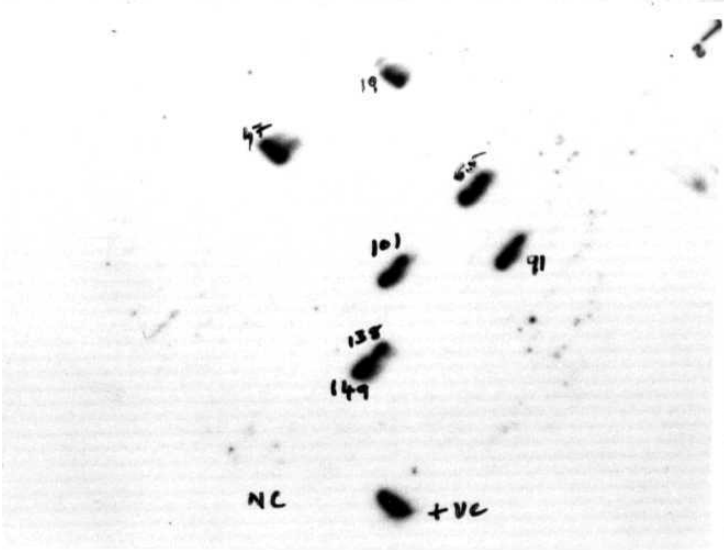

19
47
65
101
91
135
149
NC
+VC

Fig. 5d. Identification of the orientation of the eIF2a insert in the vector pBacPAK8.

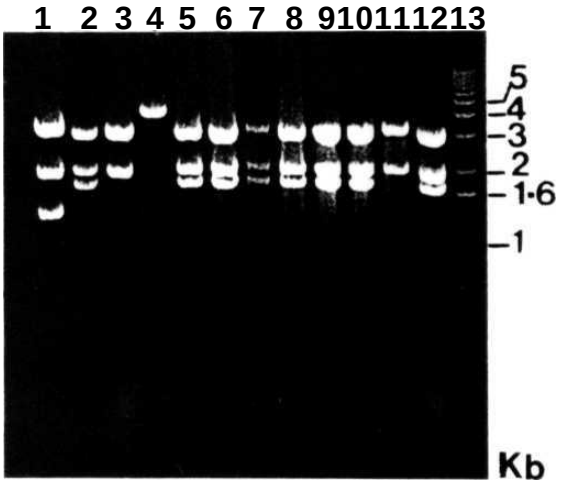
Positive colonies, identified by colony hybridization, were randomly picked and their plasmid DNA was digested with Mlu I and Sca I and then loaded on a 1% agarose gel. Right orientation yields three fragments of the following sizes: 1306, 3736 and 2096bp (total 7138bp). Wrong orientation results in the following fragments: 1806, 3236 and 2096bp (7138bp).

Upper panel 51D

Lane 1. Right orientation. Lanes 2-12 contain plasmid DNA from colonies, which have the insert in the wrong orientation. Lane 13 is the marker (1 Kb **DNA** ladder).

Bottom Panel 51A

Lane 1, 1kb DNA ladder; Lanes, 2 and 3, 51A in the right orientation; Lanes 4-7, wrong orientation of 51A; and lanes 8-13 were 51D wrong orientation.


1 2 3 4 5 6 7 8 9 10 11 12 13
5
4
3
2
1·6
1
Kb

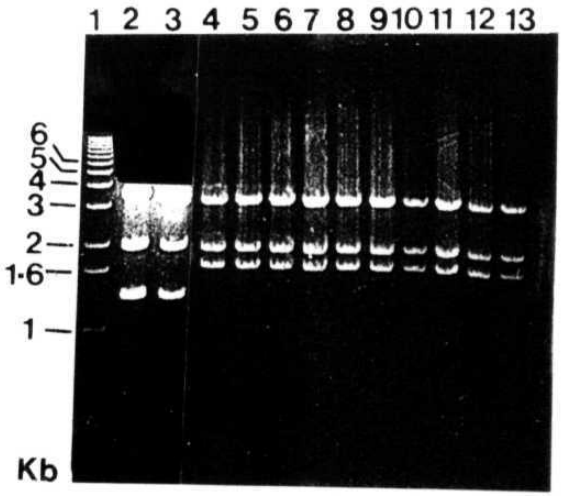

1 2 3 4 5 6 7 8 9 10 11 12 13
6
5
4
3
2
1·6
1
Kb

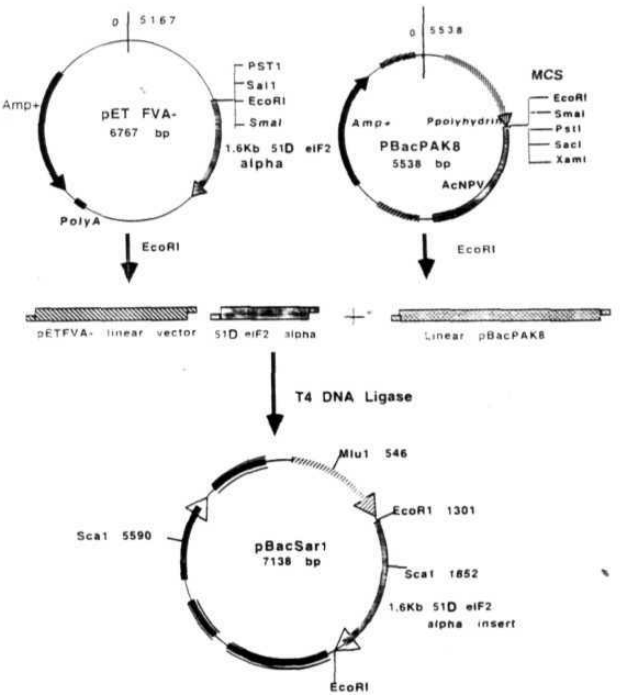
Fig. 6. Construction of recombinant pBacSar1 **and pBacSar2 vectors harboring eIF2α 51D and 51A** elF2a

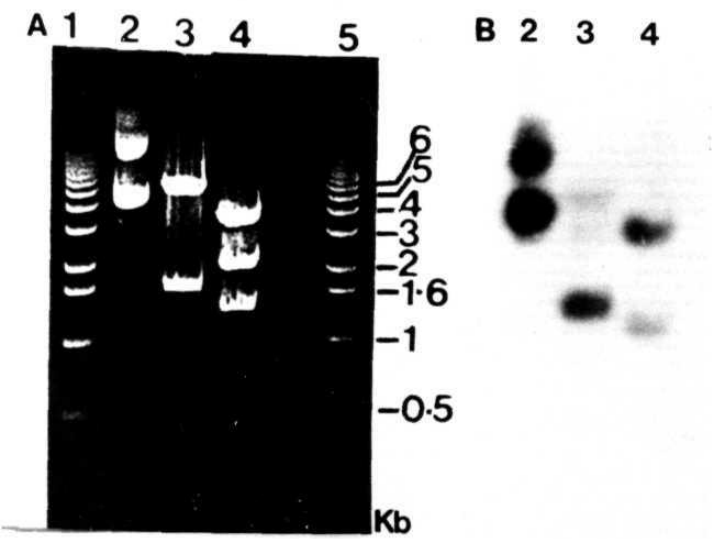
Schematic representation of the pBacSar1 or pBacSar2 construct with 7138 bp. PETFVA is the parent vector with 5167 bp. 1.6kb fragment of eIF2α 51A or 51D was cloned initially into this vector by Davies et al (1993). This insert was released by EcoR I digestion and is ligated to pBacPAK8 transfer vector (5538 bp and obtained from Clonetech). The resulting construct were named as pBacSar1 or pBacSar2

Fig. **Panel** A: Restriction digestion pattern of pBacSar1 or pBacSar2 harboring 51D and 51A eIF2α. The plasmid is transformed into DH5a cells and the plasmid DNA was isolated and treated with or without EcoR I to release the insert. Also the DNA was double digested with Sca I and Mlu I to determine the orientation. Lanes1 and 5, 1 Kb DNA ladder as a marker; lane 2, uncut pBacSar1 plasmid DNA; lane 3, Plasmid DNA treated with EcoR I; lane 4, Plasmid DNA treated with Sca I and Mlu I.

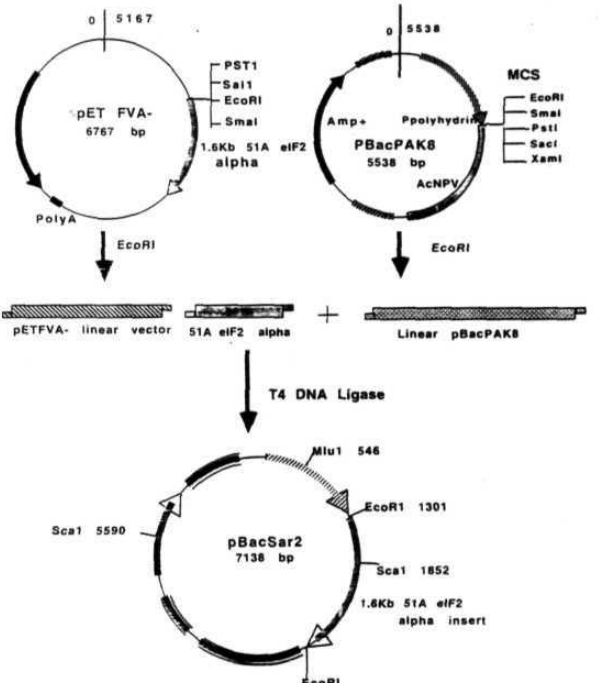
Panel B: Southern hybridization of this above gel using labelled 1.6 kb 51D or 51A elF2a cDNA probe.

Cloning of 51D eIF2 alpha in Bacculovirus transfer vector





Cloning of 51A eIF2 alpha in Bacculovirus transfer vector



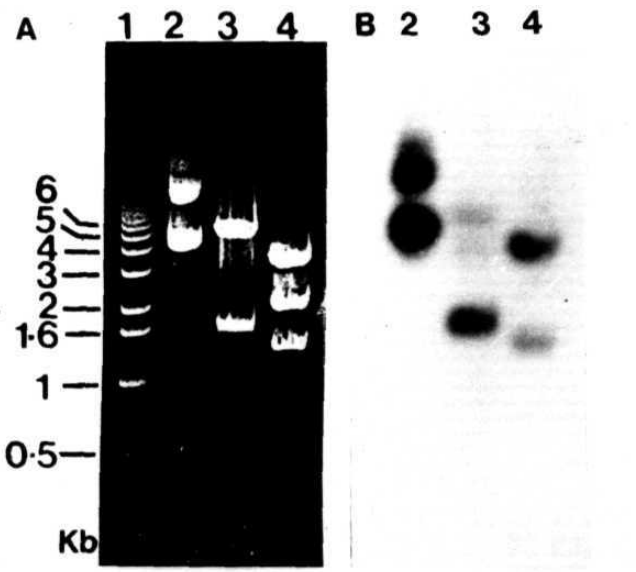
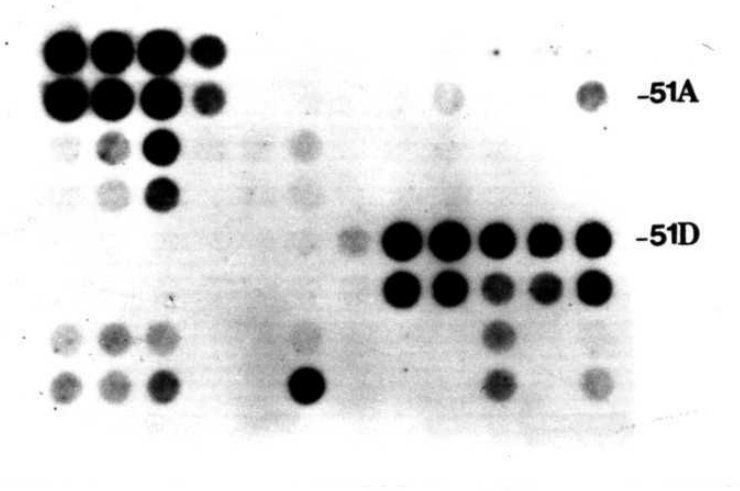


Fig. 7. Dot blot hybridization.

A radioactive probe against 1.6 kb eIF2α cDNA was used to identify **the** plaques containing recombinant virus expressing eIF2α 51A and 51D mutants. The supernatants from the plaques were used to infect Sf9 cells in a 96 well **plate as** described under Materials and Methods. Out of 48 plaques used for 51A eIF2α **(upper panel),** 10 of them were found to be positive. **For** the elF2a 51D, out of 48 plaques used (lower panel), 9 of them were found to be positive. The wells 3 and 7, 4 and 8 in lane 13 contain uninfected and non-recombinant AcNPV virus infected cell extracts as negative controls. A positive controls of eIF2α was used in the well 8 of lane 6.

DOT BLOT

1 2 3 4 5 6 7 8 9 10 11 12 13



Typically the polyclonal antibody recognizes four of the five subunits. In addition to eIF2B subunits, the fractions of eIF2B also contain the three subunits of eIF2. This is consistent with the idea that eIF2 and 2B comigrate together (Matts et al., 1983). Since eIF2B is **a** rate-limiting protein **and** is found to rescue protein synthesis inhibition in reticulocyte lysates caused by heme-deficiency and or double stranded RNA or poly (IC), the ability of various purified fractions of eIF2B were tested for the same here in Figs 4b and 4C respectively. It was observed that eIF2B can restore partially the inhibition in protein synthesis caused by the above agents and the relief in the inhibition is related to the relative purity of the fraction (Fig. 4b and 4c).

3. CLONING OF 51A AND 51D HUMAN eIF2α IN BACULOVIRUS.

Previously, eIF2a wt and 48A mutant were cloned and expressed using baculovirus system (Thanuja Krishnamoorthy Ph.D. Thesis). Here, eIF2a **51A** and 51D were cloned into a baculovirus vector and expressed as described in Figs.5, 5a - 5d, 6 and 7.

4.0. EXPRESSION, PARTIAL PURIFICATION, FUNCTIONAL CHARACTERIZATION OF RECOMBINANT eIF2u Wt AND MUTANT PROTEINS AND INTERACTION BETWEEN eIF2 AND eIF2B.

4.1. Introduction.

Expression of mutants of eIF2α, in which the putative phosphorylation sites, serine residues at 48 and 51, were changed to alanine, mitigates the inhibition of protein synthesis (Choi et al., 1992 and Murtha-Riel et al., 1993) and the reduction in GNE activity of eIF2B (Ramaiah et al., 1994) caused by eIF2α phosphorylation **in** cultured mammalian cells. While 51A mutant was not phosphorylated, 48A mutant was used as a substrate. In contrast,

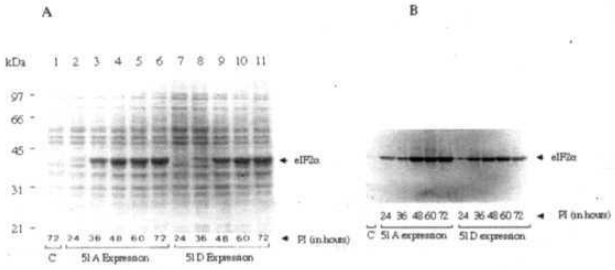
expression of a serine 51 to aspartic acid mutant (51D) inhibited protein synthesis. The mutants of eIF2α were useful in resolving the phosphorylation sites in mammalian eIF2cx (Pathak et al., 1988 and Choi et al., 1992), in elucidating the protein synthesis defects caused due to eIF2α phosphorylation in such cases as heat shock and calcium sequestration (Murtha-Riel et al., 1993 and Srivastava et al., 1995), in determining the importance of eIF2a phosphorylation in growth and development and in apoptosis (Donze et al., 1995 and Qu et al., 1997) and also in expressing eIF2α kinases that are inhibitory for protein synthesis (Chefalo et al., 1994).

