REGULATION OF THE FUNCTION OF EUKARYOTIC INITIATION FACTOR 2 (eIF2): EFFECTS OF VANADIUM COMPOUNDS; CLONING AND CHARACTERIZATION OF THE BACULOVIRUS-EXPRESSED HUMAN eIF2 ALPHA SUBUNIT

Thesis submitted for the Degree of

DOCTOR OF PHILOSOPHY

By

THANUJA KRISHNAMOORTHY

Department of Biochemistry
School of Life Sciences
UNIVERSITY OF HYDERABAD
Hyderabad - 500046
INDIA
1997

Enrollment No. 93LBPH01

Department of Biochemistry
School of Life Sciences
UNIVERSITY OF HYDERABAD
Hyderabad - 500 046
INDIA

CERTIFICATE

This is to certify that the thesis entitled, **'Regulation of Eukaryotic Initiation Factor 2 (eIF2): Effects of Vanadium Compounds; Cloning and Characterization of the Baculovirus-expressed Human eIF2 Alpha subunit'** is based on the results of the work done by Ms **Thanuja Krishnamoorthy** for the degree of **Doctor of Philosophy** under my supervision. This work has not been submitted for the award of degree or diploma of any other University or Institution.

K. Subba Rao 24/11/97
Prof. K. Subba Rao
Head
Department of Biochemistry

K. V. A. Ramaiah
Supervisor
Department of Biochemistry

Prof. A. R. Reddy
Dean
School of Life Sciences

Department of Biochemistry
School of Life Sciences
UNIVERSITY OF HYDERABAD
Hyderabad - 500 046
INDIA

DECLARATION

I here by declare that the work presented in the present thesis entitled, **'Regulation of the Function of Eukaryotic Initiation Factor 2 (eIF2): Effect of Vanadium Compounds; Cloning and Characterization of the Baculovirus-expressed Human eIF2 Alpha Subunit',** is entirely original and was carried out by me under the guidance of **K. V. A. Ramaiah,** Ph. D., Department of Biochemistry, School of Life Sciences, University of Hyderabad, Hyderabad, INDIA. I also declare that this has not been submitted before for the award of degree or diploma of any other University or Institution.

Date: November 1997

K. Thanuja
Thanuja Krishnamoorthy
Candidate

Akeela Ramaiah 21 NOV 97
K. V. A. Ramaiah, Ph. D.
Supervisor
Department of Biochemistry

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Thanuja..

ABBREVIATIONS

A, Ala	:Alanine
AcNPV	:*Autographa californica* nuclear polyhedrosis virus
AP	:Alkaline phosphatase
Asp	:Aspartic acid
ATP	:Adenosine 5' triphosphate
BCIP	:5-bromo-4-chloro-3-indolyl 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-Sephadex	:Carboxy methyl sephadex
CK II	:Casein kinase II
CP	:Creatine phosphate
CPK	:Creatine phosphokinase
cpm	:Counts per minute
dCTP	:Deoxy cytosine triphosphate
DEAE	:Diethyl ribonucleic acid
DMSO	:Dimethyl sulphoxide
DNA	:Deoxyribonucleic acid
cDNA	:Complemtary DNA
dNTP	:Deoxy nucleotide triphosphate
DTT	:Dithiothreitol
EDTA	:Ethylene diamine tetra acetic acid
eEF	:Eukaryotic elongation factor
EGTA	:Ethylene-bis(p-aminoethyl ether) n, N, N', N'-tetraacetic acid

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	:Guanine nucleotide exchange factor of eIF2 or reversing factor
EPR	:Electron paramagnetic resonance
eRF	:Eukaryotic releasing factor or termination factor
FCS	:Fetal calf serum
Fig.	:Figure
GCN	:General control non-derepressible
GDP	:Guanosine 5' diphosphate
GSH	:Reduced gluthathione
GSSH	:Oxidized gluthathione
GTP	:Guanosine 5' triphosphate
h :	Hours
HRI	:Heme regulated inhibitor
HEPES	:N-[2-hydroxyethyl] piperzine-N'-[2-ethane-sulfonic acid]
Hsp	:Heat shock protein
I.U.	:International units
kDA	:Kilo daltons
1 :	Litre
LB	:Luria-Bertani
LGTA	:Low gelling temperature agarose
M	:Molar; Molecular weight markers
mg	:Milligram(s)
min	:Minutes
MOI	:Multiplicity of infection
NADPH	:Nicotinamide adenine dinucleotide phosphate, reduced
NBT	:Nitro blue tetrazonium