Here we have used baculovirus expression system to express human eIF2α wt and three of the mutants and partially purified the recombinant proteins. The ability of these recombinant eIF2α wt and mutants, to serve as substrates for phosphorylation were tested. To further understand the importance of serine 51 phosphorylation in the formation of eIF2a(P).eIF2B complex and intersubunit protein interactions, we studied the phosphorylation of eIF2α, GNE activity of eIF2B and formation of eIF2α(P).eIF2B complex in hemin or hemin and poly (IC)-treated reticulocyte lysates in the presence of partially purified recombinant human eIF2α wt, and mutants of eIF2α. Our findings support the hypothesis that phosphorylation of serine 51 in wt or native eIF2α promotes complex formation between eIF2 and eIF2B, and thereby impairs the GNE activity of eIF2B (Ramaiah et al., 1994). Further, the serine 48 residue which is not phosphorylated, is however required for the formation of a complex between eIF2α(P) and eIF2B when the serine residue in 51 is phosphorylated. In addition, we have other observations suggesting that insect cells carry a potent eIF2(x kinase and viral infection of these cells leads to a diminution in the eIF2α phosphorylation.

Fig. 8. Kinetics of eIF2a 51A and **51D** expression in insect **cells**

Panel A: Expression and immuno reactivity of recombinant human eIF2a 51A and 51D in Sf9 insect cells using baculovirus. Kinetics of eIF2α 51A and 51D protein expression at different time points, 24 to 72 hours post infection (PI), can be seen in lanes 2-6, and 7-11 in panel A respectively. Uninfected control Sf9 cell extracts were prepared and loaded in lane, 1 (C). Each lane contains approximately 30 μg of extract protein and the figure is a coomassie stained 10% SDS-PAGE gel.

Panel B: Western immunoblot analysis of recombinant eIF2α 51A and 51D. The recombinant proteins expressed in Sf9 insect cells were separated by 10% SDS-PAGE as shown in Panel A and transferred to a nitrocellulose membrane. Membrane was then probed with an eIF2a monoclonal antibody. The immunoreactivity of the recombinant eIF2α was detected with the help of rabbit anti-mouse alkaline phosphatase conjugated secondary antibody (Promega). Lanes 1-11 represent as in panel A.


A
B
kDa
1 2 3 4 5 6 7 8 9 10 11
97
66
45
31
21
eIF2α
72 24 36 48 60 72 24 36 48 60 72
PI (in hours)
C
51A Expression
51D Expression
24 36 48 60 72 24 36 48 60 72
C
51A expression
51D expression

SCHEME FOR PURIFICATION OF BACULOVIRUS EXPRESSED *eIF2α*

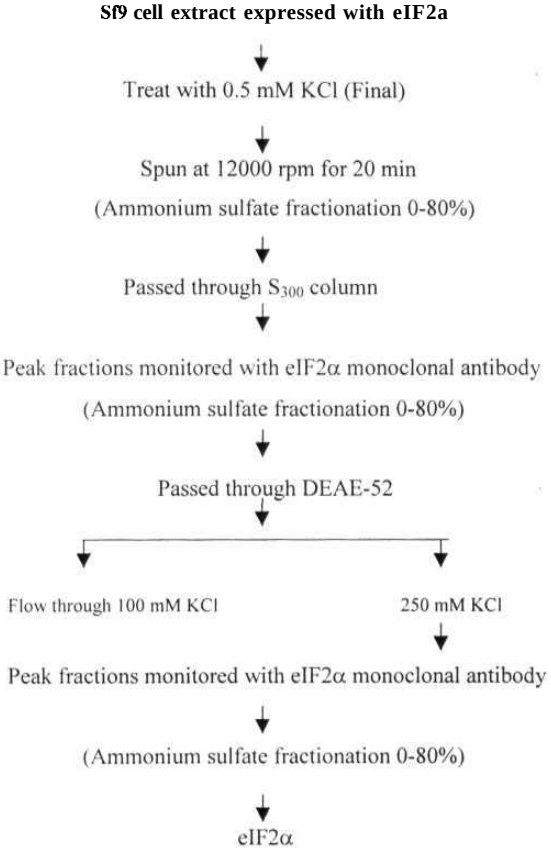


Fig. 9 . Scheme for Baculovirus expressed eIF2a purification

The recombinant eIF2α wt, 48A, 51A, and 51D protein expressed by Sf9 cells were partially purified using a gel filtration and DEAE-52 ion-exchange chromatography as described in 'Materials and Methods'.

Fig. 9a. Partial purification of recombinant eIF2a.

Extracts of Sf9 cells expressing 51A, 51D, 48A and wt eIF2α mutant proteins were prepared and passed through Sephacryl S-300 (Pharmacia) and DEAE Cellulose-52 columns as described in Materials & Methods. The figure is a coomassie stained 10% SDS-PAGE gel. Lane 1 contains purified reticulocyte eIF2[eIF2(R)]. Lanes 2-6 contain extracts of Sf9 cells as indicated . Lanes 7-10 contain proteins of S-300 fractions of the cell extracts expressing 51A, 51D, 48A and wt eIF2α respectively. Lanes 11-14 contain proteins of S-300 fraction that was eluted from DEAE-52 with 0.25 M KCl.

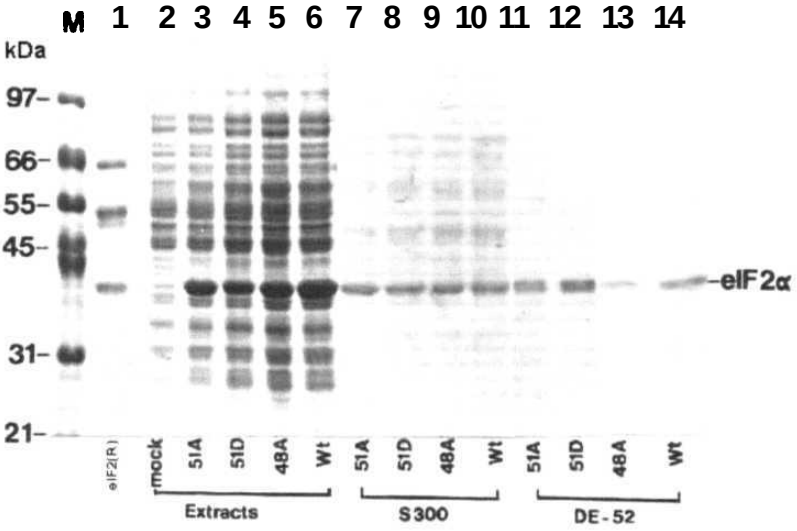
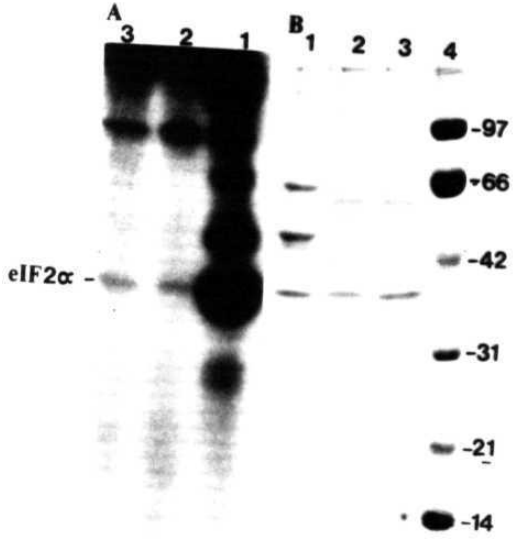

M
1
2
3
4
5
6
7
8
9
10
11
12
13
14
kDa
97-
66-
55-
45-
31-
21-
eIF2α
eIF2(R)
mock
51A
51D
48A
Wt
51A
51D
48A
Wt
51A
51D
48A
Wt
Extracts
S300
DE-52

Fig. 10. Phosphorylation of recombinant wt and 48A eIF2α and purified reticulocyte eIF2 *in vitro.*

In vitro phosphorylation of eIF2α 48A mutant and wild type in comparison with the three subunit reticulocyte eIF2. Partially purified eIF2α wild type and 48A mutant and reticulocyte eIF2 were supplemented with 30 μM ATP, HRI [eIF2a kinase] and 10 μCi of [γ-^{32}P] ATP and incubated at 30 ^{0}C for 10 min. The reactions were terminated by addition of 4X SDS sample buffers. The samples were resolved on 10% SDS-PAGE gel and analyzed by autoradiography. Panel A is an autoradiography and panel B coomassie gel. Lane 1 in panel A and B corresponds purified reticulocyte eIF2, lane 2 corresponds to 48A mutant eIF2α and lane 3 corresponds to wild type eIF2α. Lane 4 is molecular weight marker.


A
3
2
1
B
1
2
3
4
eIF2α
-97
-66
-42
-31
-21
-14

4.2. Expression, immunoreactivity and partial **purification of recombinant 51A and 51D mutants of** eIF2α **[Figs. 8/9/9A].**

Extracts of Sf9 cells transfected with recombinant AcNPV harboring 51 A and 51 D mutants of human **eIF2a** were prepared at different time points post-infection (24, 36, 48, 60 and 72h) and were analyzed by 10% SDS-Polyacrylamide gel (Fig. 8, Panel A) to monitor the expression of eIF2cx. A single protein with a molecular mass of 38 kDa was detected that increased with time up to 72h. Uninfected or AcNPV infected cells do not produce a protein of similar molecular mass in large amounts where expression is increased with time post-infection. Expressed recombinant protein cross reacts with a monoclonal anti-eIF2α antibody in the western immunoblot analysis and the intensity of the eIF2α signal is related to the level of protein expression which increases with time post-infection (Fig. 8 Panel B). Although moderate levels of 51 D protein is produced in Sf9 cells, it is noted however that 51 D expression is slightly lower than 51 A protein (Fig. 8). The expression level is dramatically increased over previous methods (Kaufman et al., 1989 and Srivatava et al., 1998).The recombinant eIF2α wt, 48A, 51 A and 51 D proteins expressed by Sf9 cells were partially purified using a gel filtration and DEAE 52 ion-exchange columns (Figs. 9 and 9A). The protein was eluted in the 200 and 250 mM KCl fraction of DEAE cellulose column.

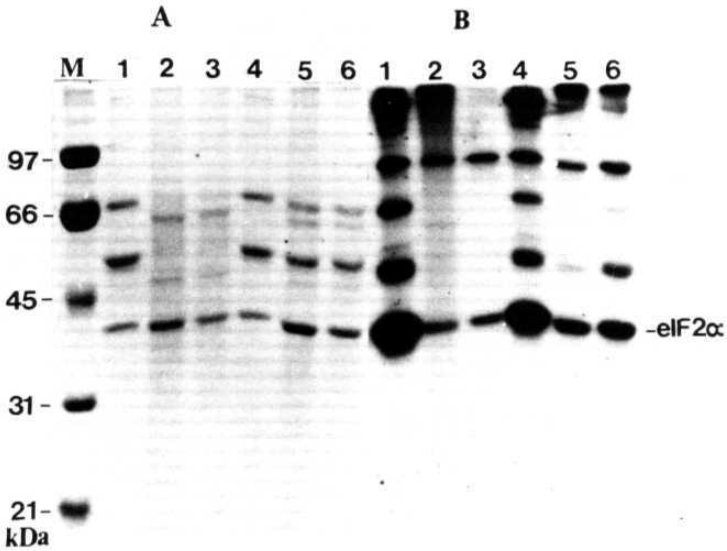
4.3. Human recombinant eIF2α protein is less **efficiently phosphorylated than the** eIF2<x **present in the purified trimeric reticulocyte eIF2 [** Fig. 10].

Initially, the above DE-52 fractions containing recombinant eIF2α wild type or 48A mutant proteins were phosphorylated *in vitro* **in** the presence of reticulocyte heme-regulated eIF2α **kinase** (Fig. 10). In panel B, lane 1 contains three subunit purified reticulocyte eIF2. Lanes 2 and 3 contain partially

Fig. 11. Phosphorylation of the alpha **subunit in purified rabbit reticulocyte** trimeric **eIF2 in the presence and absence of recombinant subunit eIF2α (wt or 48A mutant) and by purified HRI and [γ-P^{32}] ATP *in vitro.***

Purified eIF2 and partially purified recombinant subunits of eIF2a wt and 48A mutants were phosphorylated as described (Ramaiah et al., 1997) and **the** reaction mixtures were loaded on 10% SDS-PAGE. **Panel A** is a stained gel and **Panel B** is the corresponding autoradiogram indicating HRI and eIF2α phosphorylation. The various lanes are as follows:

1, Purified rabbit reticulocyte eIF2 complex; 2, partially purified wt recombinant eIF2α; 3, partially purified 48A mutant eIF2α; 4, purified rabbit reticulocyte eIF2; 5, purified rabbit eIF2 + wt eIF2α; 6, purified rabbit eIF2 + 48A mutant eIF2cx. HRI is present in all reactions.


A
B
M
1
2
3
4
5
6
1
2
3
4
5
6
97
66
45
31
21
kDa
eIF2α

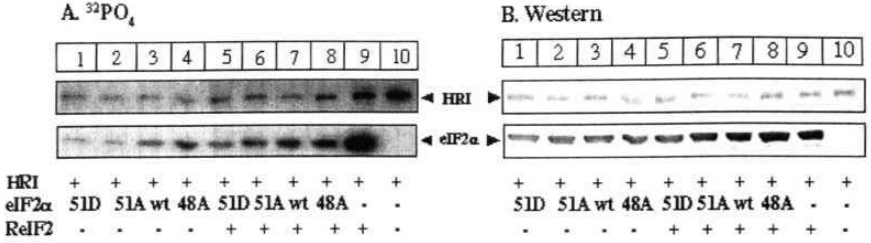
purified DE-52 fractions of recombinant wild type eIF2a and 48A mutant respectively. The fractions were stained with coomassie stain. Based on the stain intensity, eIF2α protein in these three fractions were found to be approximately similar. However, their ability for phosphorylation differed markedly (Fig. 10, Panel A). eIF2α present in the three subunit purified eIF2 preparation was phosphorylated much more efficiently than the recombinant eIF2α subunit (compare lane 1 of panel A to lanes 2 and 3). Both the wild type and 48A mutant eIF2α were phosphorylated to the same extent suggesting that 48A mutation does not inhibit or affect eIF2α phosphorylation on 51 serine residue.

4.4. Recombinant wt and 48A mutant eIF2α decreases the phosphorylation of reticulocyte eIF2α in the trimeric preparation *in vitro* [Fig. 11).

In order to understand if the phosphorylation of recombinant eIF2a, wt or 48A mutant was in any way different from that of the alpha subunit in the trimeric complex of eIF2, the phosphorylation of partially purified recombinant eIF2α (purified using Sephacryl-300 and the DEAE cellulose 52 column) was studied *in vitro* by HRI kinase (Fig. 1 1 A & B). Interestingly, it is observed here that the recombinant eIF2α (both wt and 48A mutant) is less efficiently phosphorylated than the eIF2α present in the purified reticulocyte eIF2 complex (Fig. 1 1 B, lanes 2, 3 vs. lane1). In addition, the phosphorylation of HRI kinase is also decreased in the presence of recombinant wt or 48A mutant protein (Fig. 1 1B). These experiments were carried out in such a way that protein levels of recombinant eIF2α and the eIF2α in the purified complex were fairly similar as judged by the stain intensity in the gels (Fig. 11 A). These findings raise the possibility that virus infection may inhibit HRI kinase and there by eIF2α phosphorylation. This possibility is supported by the observation that phosphorylation of eIF2α in the purified trimeric reticulocyte

Fig. 12. **Phosphorylation** of recombinant eIF2cx (51D, 51A, wt **and 48A)** in **the** presence and **absence** of purified reticulocyte eIF2 *in* ***vitro.***

Phosphorylation of a) purified reticulocyte eIF2 (lane 9, panel A, RelF2), b) recombinant eIF2α 51D, 51A, wt and 48A (lanes 1-4, panel A), and c) purified reticulocyte eIF2 in the presence of recombinant eIF2cx 51D, 51A, wt and 48A (lanes 5-8, panel A) was performed in the presence of HRI and [γ-^{32}P]ATP at 30 °C for 10 min as described in Materials and Methods. The reaction mixtures were separated by 10% SDS-PAGE. Lane 10 contains HRI alone. Panel A is an autoradiogram showing the phosphorylation of eIF2α and HRI. Panel B is an immunoblot of panel A indicating eIF2α and HRI protein levels used in the reaction mixtures as judged by their respective monoclonal antibodies.


A. $^{32}PO_4$
B. Western
1 2 3 4 5 6 7 8 9 10
HRI
eIF2α
HRI + + + + + + + + + +
eIF2α 51D 51A wt 48A 51D 51A wt 48A - -
ReIF2 - - - - + + + + + -
+ + + + + + + + + +
51D 51A wt 48A 51D 51A wt 48A - -
- - - - + + + + + -

eIF2 complex is not enhanced but decreased in the presence of the recombinant eIF2a wt or 48A mutant *in vitro* (Fig. 1 1B lane 4 vs. lanes 5 & 6). However, the decrease in phosphorylation of eIF2α in the purified complex is similar in the presence of partially purified recombinant wt and or 48A mutant eIF2α (lanes 5 and 6). Also, the phosphorylation of partially purified recombinant wt eIF2α subunit is not different from the 48A mutant eIF2α ***in*** *vitro* **(Fig.** 1 1 B, lanes 2 and 3).

4.5. Phosphorylation of reticulocyte and recombinant eIF2a wt, 48A, 51A and 51D proteins *in vitro* by HRI kinase and γ-[^{32}P]ATP [Fig. 12].

Previous results (Figs 10 and 11) demonstrated that recombinant wt and 48A mutant eIF2α proteins expressed in Sf9 cells using baculovirus and in mammalian cells (Choi et al., 1992 and Murtha-Riel et al., 1993) could serve as substrates for eIF2α kinases such as HRI and PKR where as, the mutants of 51A and 51D expressed in mammalian cells were not phosphorylated. Since we could also express for the first time, 51A and 51D mutants of eIF2(x in Sf9 cells using baculovirus, the phosphorylation of these partially purified mutants of recombinant eIF2α was studied by addition of purified reticulocyte heme-regulated eIF2α kinase (HRI) and labelled ATP (Fig. 12A). The 51D and 51A mutants (Fig. 12A, lanes 1 and 2) of eIF2α were not phosphorylated whereas the wt and 48A mutants (lanes 3 and 4) were. The phosphorylation of trimeric purified eIF2 by HRI occurred more efficiently (Fig. 1 2A, lane 9) than the free eIF2α mutant 51A and 51D subunits (lanes 1 and 2). The phosphorylation of trimeric eIF2 was reduced in the presence of the recombinant 51D, 51A, wt or 48A mutant eIF2α (Fig. 1 2A lanes 5, 6, 7, and 8 respectively) as previously described for the 48A mutant and wild type subunits (Figs 10 and 11). The differences observed in the phosphorylation could not be explained by the levels of HRI and eIF2 present in the reactions

Fig. 13. Phosphorylation of recombinant eIF2α in hemin and poly (IC)-treated reticulocyte lysates.

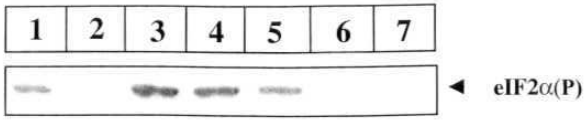
Panel A: Partially purified recombinant **eIF2a** wt, 48A, 51A and 51D proteins (~6 μg) were added to hemin and poly (IC)-treated reticulocyte lysates and incubated at 30 °C for 10 min. 10 μl of the respective lysate reaction mixtures was precipitated at pH 5.0 and separated by 10% SDS-PAGE. Phosphorylated eIF2a, eIF2a(P), was analysed by western immunoblotting using a phophospecific eIF2α antibody.

The various lanes are as follows: lane 1, with out hemin; lane 2, with hemin ; lane 3, heme and poly(IC); lane 4, herne, poly (IC) and wt eIF2α; lane 5, heme, poly (IC) and 48A eIF2α; lane 6, heme, poly (IC) and 51A **eIF2a;** lane 7, heme, poly (IC) and 51D eIF2α.

Panel B : **Kinetics of** reticulocyte eIF2α phosphorylation **in hemin and poly(IC)-treated lysates in the presence and absence of partially purified recombinant wt, 48A, 51A and 51D eIF2a.**

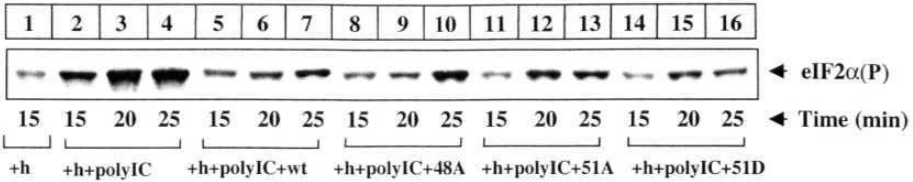
Reaction mixtures were processed and eIF2α phosphorylation of the reactions were analysed as stated in panel A. Various lanes are as follows: lane 1, heme; lanes 2, 3 and 4, heme and poly (IC)-treated lysates for 15, 20 and 25 min respectively; lanes 5-16 represent heme and poly (IC)-treated lysates for 15, 20 and 25 min with wt or mutants of 48A, 51A and 51D eIF2α respectively.

Panel A



h	-	+	+	+	+	+	+
polyIC	-	-	+	+	+	+	+
eIF2α	-	-	-	wt	48A	51A	51D

Panel B



as analysed by their respective monoclonal antibodies (Fig. 12B). In addition, the phosphorylation of HRI kinase that occurred in the presence **of** trimeric eIF2 (lane 9, Fig.12A) was decreased **in** the presence of partially purified recombinant eIF2α protein (lanes 1-8) and was not proportional to the actual levels of **HRI** present as analysed by western blot analysis in the reactions (lanes 1-8, Fig. 12B).

4.6. Kinetics of reticulocyte eIF2α phosphorylation in hemin and poly(IC)-treated lysates in the presence and absence of partially purified recombinant eIF2a wt and mutants [Fig.13].

The phosphorylation of reticulocyte eIF2α in heme-deficient, hemin or hemin and poly (IC)-treated reticulocyte lysates was also evaluated **in** the presence and absence of partially purified recombinant eIF2a by immunoblot analysis **using a phosphopeptide-specific anti-eIF2α antibody** (Fig. 13). The levels **of** eIF2α and HRI in the reactions were analysed by their respective monoclonal antibodies (data not shown). Results demonstrate that eIF2α phosphorylation (eIF2α(P)) decreased **substantially in** the presence of hemin as compared with the absence of hemin or heme-deficiency (Fig. 13, compare lane 2 vs. lane 1). In the absence of hemin, HRI kinase is activated and phosphorylates eIF2α. Similarly, addition of poly (IC) to hemin-supplemented lysates activates yet another eIF2α kinase called PKR and increases eIF2α phosphorylation (lane, 3). Addition of recombinant eIF2a wt and 48A to hemin and poly (IC)-treated lysates decreased the phosphorylation of lysate eIF2α to some extent (lanes 4 and 5 vs. lane 3) but addition of recombinant mutant proteins of eIF2α 51A and 51D decreased the phosphorylation of reticulocyte lysate eIF2α much more significantly (lanes 6 and 7 vs lane, 3).

Fig. 14. Reticulocyte eIF2a phosphorylation by HRI in the presence of uninfected and AcNPV-infected cell extracts (using [γ-^{32}P] ATP).

Phosphorylation of reticulocyte eIF2 was carried out ***in vitro*** by HRI and [γ-^{32}P] ATP (10 μCi) in a total volume of 20 μl as described (Ramaiah et al., 1997) in the presence of different concentrations of uninfected and virus-infected cell extracts. The samples were separated on 10% SDS-PAGE and transferred to nitrocellulose membrane. The eIF2 and HRI levels were analysed by using respective monoclonal antibodies as described (Ramaiah et al., 1994 and Pal, J. K et al., 1991) and the immunoblot was analysed by autoradiography to determine eIF2 and HRI phosphorylation. **Panel A** is an immunoblot indicating eIF2α and HRI levels present in the reaction mixtures. **Panel B** is an autoradiogram of the same gel indicating the phosphorylation **of** eIF2α and HRI in the presence of insect cell extracts in vitro. The different lanes are:

Lanes 1-5, Uninfected cell extracts containing 10, 15, 20, 25 and 30 μg of protein respectively; lanes 6-10 AcNPV-infected cell extracts containing 10, 15, 20, 25 and 30 μg of protein; Lane 11, Control lane containing HRI and reticulocyte eIF2.


A
B
1 2 3 4 5 6 7 8 9 10 11
1 2 3 4 5 6 7 8 9 10 11
<-- HRI -->
<-eIF2α->

The results on the kinetics of lysate eIF2α phosphorylation in heme and poly (IC)-treated lysates in the presence and absence of partially purified wt, and mutants of elF2(x proteins are shown in Fig. 1 3B. In this batch of lysates a higher basal level of eIF2α phosphorylation is observed in the presence of hemin (lane 1, Fig. 13B) compared to earlier Fig. 1 3A. Addition of poly (IC) increases the phosphorylation of lysate eIF2α with time (lanes 2, 3 & 4) as expected. Addition of the recombinant subunit of eIF2α wt or mutants to poly (IC)-treated lysates however decreases significantly the phosphorylation of lysate eIF2α (lanes 5-16 vs. lanes 2-4). In reactions where the non-phosphorylatable eIF2α 51A or 51D is supplemented, a time-dependent increase in phosphorylation of lysate eIF2α is also evident. This is possible if the exchange of recombinant human eIF2α 51A or 51D into the trimeric endogenous lysate elF2 is not complete as has been shown earlier (Choi et al., 1992). Further, the phosphorylation of lysate eIF2α is not different in the presence of partially purified recombinant wt and 48A mutant eIF2α (Fig. 4B, lanes 5-7 and 8-10 respectively). Similarly, the phosphorylation of reticulocyte lysate eIF2α in the presence of 51 A and 51 D mutants appear to be similar (lanes 11-13 and 14-16 respectively). These differences in phosphorylation cannot be explained by the levels of HRI and elF2a present in the reaction mixtures by two methods of analysis (See Discussion).

4.7. Reticulocyte eIF2α phosphorylation by HRI is decreased in the presence of AcNPV- infected ceil extracts (using γ-[^{32}P]ATP) [Fig.14].

To further understand if virus infected cell extracts reduce eIF2α phosphorylation, different amounts (10-30 μg protein) of uninfected and AcNPV-infected insect cell extracts were added to the phosphorylation reaction mixtures containing purified reticulocyte elF2 and HRI (Fig. 14). It is observed that reticulocyte elF2 alpha phosphorylation is reduced significantly

Fig. 15. Phosphorylation of Sf9 and reticulocyte eIF2 in uninfected and virus-infected extracts without added kinase (using eIF2α phosphospecific antibody).

1) Uninfected (C) and AcNPV-virus infected (V) Sf9 cell extracts were prepared as described in Materials and Methods and the proteins were separated by 10% SDS-PAGE to determine the eIF2cx phosphorylation of the cell extracts by western analysis (lanes 1 and 2; panel A) The above extracts were also used to phosphorylate purified reticulocyte eIF2 in reaction mixtures containing 20 mM Tris-HCl, 1 mM Mg^{2+}, 1 mM DTT, 80 mM KCl and 100 μM unlabelled ATP at 30 °C for 10 min. The reaction mixtures were then separated by 10% SDS-PAGE and transferred to nitrocellulose membrane to determine phosphorylation of reticulocyte eIF2 [eIF2αP (R)] by western analysis (lanes 3 and 4 duplicate set of reticulocyte eIF2 phosphorylation in uninfected cell extracts and lanes 5 and 6 represent the same in the presence of virus-infected extracts. Lane 7 contains purified reticulocyte eIF2 alone. Panel A represents western analysis of phosphorylated Sf9 [eIF2αP(Sf9)] and added reticulocyte eIF2α [eIF2α P(R)] using a phosphospecific antibody. Panel B represents western analysis of reticulocyte eIF2α [eIF2α(R)] levels used in the reaction mixtures as assessed by an eIF2u monoclonal antibody.

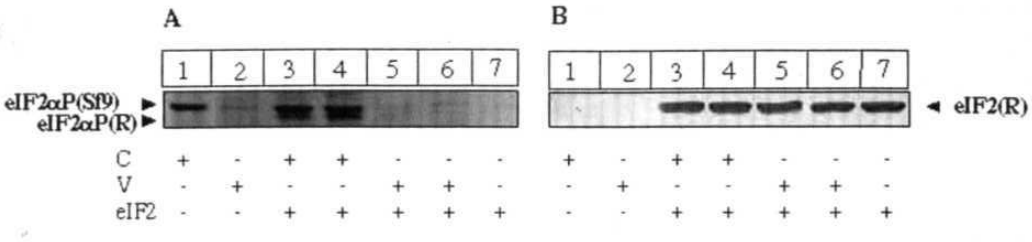
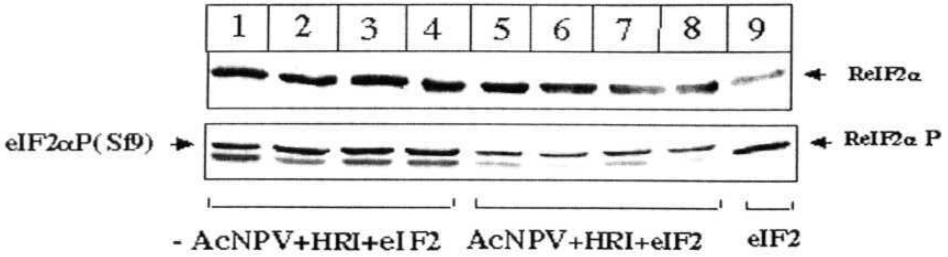

A
B
1
2
3
4
5
6
7
eIF2αP(Sf9)
eIF2αP(R)
eIF2(R)
C + - + + - - - + - + + - - -
V - + - - + + - - + - - + + -
eIF2 - - + + + + + - - + + + + +

Fig. 16. Phosphorylation of reticulocyte eIF2α by HRI kinase in the presence of increasing concentrations of uninfected and virus-infected Sf9 extracts (using eIF2u phosphospecific antibody).

Increasing concentration of uninfected (- AcNPV) and virus-infected (+ AcNPV) Sf9 extracts (15-30 μg) were added to the phosphorylation reaction mixtures containing purified reticulocyte eIF2 and HRI kinase. Phosphorylation was carried out with the addition of 20 mM Tris-HCl, pH, 7.8, 1 mM Mg^{2+}. 1 mM DTT, 80 mM KCl and 100 μM unlabelled ATP at 30 °C for 10 minutes and the mixtures were separated by 10% SDS-PAGE. Gels were analysed by western immunoblot analysis. Reticulocyte eIF2α (RelF2a) levels present in the reaction mixtures were analysed by western immunoblotting using an eIF2α monoclonal antibody. Phosphorylation of elF2a of uninfected, AcNPV-infected Sf9 cell extracts [eIF2αP(Sf9)] and of the trimeric reticulocyte elF2 (RelF2αP) were assessed by using a phosphospecific elF2a antibody. The various lanes are as follows: lanes 1-4 of the bottom panel represent phosphorylation of reticulocyte and insect cell eIF2α by purified HRI in **the** presence of increasing concentration (15, 20, 25 and 30 μg protein respectively) of uninfected Sf9 extract and lanes 5-8 represent the same in the presence of AcNPV-infected extracts. Lane 9 represents the phosphorylation of reticulocyte eIF2α alone by purified HRI kinase.