o/n	:Overnight
OV	:Occluded virus
PAGE	:Polyacrylamide
PDV	:Polyhedra derived virion
pfu	:Plaque forming units
p.i.	:Post infection
pmol	:Pmoles
PMSF	:Phenyl methyl sulfonyl fluoride
polh	:Polyhedrin
poly (IC)	:Synthetic polymer of Inosine and Cytosine
PQQ	:Pyrroline quinoline quinone
RNA	:Ribonucleic acid
dsRNA	:Double stranded RNA
Met.tRNAi	:Initiator transfer RNA
mRNA	:Messenger RNA
tRNA	:Transfer RNA
rpm	:Rotations per minute
RT	:Room temperature
S	:Svedberg
-SH	: Protein sulfhydryl groups
SDS	: Sodium dodecyl sulphate
Ser	:Serine
Sf9	:*Spodoptera frugiperda* (fall army worm) 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

V	:Volts
vadex	:Vanadyl D-glucose
vasc	:Vanadyl diascorbate

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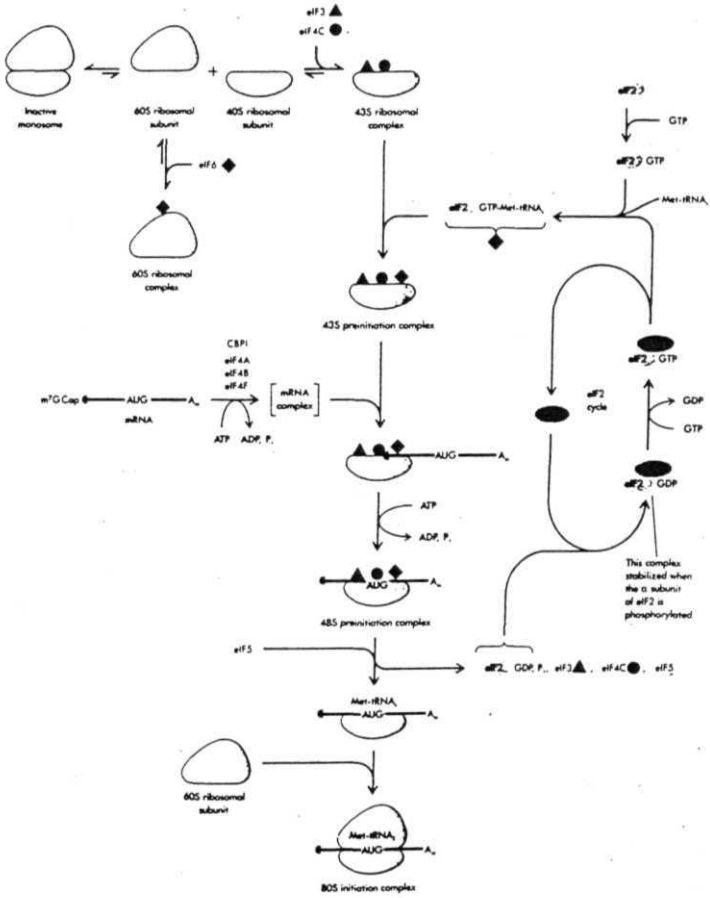
INTRODUCTION

Protein synthesis is a complex biochemical and molecular physiological process that occurs in all living cells without any discrimination and thus differs from other important physiological processes such as photosynthesis and nitrogen fixation. Proteins are polymers composed of a large number of nitrogen containing organic molecules called amino acids linearly linked together by peptide bonds. Twenty different amino acids, like the twenty six alphabets in English language, arranged in various sequence combinations are able to produce several thousands of proteins in living cells. Free amino acids are not used by the protein synthesis 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. The 'activated[1] amino acid is then accepted by a special ribonucleic acid (RNA) molecule called transfer RNA (tRNA) in the presence of amino acyl-tRNA synthetase, an enzyme that catalyzes the process.

The information that sets the sequence of amino acid residues in a polypeptide chain comes from messenger ribonucleic acid (mRNA) which in turn is synthesized based on the nucleotide sequence information available in a master molecule called deoxyribonucleic acid (DNA). The process of mRNA synthesis which is accomplished by the enzyme RNA polymerase in the presence of a DNA template is called transcription. Using mRNA as a program, and aminoacyl-tRNAs as energy rich substrates, the ribosome converts the genetic information from the nucleotide language of mRNA into the amino acid language of polypeptide chains. The decoding of information in mRNA to amino acids in proteins is called translation. Regulation of gene expression at transcriptional or translational or at both levels is a critical component in regulating cellular metabolism, orchestrating and maintaining the structural differences that exist in cells during development. Protein synthesis must be regulated so as not to trespass the bounds of available cellular energy resources (Rhoads, 1993). The view that translational controls are facts and not a fantasy any more (Hunt, 1980) can be substantiated from the mounting information available in this area and the periodic meetings held on this subject exclusively by the Cold Spring Harbor Laboratory, New York.