1
2
3
4
5
6
7
8
9
ReIF2α
eIF2αP(Sf9)
ReIF2α P
- AcNPV+HRI+eIF2
AcNPV+HRI+eIF2
eIF2

(the lower band in the autoradiogram of Fig.14B that aligns with **the** eIF2α of the immunoblot in Fig. 14A) in the presence of **AcNPV-infected cell extracts** containing 15-30 μg of protein (Fig. 14B, lanes, 7-10) where as, a similar decrease in eIF2α phosphorylation is not seen in the presence of uninfected cell extracts (Fig. 14B, lanes, 1-5) or in the presence of low concentration (10 μg) of virus-infected extracts (Fig. 14B lane, 6). The decrease in eIF2α phosphorylation in the presence of virus-infected extracts appears to be due to a decrease in HRI kinase phosphorylation. Corresponding immunoblot indicating **HRI** and eIF2 levels are presented in Fig. 14A.

4.8. **Uninfected Sf9 cell extracts show higher cellular eIF2α phosphorylation and can phosphorylate efficiently purified reticulocyte eIF2 compared to virus-infected cell extracts (using phospospecific anti-eIF2α antibody) [Figs.15 & 16].**

Earlier observations suggested that AcNPV-infection leads to the synthesis of PK2 protein, a truncated protein which is homologous to eIF2α kinases and blocks PKR kinase autophosphorylation and thereby eIF2u phosphorylation (Dever et al., 1998). It was suggested that the presence of PK2 like protein enables the virus to withstand activated eIF2α kinase(s) like GCN2 probably present in insect cells (Santoyo et al., 1997). Since it is observed here that purified reticulocyte eIF2α phosphorylation decreased in the presence of partially purified recombinant eIF2α preparations, we have analysed eIF2α phosphorylation in the mock and AcNPV-infected **Sf9** cell extracts and the ability of these extracts to phosphorylate purified reticulocyte eIF2α (Fig. 15). The phosphorylation status of both **the** cellular and added eIF2α were assessed by a phosphopeptide-specific anti-eIF2α antibody as above. Results indicate that the phosphospecific polyclonal anti-eIF2α antibody recognised the phosphorylated eIF2α of the reticulocytes **and**

SO cells (Fig.15A) whereas the eIF2α monoclonal antibody used here recognises only mammalian eIF2α (Fig. 15B). The phosphorylation of insect cell eIF2a was significantly reduced upon AcNPV infection (Fig. 1 5A, lane 1 vs.lane2). Further, added purified reticulocyte eIF2α was efficiently phosphorylated (lanes 3 and 4) in insect cell extracts which were not infected with the virus and in the absence of any added **kinase**. In contrast, AcNPV-infected cell extracts were unable to efficiently phosphorylate added purified reticulocyte eIF2α (lanes 5 and 6). Purified eIF2a is not recognised by the phoshospecific anti-eIF2α antibody (lane, 7) suggesting that this purified preparation of trimeric reticulocyte eIF2 is not phosphorylated with out the addition of eIF2α kinase and ATP. These results are thus consistent with the idea that the uninfected insect cells contain an active eIF2a kinase that can phosphorylate added eIF2 and produce an eIF2α kinase inhibitor (Dever et al., 1998) upon baculovirus infection.

Since the eIF2α kinase activity of uninfected insect cells was decreased upon viral infection, we also studied the phosphorylation of purified reticulocyte eIF2α *in vitro* by added HRI kinase or in the presence of increasing concentrations of mock and AcNPV-infected cell extracts (Fig. 16). Phosphorylation of reticulocyte and insect cell eIF2a was assessed by using a phosphopeptide-specific anti-eIF2α antibody (Fig. 16, lower panel). Our results show that the phosphorylation of reticulocyte and insect cell eIF2α was decreased in the presence of increasing concentrations of AcNPV-infected extracts significantly greater than in the presence of uninfected extracts (Fig. 16, compare lanes 5-8 vs. 1-4). These observations are consistent with our earlier findings (Fig. 14) that virus-infected extracts decrease eIF2α phosphorylation by blocking the autophosphorylation of HRI as measured by γ- [^{32}P] labelling.

Fig. 17. Early expression of kinase inhibitor upon viral infection.

Panel A: Kinetics of AcNPV infected cell extracts at different time points, 6, 12, 24, 36 and 72 hours of post infection, can be seen in lanes 4-8. Uninfected cell extracts were prepared and loaded in lanes, 2 and 3 (controls) and lane 1 contains molecular weight marker, lane 9 contain reticulocyte eIF2 Each lane contains approximately 30 μg of cell extract loaded and the figure is a coomassie stained 10% SDS-PAGE gel.

Panel B: Western immunoblot analysis of panel A. Proteins were separated on 10% SDS-PAGE and transferred to nitrocellulose membrane and probed with eIF2a phosphospecific antibody. The immunoreactivity of the phosphorylated eIF2α protein in the Sf9 cells during post infection time **is** shown in lanes 1-8 represent as in Panel A.

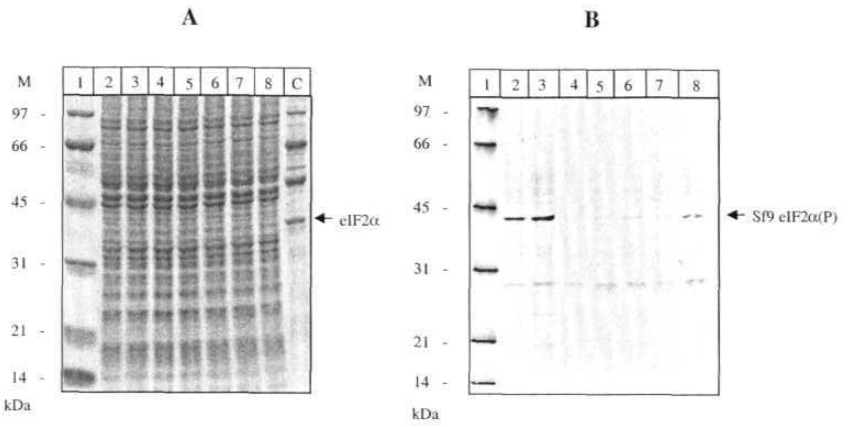
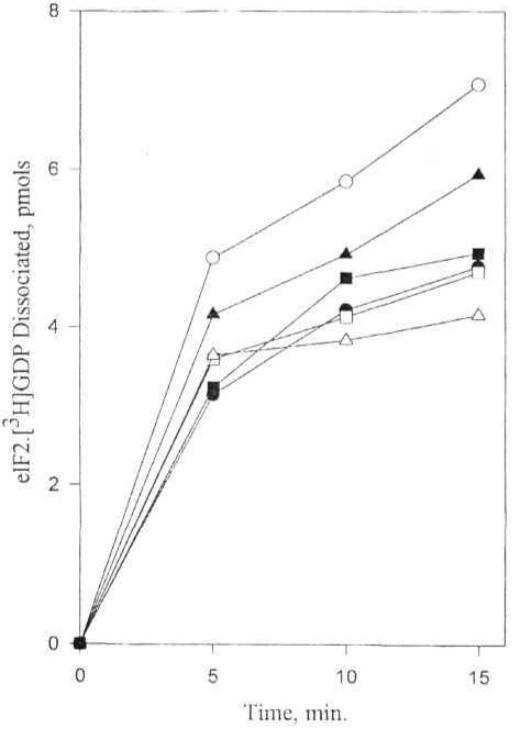

A
M
1
2
3
4
5
6
7
8
C
97
66
45
31
21
14
kDa
eIF2α
B
M
1
2
3
4
5
6
7
8
97
66
45
31
21
14
kDa
Sf9 eIF2α(P)

Fig. 18. Kinetics of eIF2.[^{3}H]GDP dissociation in hemin or hemin and poly(IC)-treated reticulocyte lysates in the presence of insect cell extracts expressing eIF2α wt or 48A mutant.

Protein synthesis was carried out in 70 μl rabbit reticulocyte lysates at 30 °C for 10 min as described (20, 23) under the following two conditions: 1) 0-0, + heme (20 μM); 2) •-•, + heme+ poly (IC) (300 ng/ml); 3) A-A, + heme + poly (IC) + uninfected cell extract; 4) - , +heme+ poly (IC) + virus infected cell extract; 5) •• +heme+ poly (IC) + virus infected cell extract expressing wt eIF2α ; 6) A-•, + heme + poly (IC) + virus infected cell extract expressing 48A eIF2α.

The insect cell extracts (175 μg in 35.25 μl) were prepared 48h p.i. 70 μl (35.0 pmol) of the preformed binary complex was added to the above reticulocyte lysate reactions) 70 |il) and incubated at 30 °C. At various time intervals, 50 μl aliquots were taken to determine the amount of eIF2.[^{3}H]GDP dissociated as described in Materials and Methods.


8
6
4
2
0
eIF2.[3H]GDP Dissociated, pmols
0
5
10
15
Time, min.

4.9. Insect cell eIF2α phosphorylation is decreased more efficiently in the early stages of virus infection than in the later stages [Fig. 17].

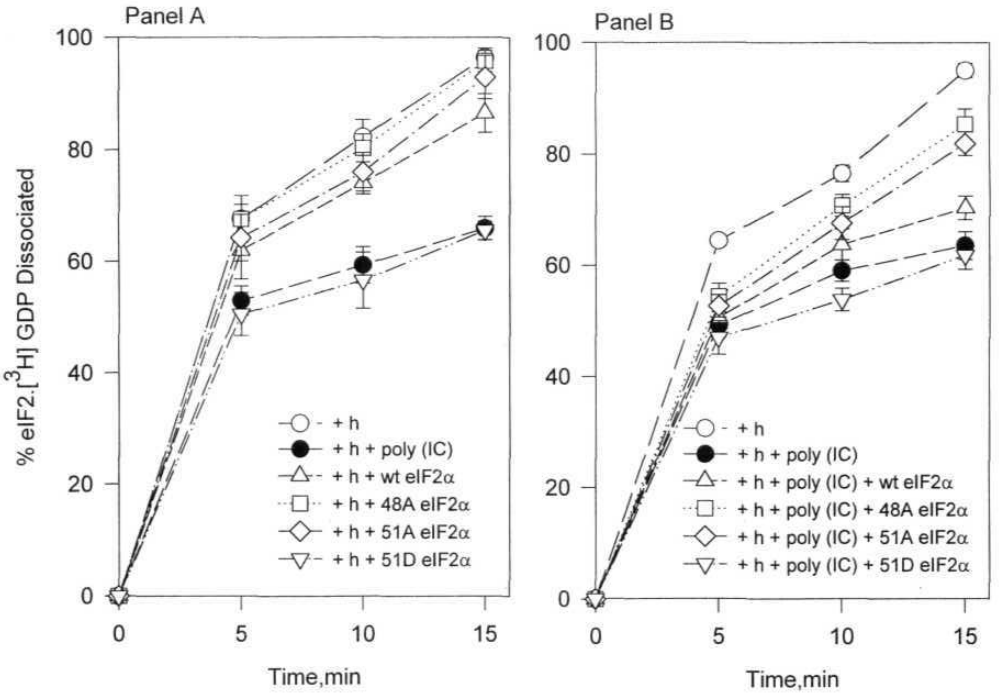
Since the results presented above indicate that the virus infection may be producing an inhibitor of elF2(/. kinase in insect cells, we have studied here to obtain some information with regard to its expression during the various stages of viral infection (Fig. 17). Cell extracts were prepared at 6, 12, **24,** 36 and 72 hours of post infection and elF2a phosphorylation was studied using the phosphospecific anti-eIF2α antibody. The findings indicate the eIF2α phosphorylation of mock infected insect cells is very high (lanes 2 and 3, Panel B , Fig. 17) compared the viral-infected extracts. However, with increase in post infection time one can see a gradual increase in the elF2a phosphorylation (compare lanes 4 vs. 8 at 6h and 72h p.i respectively). These findings suggest probably that the kinase inhibitor may be produced soon after the infection and its concentration may be reduced in the late stages of infection.

4.10. 48A mutant decreases the inhibition of **elF2B activity in poly** (IC)-**treated reticulocyte lysate** [Fig. 18].

Earlier studies with Chinese hamster ovary cells (Ramaiah et al., 1994) have shown that the inhibition of elF2B activity via phosphorylation of eIF2α, either by purified reticulocyte HRI or by endogenous eIF2α kinase activated by heat shock, was reduced by mutations replacing serine residues at 48 and 51 with alanine compared to wild type elF2cx. The functional characteristics of baculovirus-expressed human wild type and 48A mutant elF2a, and their effects on the inhibition of elF2B activity that occurs in poly (IC)- treated reticulocyte lysates was also studied (Fig. 18). The kinetics of elF2B GDP-GTP exchange activity in poly (IC) and hemin-supplemented lysates is shown

Fig. 19. Kinetics of eIF2.[^{3}H]GDP dissociation in reticulocyte lysates in the presence of partially purified recombinant wt and mutants of eIF2α.

Dissociation of preformed labelled eIF2.GDP binary complex was studied in hemin (+ h) or hemin and poly(IC)-treated reticulocyte lysates in the presence of partially purified recombinant wt and or mutants of eIF2α as indicated in panels A and B respectively. The experiment was carried out as described earlier (Sudhakar et al., 1999) but in the presence of 6 μg of partially purified recombinant eIF2α protein. 70 μl of preformed eIF2.[^{3}H]GDP binary complex was added to 70 μl translating lysates. The amount of labelled binary complex present **in the three** independent experiments was 30.8 , 29.75 and 28.87 pmols (panel A) and was 28.35, 30.1 and 27.3 pmols (panel B) respectively. At every time point, 40 μl aliquots were taken out and the percentage of eIF2.[^{3}H]GDP dissociated was measured as described in Materials and Methods. Data are presented as a percentage mean of three independent experiments and standard errors are shown against each value.


Panel A
Panel B
% eIF2.[3H] GDP Dissociated
Time,min
+ h
+ h + poly (IC)
+ h + wt eIF2α
+ h + 48A eIF2α
+ h + 51A eIF2α
+ h + 51D eIF2α
+ h + poly (IC) + wt eIF2α
+ h + poly (IC) + 48A eIF2α
+ h + poly (IC) + 51A eIF2α
+ h + poly (IC) + 51D eIF2α

in Fig. 18. The guanine nucleotide exchange activity of eIF2B in lysates is measured from the dissociation of labelled GDP in the preformed eIF2.[^{3}H]GDP binary complex. Dissociation of the labelled GDP is higher in hemin-supplemented lysates than in poly (IC)-treated lysates. The inhibition of eIF2B activity in hemin and poly (IC)-treated reticulocyte lysates is consistent with the earlier findings that poly (IC) induces eIF2a phosphorylation via double-stranded RNA dependent kinase (PKR) and causes inhibition of eIF2B activity in reticulocyte lysates (Matts et al., 1984; Babu et al., 1996 and Ramaiah et al., 1997). Poly (IC)-treated reticulocyte lysates were supplemented with insect cell extracts (50 μg) **prepared from control Sf9 cells, wild type** AcNPV-infected cells or **recombinant virus-infected cells expressing the wild type** or mutant eIF2α. It is observed that the virus infected cell extracts than the uninfected extracts can relieve to some extent the inhibition in eIF2B activity of reticulocyte lysates caused by the addition of poly (IC). However, the decrease in the inhibition of eIF2B activity is relatively higher in the presence of cell extracts expressing the 48A mutant than wt eIF2α (Fig. 18). These experiments were performed with equal amounts of extract protein in each of the reactions that contained fairly equal amount of expressed eIF2α protein.

4.11. eIF2.[^{3}H]GDP dissociation in hemin or **hemin and poly (IC)-treated** reticulocyte lysates in the presence of partially purified recombinant eIF2a **wt** and the three mutants [Fig. 19].

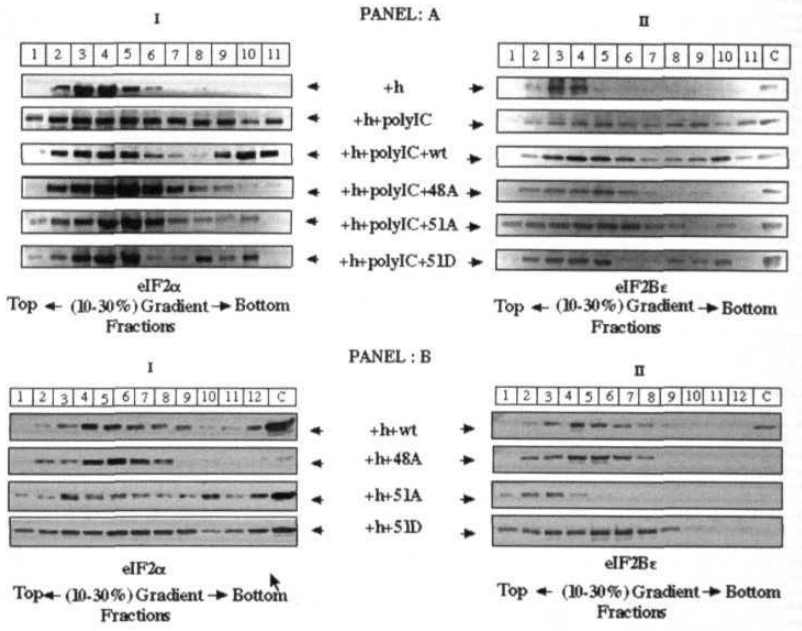
Previous studies (Ramaiah et al., 1994), in heat-shocked mammalian cells, have shown that the inhibition of eIF2B activity that occurred due to eIF2α phosphorylation could be partially reduced by over expression of the 48A and 51A mutant eIF2α. Previously it has not been possible to study eIF2B activity in the presence of 51 D mutant protein because the expression of this

protein leads to cell death in mammalian cells (Kaufman et al., 1989). Although the expression of 51D in insect cells is lower than the 51A protein (Fig. 8A and B), sufficient accumulation of 51D could be detected (see also discussion). Hence, we also studied the effects of **these partially purified baculovirus-expressed** recombinant **human** eIF2α **wt and the three mutants on eIF2B** activity in reticulocyte lysates treated with hemin alone or hemin and poly (IC) (Fig. 19A and B). The dissociation of preformed reticulocyte labelled eIF2.GDP binary complex is high in the presence of hemin-supplemented lysates and is low in hemin and poly (IC)-treated lysates (Fig. 19A and B) and is therefore related to the extent of lysate eIF2a phosphorylation (Fig. 13). Addition of partially purified 51D protein which reduces lysate eIF2α phosphorylation (Fig. 13), however inhibited eIF2B activity of control hemin-supplemented lysates (Fig. 19A) severely **like the** addition of poly (IC) and is consistent with the idea that it behaves **like** phosphorylated eIF2α (Choi et al, 1992). In contrast, addition of non-phosphorylatable 51A or phosphorylatable wt eIF2α to hemin-supplemented control lysates (Fig. 19A) did not inhibit eIF2B activity significantly as has been observed by the addition of poly (IC) (Fig. 19B) or partially purified 51D mutant protein (Fig. 19A and B).

The decrease in the GNE activity of eIF2B caused by addition **of poly (IC) to** hemin-supplemented lysates was mitigated partially in **the** presence of 48A and 51A proteins compared to wt eIF2α (Fig. 19B). Although 48A is phosphorylated on its 51 serine residue like the wt eIF2cx, it reduced partially the inhibition in the GNE activity of eIF2B that occurred due to eIF2α phosphorylation, consistent with our earlier studies (Fig. 18; Ramaiah **et** al., 1994). These studies support that the 48 serine residue is required for high affinity interaction between eIF2</.(P) and 2B. Addition of 51D protein

Fig. 20. eIF2a(P)-eIF2B complex formation in reticulocyte lysates.

Complex formation between eIF2 and eIF2B was studied by western analysis of eIF2a and eIF2Be subunits in the 10-30% sucrose gradient fractions of translating reticulocyte lysates. Protein synthesis reactions of reticulocyte lysates were performed in 100 μl for 15 minutes at 30 °C in the presence of hemin (20 μM) or hemin and poly(IC) (300 ng/ml) with and with out the addition of recombinant eIF2α wt, 48A, 51A and 51D proteins (~6 μg) as indicated. The complex, eIF2-eIF2B was fractionated and fractions were collected using a ISCO-gradient fractionator as described in Materials and Methods. Proteins were separated by 10% SDS-PAGE and analysed by western analysis. Panel A represents western analysis of eIF2α (I) and eIF2Bt: (II) subunits in the fractions of 10-30% gradients containing reaction mixtures of hemin and poly(IC)-treated reticulocyte lysates in the presence of partially purified recombinant eIF2α wt, 48A, 51A or 51D proteins as shown in the figure. Panel B represents western analysis of eIF2a (I) and eIF2Bε (II) subunits in the fractions of 10-30% gradients containing reaction mixtures of hemin-supplemented reticulocyte lysates treated with the recombinant eIF2α wt, 48A, 51A or 51 D proteins.


PANEL: A
I
1 2 3 4 5 6 7 8 9 10 11
+h
+h+polyIC
+h+polyIC+wt
+h+polyIC+48A
+h+polyIC+51A
+h+polyIC+51D
eIF2α
Top ← (10-30%) Gradient → Bottom
Fractions
II
1 2 3 4 5 6 7 8 9 10 11 C
eIF2Bε
Top ← (10-30%) Gradient → Bottom
Fractions
PANEL : B
I
1 2 3 4 5 6 7 8 9 10 11 12 C
+h+wt
+h+48A
+h+51A
+h+51D
eIF2α
Top← (10-30%) Gradient → Bottom
Fractions
II
1 2 3 4 5 6 7 8 9 10 11 12 C
eIF2Bε
Top ← (10-30%) Gradient → Bottom
Fractions

however enhanced further the inhibition of eIF2B activity in poly (IC)-treated lysates (Fig. 19B).

4.12. Gradient analysis of eIF2cx(P).eIF2B Complex.

eIF2α(P) can form a complex with eIF2B that can be separated on a sucrose gradient (Thomas et al., 1985 and Krishna et al., 1997) or using the purified poly-histidine tagged phosphorylated and unphosphorylated eIF2 that binds to Ni-NTA-agarose affinity resin (Pavitt et al., 1998). The latter study demonstrated that eIF2 can form a stable complex with the over expressed yeast eIF2B subunits that can be captured using the affinity matrix. It was shown that the binding of all five subunits of eIF2B to eIF2α(P) was about 2-fold higher than to unphosphorylated eIF2. Here, we have analysed here the eIF2α(P).eIF2B complex formation in hemin or hemin and poly (IC)-treated reticulocyte lysates in the presence and absence of recombinant eIF2α wt, 48A, 51A and 51D mutants on 10-30% gradients (Figs. 20A and B) as previously described. The eIF2 signal in the gradient fractions is detected by using a monoclonal anti-eIF2α antibody that cross reacts with the u-subunit of eIF2(Figs. 20A and B, I). Lysate eIF2 migrated mostly in the top of the gradient fractions (Fig. 20A, I, + h), if it was not phosphorylated, as in hemin-supplemented lysates (Fig. 13). This is because free eIF2 has a lower molecular mass than eIF2</.(P).eIF2B complex. In addition to analysing the presence of eIF2, we have also analysed the gradient fractions for the presence of eIF2B using a monoclonal antibody against eIF2Bε subunit (Fig. 20A and B, II). Our observations suggest that eIF2 (I) and eIF2B (II) are present mainly as a single peak spreading about top 2 to 6 fractions in control hemin-supplemented lysates (Panel A, + h). These lysates are translationally active (Fig. 2), show high eIF2B activity (Fig. 19) and display little eIF2α phosphorylation (Fig. 13A, lane, 2). In contrast, eIF2 and 2B

presence were detected at either the top and or the bottom of the gradient fractions of hemin-supplemented lysates which were treated with poly (IC) (Fig. 20A, +h + poly (IC). Typically, two peaks of eIF2α are observed in the gradient fractions of lysates that are treated with hemin and poly (IC). The first peak comes between fractions 2 and 6 and the second peak is seen at the bottom between fractions 8 and 1 1. These lysates showed enhanced eIF2α phosphorylation (Fig. 1 3) and low levels of eIF2B activity (Fig. 1 9).

Addition of 48A or 51 A recombinant proteins to hemin and poly (IC)-treated lysates decreased the amount of eIF2α (I) and 2Be (II) protein present at the bottom of the gradient fractions, the second peak of fractions in the gradient (Fig. 20A). Addition of recombinant wt **eIF2a,** however, to hemin and poly (IC)-treated lysates resolved the bottom and top peaks of eIF2a and 2Bε, thereby suggesting that complex formation between eIF2α(P) and 2B occurred in the presence of wt eIF2α. The signal intensity of eIF2 and 2B in these fractions was reduced significantly in the presence of 48A and 51 A mutants of eIF2α (Fig. 20A). The findings suggest that the complex formation between eIF2 and 2B requires the phosphorylation of serine 51 residue and also the presence of adjacent unphosphorylated serine 48 residue. This was further substantiated by using 51 D mutant protein, which does not get phosphorylated but mimics the phosphorylated form with the 48 serine residue being conserved. Addition of this mutant protein to hemin and poly (IC)-treated lysates, enhanced the eIF2α and eIF2Bε signal in the bottom fractions of the gradients like wt eIF2α (Fig. 20A, + h + poly (IC) + 51D / or wt).

Complex formation was also analysed in control hemin-supplemented lysates treated with the recombinants of eIF2α. Addition of wt eIF2α, 48A and 51 A produced a different effect compared to 51 D mutant protein in the control

hemin-supplemented lysates which were not treated with poly (IC) (Fig. 20B). In control hemin-supplemented lysates, eIF2cx and 2Bε sedimented in the gradient as a single peak in the presence of either recombinant wt, 48A or 51A eIF2α . While eIF2α was detected in both the top and the bottom of the gradient fractions with out significant separation, eIF2Bε was found mostly in the top fractions. This may have resulted from the absence of eIF2(x phosphorylation in hemin-supplemented lysates that reduces the interaction between eIF2 and 2B (Pavitt et al., 1998) and/or from the presence of high concentrations of added overexpressed variant subunits of eIF2a in the reactions. In contrast, addition of 51D mutant protein to control hemin-supplemented lysates produced two separate peaks detected by increased intensity of the eIF2α and 2Bε signal in the bottom gradient fractions (Fig. 8B).

DISCUSSION

Regulation of translation mediated by eIF2α phosphorylation is one of **the** best characterized control mechanisms operating at the initiation step of protein synthesis (reviewed in refs. Hershey, 1989, 1991; Clemens, 1996; London et al., 1987 and Jackson, 1991). Several studies suggested that phosphorylation of a small portion of eIF2α can inhibit protein synthesis by inhibiting the GNE activity of limiting amounts of eIF2B (reviewed in refs. Webb and Proud, 1997 and Jackson, 1991). Active eIF2B is required to convert inactive eIF2.GDP binary complex to active eIF2.GTP complex that can interact with Met-tRNAi and 40S ribosomal subunits, and to facilitate the recycling of eIF2 (Thomas et al., 1985; Ramaiah et al., 1992 and Gross et al., 1987). Further, earlier studies suggested that phosphorylated eIF2α sequesters eIF2B into a complex in which eIF2B is non-functional (Thomas et al., 1985). By use of enzyme kinetic methods, it was demonstrated that

eIF2α(P) is not a substrate for eIF2B and that eIF2B has a higher affinity for the inhibitor eIF2cx(P) than for the substrate, eIF2 (Rowlands et al., 1988a and Goss et al., 1984). Using an affinity matrix assay, it has been shown recently that binding of all five subunits of yeast eIF2B to yeast polyhistidine-tagged eIF2a(P) is about two fold higher than to unphosphorylated eIF2 (Pavitt et al., 1998). Using mutants of human eIF2α, earlier studies (Ramaiah et al., 1994) hypothesized that phosphorylation of serine 51 promotes the complex formation between eIF2 and 2B.

This hypothesis was tested here by studying complex formation between eIF2rx(P) and eIF2B that occurs in inhibited hemin and poly (IC)-treated reticulocyte lysates in the presence of recombinant human eIF2α wt and 48A, 51A and 51D mutants of eIF2α. The results presented here suggest that eIF2α subunit in the purified trimeric reticulocyte eIF2 complex is phosphorylated more efficiently than the baculovirus-expressed single subunit wt or variant eIF2α forms (Fig. 12A). This is probably because the single subunit of eIF2(x is a poor substrate for eIF2α kinases as has been suggested previously (Choi et al., 1992). However, addition of partially purified recombinant subunits of wt and or mutants of eIF2cx is found to inhibit HRI autokinase activity *in vitro* and thereby inhibits reticulocyte eIF2α phosphorylation (Fig. 12A). The diminution in the autokinase activity of HRI in the presence of baculovirus-expressed wt and mutant forms of eIF2α suggests that these partially purified recombinant preparations carry a contaminant inhibitor of eIF2α kinases. This idea is consistent with the results presented here in figs 12-17. It is observed here that eIF2α phosphorylation is significantly higher in uninfected Sf9 cells than in virus-infected cells (Fig. 15). This is due to a potent eIF2α kinase activity in uninfected Sf9cells which can also phosphorylate reticulocyte eIF2α *in vitro* (Fig. 16). Virus infection reduces cellular eIF2α phosphorylation (Fig. 15) and

the virus-infected cell extracts also reduce the phosphorylation of reticulocyte eIF2a that is mediated by HRI kinase *in vitro* (Fig. 16). All these results are consistent with the idea that baculovirus infection produces a truncated eIF2α kinase homolog that inhibits eIF2α kinase activity of Sf9 cells (Dever et al., 1998). Apparently the inhibitor is comigrating with the partially purified baculovirus expressed wt and mutants of eIF2α. This is evident because the eIF2a phosphorylation that occurs in hemin and poly (IC)-treated reticulocyte lysates due to the activation of PKR is reduced in the presence of baculovirus expressed eIF2α wt and or mutants (Fig. 13B).

However, reticulocyte eIF2α phosphorylation in these above lysates is found similar in the presence of wt and 48A mutant eIF2α and is significantly higher than in the presence of non-phosphorylatable 51A and 51D mutants of eIF2α. The decrease in reticulocyte eIF2 phosphorylation in the presence of 51A and 51D eIF2αlpha subunit is due to formation of a mutant reticulocyte eIF2 complex which is generated by an exchange mechanism that replaces the reticulocyte eIF2α subunit with the recombinant variant form as has been suggested previously (Choi et al., 1992). With increasing time, however, a significant increase in lysate eIF2 phosphorylation is evident even in the presence of 51A and 51D mutants of eIF2a (Fig. 13B). This is very likely due to the incomplete exchange of recombinant variant form of eIF2 into the reticulocyte endogenous eIF2 complex.

Further, the results presented here suggest that phosphorylation of the 48A mutant eIF2α is comparable to the wt form but the former reduces significantly the inhibition in the GNE activity of eIF2B (Fig. 19B) and the complex formation between eIF2α(P) and eIF2B that occurs in hemin and poly (IC)-treated reticulocyte lysates (Fig. 20A). These findings suggest that the serine 48 residue is required for high affinity interaction between

eIF2α(P).eIF2B. In contrast, the nonphosphorylatable 51D mutant but not 51A and wt, inhibited the GNE activity of eIF2B (Fig. 19A) and enhanced significantly the complex formation between eIF2 and eIF2B in uninhibited or hemin-supplemented control reticulocyte lysates (Fig. 20B). While 51A mutant reduced significantly the complex formation between eIF2a(P) and 2B in hemin and poly (IC)-treated reticulocyte lysates, 51D mutant and wt eIF2α proteins were unable to cause a similar effect (Fig. 20A), thereby suggesting that the aspartic acid in the place of 51 serine residue mimcs the charge of a phosphorylated serine. Previously (Ramaiah et al., 1994), and also here it is observed that 48A mutant is somewhat better and offers more protection to the GNE activity of eIF2B in poly (IC)-treated lysates than the non-phosphorylatable 51A mutant of eIF2α. It is not known if this is due to the presence of trimeric mutant 48A in reticulocyte lysates that results from the exchange of free 48A subunits into endogenous trimeric eIF2 (Choi et al., 1992). This chimeric eIF2 may be able to perform the GDP/GTP exchange independent of eIF2B.

Surprisingly, in spite of significant levels of eIF2α phosphorylation, the uninfected cells were able to survive. As has been suggested by Dever et al (1998), this may be due to species differences. eIF2B activity of Sf9 cells is less sensitive to regulation by phosphorylated eIF2 than the eIF2B from mammalian systems where small changes (10-20%) in eIF2α phosphorylation can drastically inhibit the GNE activity of eIF2B. This is also consistent with species specific reactivity of the eIF2 monoclonal antibody. This species specificity may also explain the better ability of Sf9 cells to tolerate moderate expression of 51D mutant of eIF2α than mammalian systems.

CHAPTER IV

SUMMARY

SUMMARY

1) Serine 48A in eIF2<x is required for high affinity interaction between eIF2α(P) and eIF2B.

2) Phosphorylation of serine 51 in eIF2cx promotes complex formation between eIF2α(P) and eIF2B and causes inhibition in the guanine nucleotide exchange activity of eIF2B.

3) Recombinant eIF2a is less efficiently phosphorylated compared to eIF2a in the trimeric purified eIF2 complex.

4) eIF2α phosphorylation in uninfected insect cells is higher than in virus-infected cells.

5) Baculovirus infected cell extracts or partially purified recombinant eIF2α preparations inhibit the phosphorylation of reticulocyte HRI kinase and the eIF2α phosphorylation suggesting that the virus infection produces an eIF2α kinase inhibitor.

CHAPTER V

REFERENCES

REFERENCES

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Serine 48 in Initiation Factor *2a* ($eIF2\alpha$) Is Required for High-Affinity Interaction between $eIF2\alpha(P)$ and eIF2B

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Serine 48 in Initiation Factor 2a (eIF2a) Is Required for High-Affinity Interaction between eIF2a(P) and eIF2B[†]

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ABSTRACT: Phosphorylation of the serine 51 residue in the α-subunit of translational initiation factor 2 in cukaryotcs (eIF2α) impairs prolcin synthesis presumably by sequestering cIF2B, a rate-limiting pcnlamcric guaninc nucleotide exchange protein which catalyzes the exchange of GTP for GDP in the eIF2−GDP binary complex. To further understand the importance of cIF2a phosphorylalion in the interaction between eIF2α(P) and cIF2B proteins and thereby the regulation of cIF2B activity, we expressed the wild type (wt) and a mutant cIF2a in which the scrinc 48 residue was replaced with alaninc (48A mutant) in the baculovirus system. The findings reveal that the expression of both of these recombinant subunits was very efficient (15-20% of the total protein) and both proteins were recognized by an eIF2a monoclonal antibody and were phosphorylated to the same extent by reticulocyte cIF2a kinases. However, partially purified recombinant subunits (wt or 48A mutant) were not phosphorylatcd as efficiently as the cIF2a subunit present in the purified rcticulocytc trimcric cIF2 complex and were also found to inhibit the phosphorylation of eIF2a of the trimcric complex. Furthermore, the extents of inhibition of cIF2B activity and formation of the eIF2α(P)−eIF2B complex that occurs due to eIF2α phosphorylation in poly(IC)-treated rabbit reticulocyte lysates were decreased significantly in the presence of insect cell extracts expressing the 48A mutant cIF2a compared to those for wt. These findings support the hypothesis that the serine 48 residue is required for high-affinity interaction between cIF2a(P) and cIF2B.

Translational initiation factor 2 in cukaryotcs (cIF2)' is a heterotrimer composed of a-, β-, and γ-subunits. It plays a central role in the translational initiation, forming a ternary complex with GTP and Met-tRNAi which then joins 40S ribosomes to form the 43S initiation complex. Following the joining of messenger RNA, the GTP in the ternary complex is hydrolyzed and the inactive eIF2−GDP binary complex is released at the end of the initiation step in protein synlhcsis (reviewed in refs *1*−*3*). For cIF2 to enter another round of initiation, the GDP in the cIF2 binary complex must be exchanged for GTP, a reaction that is catalyzed by a heteropentameric protein called eIF2B (previously called the reversing factor or guaninc nuclcotidc exchange factor) *(4*—*8).* One of the most important ways through which the recycling of cIF2 and thereby protein synlhcsis is regulated occurs through phosphorylation of the small or a-subunit in eIF2 (cIF2a) (reviewed in refs *9*−*12*).

Several physiological stimuli such as hcmc deficiency, viral **infection,** amino acid starvation, heavy metal stress, heal shock, scrum and calcium deprivation, or mobilization of intracellular calcium inhibit prolcin synthesis through the activation of cIF2a kinascs which stimulate cIF2a phosphorylalion (reviewed in refs *13*−*15*). Some of the eIF2a kinascs such as hcme-regulated kinasc (HR1) (reviewed in refs *13* and *14),* the double-stranded RNA-induced inhibitor (PKR) *(16),* and the GCN2 kinasc in yeast and higher cukaryolcs which become activated in response to amino acid starvation (reviewed in rcf /.5) have been well characterized. In addition, two more cIF2a kinascs have been recently characterized. These arc PEK, a pancreatic kinasc, and PERK, an cndoplasmic reticulum resident kinasc *(43, 44).*

Phosphorylation of a small portion of the total cIF2a inhibits the catalytic ability of purified and lysatc cIF2B lo exchange guaninc nucleotides on cIF2 in vitro (6, *17*−*23*). Phosphorylaled cIF2a is a strong **competitive** inhibitor of eIF2B (*24, 25).* The affinity **of** eIF2B for phosphorylaled cIF2 (in which the a-subunil is phosphorylaled) is found to be much higher than that for unphosphorylatcd cIF2 *(24),*

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' Abbreviations: eIF2α, a-subunil (38 kDa) of cukaryolic translational initiation factor 2; cIF2a(P), phosphorylatcd eIF2α; dsRNA, double-stranded RNA; HRI, hcme-regulated inhibitor; PKR, double-stranded RNA-dependent protein kinasc; Met-tRNAi, initiator methionyl tRNA; MOI, multiplicity of infection; AcNPV, *Autographa californica* nuclear polyhcdrosis virus; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; wl, wild type.

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and phosphorylated eIF2 forms a tight complex with eIF2B in which eIF2B becomes nonfunctional *(26)*. Since eIF2B does not exceed 20—30% of the total eIF2, a small proportion of eIF2α phosphorylation sequesters all of the available eIF2B into an inactive complex and prevents the recycling of eIF2 (26, 27).

The availability of site-specific mutants of eIF2a like the 48A or 51A mutant in which the serine residues in the respective positions of eIF2α have been replaced with alanine has advanced our understanding in identifying that (a) the serine 51 residue in eIF2α is the only site for phosphorylation in mammalian eIF2a (28), (b) the translational block caused by adenoviral mRNAs, plasmid-derived mRNAs, heat shock, or calcium release from the endoplasmic reticulum is due to an increased level of eIF2a phosphorylation *(29—32)* or to localizing the translational inhibition caused by eIF2a phosphorylation, and (c) phosphorylation of eIP2a plays a critical role in cell proliferation and development *(33, 34)*. In addition, the coexpression of a mutant eIF2a which cannot be phosphorylated has facilitated the expression of mammalian eIF2α kinases such as the heme-regulated kinase in insect cells *(35)*.

To further understand the mechanism of regulation of eIF2B activity by phosphorylated eIF2a and the protein-protein interactions, we have used the baculovirus system to produce the eIF2a wild type (wt) and the 48A mutant. The latter can be phosphorylated on its serine 51 residue and has been shown to rescue protein synthesis inhibition caused by PKR and heat shock *(29, 30)*. Consistent with these earlier observations, our findings here indicate that baculovirus-expressd 48A mutant eIF2a mitigates the inhibition of eIF2B activity in reticulocyte lysates caused by eIF2a phosphorylation and reduces the extent of formation of the 15S complex that occurs between eIF2 and eIF2B when the α-subunit in eIF2 is phosphorylated, thereby suggesting that the mutant eIF2a(P) cannot interact with eIF2B as **efficiently** as wt eIF2a(P). Our studies additionally demonstrate here that AcNPV-infected insect cell extracts inhibit eIF2α phosphorylation in vitro.

MATERIALS AND METHODS

Materials. The pETFVA⁻ vector harboring wild type or human mutant eIF2a has been described previously (45). pBakPAK8, pBakPAK6 (*Bsu*36I digest), lipofectin, and Sf9 cells were obtained from Clonetech. The random primer labeling kit, the hybond N^+ membrane, and [α-^{32}P]dCTP were purchased from Amersham. Poly(IC), a synthetic double-stranded RNA, was obtained from Calbiochem. Restriction enzymes, anti-mouse IgG raised as a rabbit AP conjugate, NBT, and BCIP were obtained from Promega Corp. A monoclonal anli-eIF2a antibody produced in E. C. Henshaw's laboratory and purified heme-regulated eIF2a kinase (HRI) were kindly provided by J.-J. Chen (Massachusetts Institute of Technology, Cambridge. MA). GDP. CPK, DTT, and protease solutions were purchased from Boehringer Mannheim GmbH. Fetal calf serum and antibiotics were from Gibco BRL. Insect cell culture medium (TNM-FH) and various other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). [8-^{3}H]GDP (2 mM. 9 Ci/mmol) was obtained from Dupont NEN, while [γ-^{32}P]ATP (3000 Ci/mmol) was obtained from BRIT (Mumbai. India).

Preparation of the Recombinant Baculovirus Transfer Vector, DH5a cells were transformed with the parent vector harboring wt or 48A mutant eIF2a cDNA to amplify the parent vector. Vector DNA was isolated using the Qiagen column and digested with *Eco*RI lo separate the 1.6 kb eIF2a insert. The insert was electroeluted from a 1% agarose gel and was purified by Qiaquick Spin columns. PBacPAK8, a baculovirus transfer vector, was linearized with *Eco*RI and ligated lo the eIF2α cDNA. DH5α cells were transformed with the recombinant pBacPAK8 vector, and the positive colonies containing the vector wilh the eIF2a insert were identified by using the colony hybridization technique. The insert orientation in the vector was checked by double-restriction digestion using *Mlu*I and *Sca*I, or *Sph*I and *Bam*HI, enzymes for pBacPAK8 vector carrying wl or 48A mutant eIF2α cDNA, respectively, and the recombinant constructs with the insert in the right orientation were used for further work.

Maintenance of Insect Cells, Cotransfection, and Identification of Recombinant Baculoviruses. The *Spodoptera frugiperda* (Sf9) cell line was maintained in complete TNM-FH medium containing 10% fetal calf scrum and antimycotic and antibiotic solutions as described previously *(35)*. Recombinanl baculoviruses were generated in vitro by transfecting *Bsu*36I-digested AcNPV virus DNA (Clonetech) into Sf9 cells as described previously *(37)*.

Plaque assays were carried out to obtain recombinant viruses from a single clone, and the positive plaques were identified by dot blot hybridization using [α-^{32}P]dCTP-labeled cDNA. Amplification of recombinant viruses was carried out to increase the liter of the recombinanl virus in a stepwise manner.

Determination of the Level of eIF2α Expression. Uninfected insect cells as well as infected cells (infected with AcNPV or eIF2α wt or eIF2α 48A mutant recombinant viruses) were washed with ice-cold PBS (pH 6.2). The cells were lysed in ice-cold lysis buffer [20 mM Tris-HCl (pH 7.8). 1 mM Mg^{2+}, 1 mM DTT, and protease inhibitors such as pepstatin, leupeptin, and aprolcinin] and centrifuged at 10 (XX) rpm for 10 min. To the lysate supernatant were added PMSF (1 mM) and KCl (80 mM), and the mixture was immediately aliquoted and stored in liquid N_2. Samples of the concentrated extracts were separated on 10% SDS−PAGE and were also transferred to a nitrocellulose membrane lo determine the level of expression of eIF2a based on its migration on SDS-PAGE and also on its ability to interact with a monoclonal eIF2α antibody *(22, 35)*.

Preparation of Reticulocyte Lysales and eIF2. Heme-deficient reticulocyte lysales which respond to added hemin were prepared and were used as a source for measuring eIF2B activity and also for the purification of eIF2 as described previously *(20, 23)*.

Measurement of eIF2B Activity. The eIF2B activity of hemin or heme and poly(IC)-lrcalcd rcticulocyle lysatcs in the presence and absence of insect cell cxlracls expressing the wt or mutant eIF2α was measured by monitoring the level of dissociation of the preformed labeled reticulocyte eIF2−[^{3}H]GDP binary complex, as described previously (20, *23, 35, 38)*.

Phosphorylalion of Recombinant eIF2α in Vitro, in Poly-(IC)-Treated Reticulocyte Lysates, and in Insect Cell Extracts. Purified heme-regulated kinase (HRI) and [γ-^{32}P]ATP

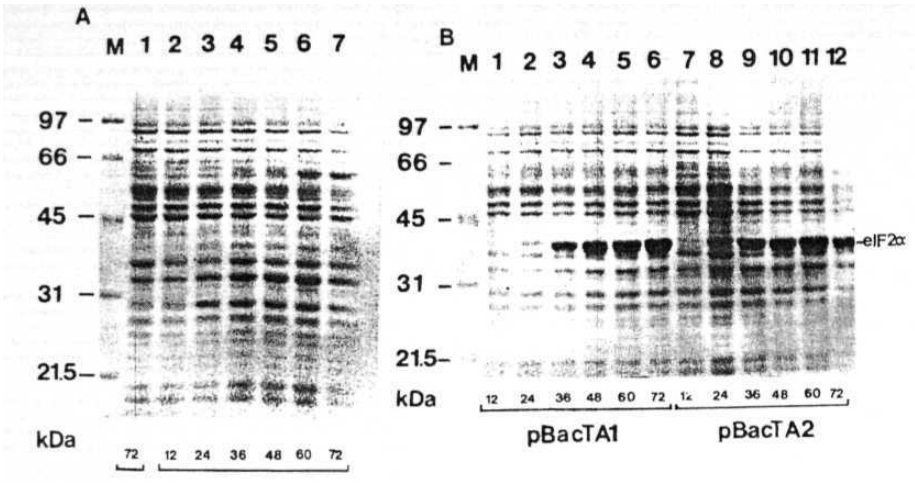

FIGURE 1: Expression of wild type and 48A mutant human eIF2α in insect cells using recombinant baculovirus. Time course of protein expression. Insect cell extracts were prepared as described in Materials and Methods from Sf9 cells infected al a multiplicity of infection (MOI) of 10, with recombinant viruses harboring wt or 48A mulanl eIF2α (B) or with wild type AcNPV (A) at different time points as shown in the figure. Extract from uninfected cells (control, C, in panel A) was prepared for only one time point (72 h postinfection). Each extract was prepared from 2 x 10^6 Sf9 cells. The viruses had an MOI of 10 in each case. An equal amount of protein extract (25 /<g) was loaded in each well of a 10% SDS-PAGE and stained with Coomassie bluc.

were used to phosphorylatc the expressed rccombinanl eIF2α in insect cell extracts. The phosphorylated extracts were then resolved by 10% SDS-PAGE and analyzed by autoradiography (*20*–*23*). Also, the insect cell extracts or the partially purified cIF2a wt or 48A mulanl (purified using Scphacryl 300 and DEAE cellulose 52) was phosphorylatcd with or without HRI, respectively, in the presence of [γ-^{32}P]ATP. In addition, phosphorylation of poly(IC)-trcatcd hemin-supplemented reticulocyte lysates was carried out in the presence and absence of insect cell extracts expressing wt or 48A mutant cIF2a.

Analysis of the eIF2α(P)–*eIF2B Complex in Reticulocyte Lysates.* Reticulocyte lysatc protein synthesis reactions (100 μL) were carried out al 30 °C for 15 min in 20 μM heme or heme and poly(IC)-lrcalcd (20 μM and 300 ng/mL) rcliculocytc lysates in the presence of nonrecombinant or rccombinant virus-infected cell extracts (25 μg of prolcin) as described in the legend of Figure 5. At the end of the prolcin synthesis reaction, the lysates were diluted with an equal volume of chilled TKM buffer consisting of 20 mM Tris-HCl (pH 7.6), 100 mM KC1, and 2 mM $Mg(OAc)_2$ to terminate the reaction. Samples were layered on 4.5 raL exponential sucrose gradients (10 to 30%) which were prepared with the dilution buffer described above. Samples were run at 40 000 rpm for 6 h al 4 °C in a SW 50.1 rotor to separate free cIF2 from the cIF2ot(P)-cIF2B complex as described previously (*22*). Fractions (400 μL) were collected by upward displacement of the gradients with the help of an ISCO gradient fractinator. Fractions were concentrated by pH 5.0 precipitation in the presence of 50 mM NaF^- and 5 mM EDTA to prevent the dcphosphorylalion of cIF2rx Samples were suspended in sample buffer, briefly boiled, and separated on 10% SDS-PAGE. Proteins were transferred to nitrocellulose membranes, and eIF2α of the various gradient fractions was detected by using an anti-human eIF2α monoclonal antibody as described previously (*22*).

RESULTS

Expression of Wild Type and 48A Mutant Human eIF2α. Extracts of uninfected and infected Sf9 cells (infected with wild type AcNPV or with rccombinant, pBacTA1, wild type cIF2a or pBacTA2, 48A mutant eIF2α) were prepared al different time points post-infection (12, 24, 36, 48, 60, and 72 h) and were analyzed by 10% SDS-PAGE (Figure 1). A protein with a molecular mass of 38 kDa is expressed in the cells infected with the rccombinanl virus from 24 h postinfcclion onward up to 72 h (Figure 1B). This protein is not found in uninfected cells or in cells infected with the wild type AcNPV virus (Figure 1A).

Immunoreactivity of the Recombinant eIF2α. Immunoreactivity of the expressed protein was tested by Western blot analysis using anli-cIF2a monoclonal antibody (Figure 2). Both the wild type (Figure 2, lanes 3—7) and the 48A mutanl (lanes 8-12) react equally well with the antibody, and the magnitude of the signal is proportional to the level of expression of the eIF2α protein. At 24 h postinfection, the extracts contain low levels of eIF2α, and accordingly, the reactivity of the antibody is poor in these lanes (lanes 3 and 8). In contrast, a strong signal appeared between 36 and 72 h (lanes 4—7 and 9-12) postinfection and is consistent with the previous results which showed that expression starts around 24 h and increases with time up to 72 h poslinfcclion. Neither control Sf9 cell extracts (lane 1) nor wild-type AcNPV-infected cell extracts (lanc 2) contain any polypcptidc that is immunorcaclivc with respect to the eIF2α monoclonal antibody described above.

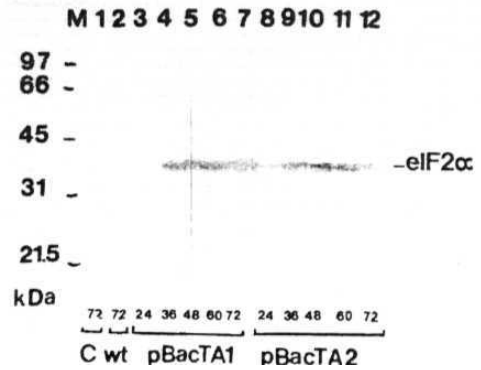

FIGURE 2: Immunoreactivity of the recombinant eIF2α. Cell extracts were prepared as described in the legend of Figure 1. The protein extracts (25 μg) were separated on a 10% SDS−PAGE, transferred to a nitrocellulose membrane, and probed with an eIF2α monoclonal antibody. The signal was detected with the help of rabbit anti-mouse alkaline phosphatase-conjugated secondary antibody (Promega). In the case of uninfected cells (C) and of cells infected with wild type AcNPV, extracts from only one time point (72 h postinfection) were used.

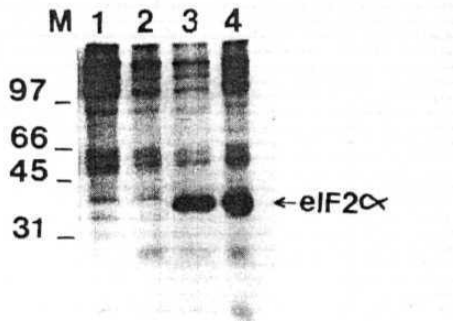

FIGURE 3: Phosphorylation of the recombinant human eIF2α wt and 48A mutant in insect cell extracts by HRI. Cell extracts (25 μg) from uninfected and infected cells (infected with wt or recombinant viruses) prepared 48 h postinfection were incubated at 30 °C for 5 min prior to the addition of HRI. HRI was preincubated in a reaction mixture containing 20 mM Tris-HCl (pH 7.6), 2 mM Mg^{2+}, 80 mM KCl, and 30 μM ATP at 30 °C for 5 min. The extracts were incubated for an additional 5 min before they were pulsed with [γ-^{32}P]ATP (10 μCi). Reactions were terminated 5 min after the addition of the labeled ATP by adding 2× SDS sample buffer and the mixtures heated for 2 min in boiling water. The samples were separated on 10% SDS−PAGE, and the gel was **analyzed** by autoradiography: lane 1, uninfected cell extract; lane 2, wild type AcNPV-infected cell extract; lane 3, recombinant virus-infected cell extract expressing the eIF2α wt; and lane 4, recombinant virus-infected cell extract expressing the eIF2α 48A mutant.

Phosphorylation of the Recombinant eIF2α wt and 48A Mutant. Since wild type eIF2α and 48A mutant eIF2α expressed in mammalian systems are shown to be substrates for eIF2α kinases *(30, 39)*, the ability of baculovirus-expressed eIF2α subunits to serve as substrates for phosphorylation in the presence of purified HRI is tested (Figure 3). Both the recombinant wild type and 48A mutant eIF2α are found to be accessible for phosphorylation (lanes 3 and 4). A similar phosphorylated protein corresponding to human eIF2α is lacking in the control and AcNPV-infected cell extracts (lanes 1 and 2). These findings suggest that baculovirus-expressed eIF2α truly represents human eIF2α. In the absence of added HRI, phosphorylation of recombinant eIF2α could not be detected in insect cell extracts (data not shown).

The 48A Mutant Decreases the Level of Inhibition of eIF2B Activity in Poly(IC)-Treated Reticulocyte Lysates. Earlier studies with Chinese hamster ovary cells *(38)* have shown that the level of inhibition of eIF2B activity via phosphorylation of eIF2α, either by purified reticulocyte HRI or by endogenous eIF2α kinase activated by heat shock, was reduced by mutations replacing serine residues 48 and 51 with alanine compared to that with wild type eIF2α. The functional characteristics of baculovirus-expressed human wild type and 48A mutant eIF2α, and their effects on the inhibition of eIF2B activity that occurs in poly(IC)-treated reticulocyte lysates, were also studied (Figure 4). The kinetics of eIF2B GDP-GTP exchange activity in poly(IC) and hemin-supplemented lysates is shown in Figure 4. The guanine nucleotide exchange activity of eIF2B in lysates is measured from the extent of dissociation of labeled GDP in the preformed eIF2−[^{3}H]GDP binary complex. The extent of dissociation of the labeled GDP is higher in hemin-supplemented lysates than in poly(IC)-treated lysates. The inhibition of eIF2B activity in hemin and poly(IC)-treated reticulocyte lysates is consistent with the earlier findings that poly(IC) induces eIF2α phosphorylation via double-stranded RNA-dependent kinase (PKR) and causes inhibition of eIF2B activity in reticulocyte lysates *(18, 20-23)*. Poly(IC)-treated **reticulocyte** lysates were supplemented with insect cell extracts (50 *fig)* prepared from control Sf9 cells, wild type AcNPV-infected cells, or recombinant virus-infected cells expressing wild type or mutant eIF2α. It is observed that the virus-infected cell extracts instead of the uninfected extracts can relieve to some extent the inhibition in eIF2B activity of reticulocyte lysates caused by the addition of poly-(IC). However, the decrease in the level of inhibition of eIF2B activity is relatively higher in the presence of cell extracts expressing the 48A mutant than in the presence of wt eIF2α (Figure 4). These experiments were performed with equal amounts of extract protein in each of the reaction mixtures that contained fairly equal amounts of expressed eIF2α protein (wild type or 48A mutant eIF2α) (data not shown).

Analysis of eIF2α(P)−eIF2B Complex Formation in Reticulocyte Lysates. Previously, it was hypothesised that the 48A mutation in eIF2α decreases the extent of interaction of eIF2α(P) with eIF2B *(38)*. To assess such a possibility, the eIF2α(P)−eIF2B complex that forms in poly(IC)-inhibited reticulocyte lysates was analyzed by sucrose gradient centrifugation, as described previously (22) in the presence of insect cell extracts containing the eIF2α wild type or 48A mutant (Figure 5). Since free eIF2 has a significantly lower molecular mass than the eIF2α(P)−eIF2B complex, the top fractions of the 10 to 30% sucrose gradient contain free eIF2, whereas the bottom fractions contain the complex. The free eIF2 and eIF2α(P)−eIF2B complex in

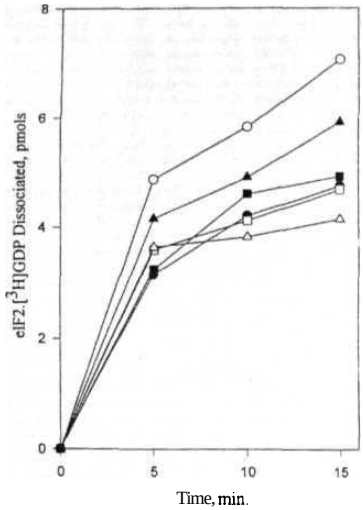

FIGURE 4: Kinetics of eIF2−[^{3}H]GDP dissociation in hemin-treated or hemin- and poly(IC)-trcated reticulocyte lysatcs in the presence of insect cell extracts expressing the eIF2α wt or 48A mutant. Protein synthesis was carried out in 70 μL of rabbit reticulocyte lysatcs at 30 °C for 10 min as described previously *(20, 23)* under the following conditions: (O) with hemc (20 μM), (•) with hemc and poly(IC) (300 ng/mL), (A) with hemc, poly(IC), and uninfected cell extract, (•) with hemc, poly(IC), and virus-infcctcd cell extract, (•) with hemc, poly(IC), and virus-infcclcd cell extract expressing eIF2α wt, and (A) with hemc, poly(IC), and virus-infected cell extract expressing the eIF2a 48A mutant. The insect cell extracts (175 μg in 35.25 μL) were prepared 48 h postinfection. Seventy microliters (35.0 pmol) of the preformed binary complex was added to the above rcliculocytc lysate reaction mixtures (70 μL) and incubated at 30 °C. At various time intervals, 50 μL aliquots were taken to determine the amount of eIF2−[^{3}H]GDP dissociated as described in Materials and Methods.

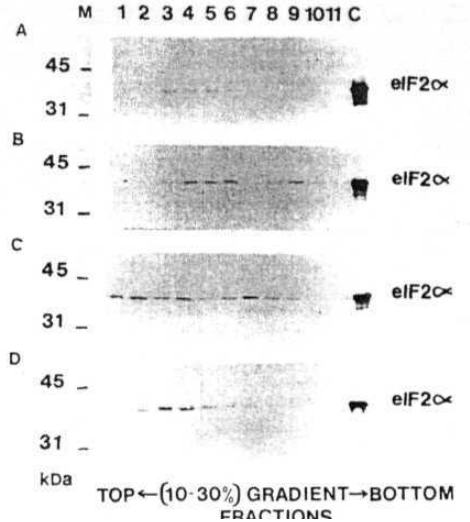

FIGURE 5: Detection of the eIF2α(P)−eIF2B complex in reticulocyte lysalcs by immunoblot analysis of clF2 in the 10 to 30% gradient fractions. Protein synthesis reactions of reticulocyte lysalcs were carried out in 100 μL for 15 min at 30 °C in the presence of (A) 20 μM hemc (h) and wild type AcNPV-infected Sf9 cell extracts (25 μg), (B) h, poly(IC) (300 ng/mL), and wild type AcNPV-infcctcd cell extract, (C) h, poly(IC), and recombinant virus-infcclcd cell extract ovcrcxprcssing eIF2α wt (25 μg), and (D) h, poly(IC), and recombinant virus-infected cell extract ovcrcxprcssing the 48A mutant. Reaction mixtures were diluted with an equal volume of TKM buffer [20 mM Tris-HCl (pH 7.8), 80 mM KCl, and 2.5 mM $Mg(OAc)_2$] and layered on a 10 to 30% sucrose gradient. The gradients were prepared in TKM buffer and centrifuged wilh the samples for 6 h at 40 OIK) rpm in a SW 50.1 rotor. The gradient fractions (400 μL) were collected, concentrated by pH 5.0 precipitation, separated on 10% SDS−PAGE, and transferred to a nitrocellulose membrane. clF2a in the transferred proteins of the different gradient fractions was identified wilh the help of anti-mouse human cIF2a monoclonal antibodies as described previously *(22).* Purified clF2 (250 ng) was loaded al the end of each gel to serve as a control.

the gradient fractions were detected by Western analysis using an eIF2α monoclonal antibody as previously described (22). The eIF2α signal could be seen only in the lop fractions of the gradient that contains hemin-supplemented lysatcs treated with wild type AcNPV-infected insect cell extracts (Figure 5A). This is because in the presence of hemin and without poly(IC) or dsRNA being included in the reaction, reticulocyte lysatcs contain very little or no cIF2a kinase activity which can phosphorylate eIF2α and facilitate the formation of a complex between eIF2α(P) and eIF2B. Hence, very little rcliculocytc clF2 is bound to eIF2B which can be detected in the bottom fractions of these gradients. In contrast, eIF2α is detected in both the top and bottom Iractions of the gradients containing lysatcs treated with hemin, poly(IC), and the wild type AcNPV-infected cell extracts (Figure 5B). A similar result was obtained, indicating the presence of the eIF2α signal in the top and bottom fractions of the gradients for the Iractions of hemin and poly-(IC)-treated lysatcs that were supplemented with insect cell extracts containing wild type eIF2α (Figure 5C). This indicates the presence of free eIF2α and eIF2α in complex with cIF2B. However, the intensity of the eIF2α signal was greater in the top fractions because the reaction mixtures contain the overexpressed wild type eIF2α subunit, and the signal can be seen even in the first fraction of the gradient (Figure 5C). This suggests that the overcxpresscd subunil form of cIF2a can also be separated from the eIF2ot present in the trimeric cIF2 complex in the lysatcs. In contrast, it was not possible to detect cIF2a in the bottom fractions of the gradient in the hemin and poly(IC)-lrcatcd rcliculocytc lysatcs which were supplcmcnlcd with insect cell extracts expressing 48A mutant clF2tt (Figure 5D). These findings suggest that the 48A mutalion in mammalian eIF2α reduces the extent of formation of the complex that exists between eIF2α(P) and cIF2B in hemin and poly(lC)-lrcalcd rcliculocylc lysalcs. These findings arc consistent wilh the eIF2B activity measurements (Figure 4).

Phosphorylation of Recombinant eIF2α, eIF2α in a Trimeric Complex in Vitro, and in ***the Presence*** *of Uninfected and AcNPV-Infected Insect Cell Extracts.* To understand if the phosphorylation of rccombinanl eIF2α, wt, or 48A mutant was in any way different from that of the α-subunit in the trimeric complex of cIF2, the phosphorylation of partially purified rccombinant eIF2α (purified using Sephacryl-300 and the DEAE cellulose 52 column) was studied in vitro

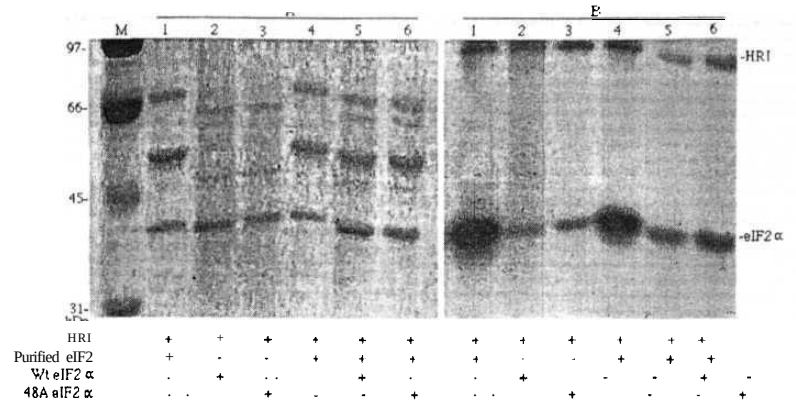

FIGURE 6: Phosphorylation of the α-subunit in purified rabbit reticulocyte trimeric eIF2 in the presence and absence of recombinanl subunit eIF2α (wt or 48A mutant) and by purified HRI and [γ-^{32}P]ATP in vitro. Purified eIF2 and partially purified recombinant subunits of eIF2α wt and 48A mutants were phosphorylated as described previously (*23*), and the reaction mixtures were loaded on a 10% SDS−PAGE gel. Panel A is a stained gel, and panel B is the corresponding autoradiogram indicating HRI and eIF2a phosphorylation: lane 1, purified rabbit rcliculocytc eIF2 complex; lane 2, partially purified wt recombinant eIF2α; lane 3, partially purified 48A mutant eIF2α; lane 4, purified rabbit reticulocyte eIF2; lane 5, purified rabbit eIF2 and wt eIF2α; and lane 6, purified rabbit eIF2 and 48A mutant eIF2a. HRI is present in all reaction mixtures.

FIGURE 7: Reliculocyle eIF2α phosphorylation by HRI in the presence of uninfected and AcNPV-infected cell extracts. Phosphorylation of reticulocyte eIF2 was carried out in vitro by HRI and [γ-^{32}P]ATP (10 μCi) in a total volume of *20* μL as described previously *(23)* in the presence of different concentrations of uninfected and virus-infected cell extracts. The samples were separated on 10% SDS−PAGE and transferred to a nitrocellulose membrane. The eIF2 and HRI levels were analyzed by using respective monoclonal antibodies as described previously *(38, 45)*, and the immunoblot was analyzed by autoradiography to determine the levels of eIF2 and HRI phosphorylalion. Panel A is an immunoblol indicating eIF2α and HRI levels present in the reaction mixtures. Panel B is an auloradiogram of the same gel indicating the phosphorylation of eIF2α and HRI in the presence of insect cell extracts in vitro: lanes 1-5, uninfected cell extracts containing 10, 15, 20, 25, and 30 μg of protein, respectively; lanes **6—10,** AcNPV-infected cell extracts containing 10, 15, 20, 25, and 30 /<g of protein, respectively; and lane 11, control lane containing HRI and reticulocyte eIF2.

with HRI kinase (Figure 6A,B). Interestingly, it is observed here that the recombinanl eIF2α (both wt and 48A mutant) is less efficiently phosphorylated than the eIF2a present in the purified rcticulocytc eIF2 complex (Figure 6B, lanes 2 and 3 vs lanc 1). In addition, the level of phosphorylation of HRI kinasc is also decreased in the presence of rccombinant wt or 48A mutant protein (Figure 6B). These experiments were carried out in such a way that protein levels of rccombinanl eIF2a and the eIF2α in the purified complex were fairly similar as judged by the stain intensity in the gels (Figure 6A). These findings raise the possibility that virus infection may inhibit HRI kinasc and thereby eIF2a phosphorylation. This possibility is supported by the observation that phosphorylation of eIF2α in the purified trimeric reticulocyte eIF2 complex is not enhanced but inhibited in the presence of the rccombinanl eIF2α wt or 48A mutant in vitro (Figure 6B, lanc 4 vs lanes 5 and ft). **However,** the decrease in the level of phosphorylation of eIF2α in the purified complex is similar in the presence of partially purified recombinant wt and 48A mutant eIF2α (lanes 5 and 6). Also, the phosphorylation of the partially purified recombinant wt eIF2α subunit is not different from that of the 48A mutant eIF2α in vitro (Figure 6B, lanes 2 and 3).

To further understand if virus-infected cell extracts reduce the level of eIF2a phosphorylation, different amounts (10—30 μg of protein) of uninfected and AcNPV-infcctcd insect cell extracts were added to the phosphorylation reaction mixtures containing purified rcliculocytc eIF2 and HRI (Figure 7). It is observed that the level of reliculocyle eIF2α phosphorylalion is reduced significantly (the lower band in the gel in Figure 7B that aligns with the eIF2ot of the immunoblol in Figure 7A) in the presence of AcNPV-infected extracts containing 15−30 μg of protein (Figure 7B, lanes 7−10), whereas a similar decrease in the level of eIF2α phosphorylation is not seen in the presence of uninfected cell extracts (Figure 7B, lanes, 1—5) or in the presence of a low concentration (10 μg) of virus-infected extracts (Figure 7B, lane 6). The decrease in the level of eIF2α phosphorylalion in the presence of virus-infected extracts appears to be due to a decrease in the level of HRI kinasc phosphorylation. Corresponding immunoblots indicating HRI and cll-2 levels arc presented in Figure 7A

Phosphorylation of Recombinant eIF2α in Inhibited Reticulocyte Lysates. In addition, the phosphorylalion of the purified rccombinant eIF2α wt or 48A mutant is found lo bc similar in translating hemin-supplemented lysatcs which

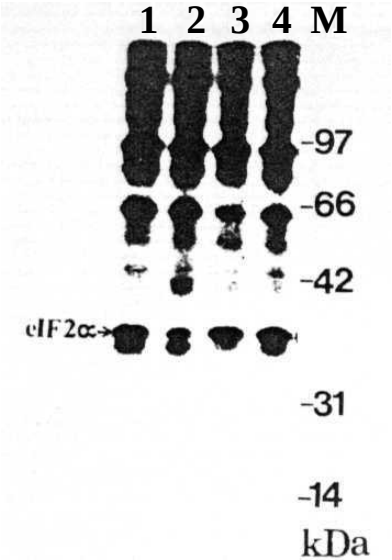

FIGURE 8: Phosphorylation of wt and 48A mutant eIF2α in heme- and poly(IC)-treated rcliculocylc lysatcs. Heme-deficient reticulocyte lysates (20 μL) were prepared, supplemented with hemin (20 μM) or hemin and poly(IC) (300 ng/mL), and incubated with the protein synthesis cocktail at 30 °C for 10 min. The lysalcs were supplemented, whereever indicated, with partially purified recombinant wt or 48A mutant eIF2α. Phosphorylalion of lysates was carried out in the presence of [γ-^{32}P]ATP (20 μCi) for 5 min between 10 and 15 min of protein synthesis. Afterward, 10 μL of the reaction mixtures was taken and precipitated al pH 5.0 as described previously *(23)*. Samples were then resuspended in SDS sample buffer and separated on 10% SDS−PAGE. The gel was dried and analyzed by autoradiography: lane 1, heme and poly(IC); lane 2, heme; lane 3, heme, poly(IC), and wt eIF2α; and lane 4, heme, poly(IC), and the 48A mutant eIF2α.

arc treated with poly(IC) (Figure 8, compare lanes 3 and 4). In the absence of poly(IC), eIF2α is less efficiently phosphorylated in hemin-supplemented lysalcs (Figure 8, lane 2) as expected, and this is consistent with the presence of higher eIF2B activity (Figure 4) and a reduced level of formation of the 15S cIF2a(P)-cIF2B complex (Figure 5A). Although the recombinant eIF2α wt and 48A mutant are phosphorylatcd to the same extent in poly(IC)-lrcalcd rcliculocylc lysalcs (Figure 8, compare lanes 3 and 4), the extents of inhibition of eIF2B activity and formation of 15S complex that occurs in poly(IC)-trealcd reticulocyte lysates are reduced significantly in the presence of extracts expressing 48A mulani eIF2α compared lo those of wt (Figures 4 and 5). These findings thus suggest that phophorylalcd 48A mutant eIF2α docs not inhibit the GDP-GTP exchange aclivily of eIF2B and is unable lo form a 15S complex with cli"2B as efficiently as the phosphorylated wt eII·2α (Figures 4 and 5).

DISCUSSION

Recombinant wild type human cIF2a and a mutant form having a serinc replaced with an alaninc al position 48 were expressed in insect cells using the baculovirus system to determine the importance of the serinc 48 residue in eIF2α in the interaction between cIF2a(P) and clF2B. Rccombinanl eIF2α was expressed as approximately 15-20% of the total protein (Figure 1B). The baculovirus system which allows simultaneous expression of multiple genes *(37)* to produce multimeric protein complexes has been used recently lo produce wt and Ser51Ala mutant forms of cIF2a for evaluating the phosphorylation site of the variant cIF2a *(39)* and for determining the requirements for the subunit assembly into a functional pentameric cIF2B protein *(40)*. This system has previously been used by us to express wild type HRI kinase by cotransfecting the cells wilh the 51A mutant of cIF2a which bypasses the protein synthesis inhibition caused by the ovcrexpression of wild type HRI *(35)*.

The importance of eIF2α phosphorylation in translalional control was highlighted through the expression of wl human cIF2a and serinc lo alaninc mutants al residues 48 and 51 (48A and 51A mutants) in mammalian and insect systems *(28—33)*. Although the 51A mutant cannot bc phosphorylaled, the 48A mutant can be phosphorylaled al its serinc 51 residue. Interestingly, expression of either mutant protects protein synthesis in mammalian cells caused by PKR and heat shock *(29−31)*These findings suggest that in addition lo serinc 51, serinc 48 is required in inhibiting translation when residue 51 is phosphorylatcd. Previous biochemical studies using cell free systems derived from rabbit reticulocytes *(18, 20)* and through genetic and biochemical experiments conducted in yeast *(24)* have shown that phosphorylalion of eIF2a results in the inhibition of guanine nucleotide exchange activity of cIF2B and thereby protein synthesis. Results presented here indicate that the baculovirus-expressed 48A mutant of cIF2a decreases the level of inhibition of eIF2B activity caused by poly(IC) treatment in reticulocyte lysalcs lhal occurs via the activation of PKR (Figure 4). Phosphorylation of rccombinanant eIF2α, wl or 48A mutant, was found not lo be different in vitro or in insect cell extracts in the presence of purified HRI kinase or in hemin- and poly(IC)-lrcalcd rcliculocylc lysalcs (Figure 3, lanes 3 and 4; Figure 6B, lanes 2 and 3; Figure 8, lanes 3 and 4).

These results arc consistent with our previous observations *(38)* showing that the level of inhibition of cIF2B activity that occurs upon heat shock in CHO cells or by addition of purified HRI lo cell extracts can bc decreased in the presence of the 48A mutant cIF2a. In this latter study *(38)*, Ramaiah et al. speculated lhal the serinc 48 residue is required for maintaining a high-affinity interaction between phosphorylatcd cIF2a and cIF2B. Therefore, we analyzed the formation of eIF2α(P)−cIF2B complexes in hemin- and poly(IC)-treated reticulocyte lysalcs. Consistent with our earlier prediction *(38)*, the extent of formation of the 15S complex between cIF2a(P) and cIF2B was decreased significantly in the presence of insect cell extracts expressing 48A mulanl eIF2α, but not in the presence of insect cell extracts from cells expressing wl eII·2α (Figure 5, compare panels D and C).

We additionally observed that the partially purified recombinant subunit of eIF2α was not phosphorylaled by HRI as efficiently as the a-subunit present in the trimeric purified rcticulocytc cIF2 complex (Figure 6B). The level of phosphorylation of HRI kinase was also decreased under these conditions. It is not clear if this is due lo the lack of a

proper conformation in the subunit form due to the absence of the other two subunits as has been suggested previously (*30*) or due to the association of some kind of an inhibitory material with the partially purified recombinant form. However, the intensity of phosphorylation of eIF2a in the three-subunit eIF2 complex was reduced in the presence of either recombinant subunit eIF2a wt or the 48A mutant, and the phosphorylation was not cumulative (Figure 6B). It is likely, therefore, that the recombinant form of eIF2et may be contaminated with an eIF2α kinase inhibitor expressed in insect cells in response to viral infection as suggested recently *(42)*. This latter interpretation is consistent with the reduction in the level of eIF2a phosphorylation of purified eIF2 in vitro by HRI kinase in the presence of virus-infected cell extracts (Figure 7B). This appears to be due to a reduction in the level of HRI phosphorylation in the presence of virus-infected extracts. However, the results do not rule out the possibility that the reduction in the level of phosphorylation of the free eIF2a subunit in vitro by HRI may also be due to lack of proper conformation comparable to that in the trimeric eIF2 complex.

The phosphorylation of the 48A mutanl was not however significantly different from that of the wt eIF2α in the presence of reticulocyte lysates. Previously, the expressed free subunits were shown to be incorporated into trimeric eIF2 *(30)*. Therefore, our results support the possibility that the reduced ability of the 48A mutant to inhibit eIF2B activity is due to the inability of the eIF2 mutant complex to interact with the reticulocyte eIF2B as efficiently as the trimeric complex containing phosphorylated eIF2a wl,

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