Fig. 1. Initiation of protein synthesis in eukaryotes (scheme from Watson et al., 1987).



The protein synthesis or translational machinery is as complex as transcriptional machinery. In addition to mRNA, ribosomes, aminoacyl-tRNA synthetases, tRNAs, the protein synthesis machinery requires the help of various protein factors, enzymes and nucleotides like ATP and GTP. The process is divided for convenience into three phases, namely, initiation, elongation and termination. The protein factors involved in each of these steps of eukaryotic translation are designated as eukaryotic initiation factors (eIFs), elongation factors (eEFs) and termination or releasing factors (eRFs) (Ochoa, 1983; Hershey, **1991; Watson *et al.*,** 1987).

Since this laboratory as well as the present thesis deals with the regulation of eukaryotic initiation factor 2 (eIF2) activity, the introduction here is focused on i) brief description about the overall protein synthesis ii) regulation of protein synthesis in general iii) current information available on the regulation of initiation factor 2 (eIF2) activity contributed by other laboratories and also by this laboratory. This thesis deals specifically with the regulation of protein synthesis and eIF2 activity of reticulocyte lysates by different vanadium compounds which differ in their oxidation states and the development of baculovirus expression system inorder to overproduce the wild type and a mutant eIF2 alpha subunit (the small subunit of eIF2) to further characterize the importance of eIF2 alpha phosphorylation in the regulation of eIF2 activity. Hence the introduction also highlights about the current information available on the effects of vanadium on various cellular activities including protein synthesis and also covers the necessity and advantages of the baculovirus expression system.

1. A BRIEF DESCRIPTION OF THE OVERALL PROTEIN SYNTHESIS OR PROTEIN SYNTHESIS IN A NUT SHELL.

1.1 *Initiation of protein synthesis:*

Initiation of protein synthesis is marked by the formation of 80S initiation complexes and the release of eIF2.GDP binary complex from initiated ribosomes. The initiator tRNA, $Met\text{-}tRNA_i$, carrying the initiator methionine amino acid residue is properly positioned on the 'start' site of mRNA. This complicated process is aided by

several initiation factors (eIFs) and can be divided into six substeps as mentioned below (reviewed in Hershey, 1991; Merrick, 1992; Rhoads, 1993).

1) Ribosomes are dissociated into their subunits at the end of protein synthesis and the subunits 40S and 60S remain separated because of their association with factors like eIF3, eIF4C (now called eIF1A) and eIF6.

2) Afterwards, eIF2 protein helps to join initiator tRNA (Met-tRNAi) to 40S ribosomes to form 43S preinitiation complex. This step requires GTP.

3) The 43S preinitiation complex (eIF2.GTP.Met-tRNA$_i$.40S ribosomes) joins the messenger RNA (mRNA) to form the 48S preinitiation complex. eIF4F, a trimeric complex, consisting of eIF4E (25 kDa, previously called eIF4α), eIF4A (22 kDa) and eIF4y (also called p220) and eIF4B proteins assist the joining of mRNA to 43S initiation complex.

4) The next step involves the recognition of 'start' site on mRNA by Met-tRNA$_i$. Prokaryotic mRNAs possess distinct structural features (Shine-Dalgarno sequence) in the mRNA for it to facilitate a direct hydrogen bonding interaction with the 16S rRNA of ribosomes preceding the AUG or 'start' codon. In contrast, eukaryotic mRNAs do not carry a comparable recognition sequence. The 43S complex 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' side 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 start AUG reduces the translation. Sine many mRNAs lack such purine residues before the start AUG, the mechanism by which the 'start codon' in mRNA is recognized 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 mRNA preceding the start site, so that the 48S complex can easily scan the 5' untranslated region and reach the start site (Kozak, 1992).

5) The joining of 60S ribosomal subunit to the 48S initiation complex to form 80S initiation complex occurs in the next step. This step requires the dissociation or release of

anti-association factors like eIF3, eIF4C and eIF6 from 40S and 60S subunits. The formation of 80S initiation complex is assisted by eIF5 protein which has an intrinsic GTPase activity. Hydrolysis of GTP in the ternary complex, eIF2.GTP.Met-tRNA$_i$ facilitates the formation of eIF2.GDP binary complex. Previously, it has been thought that eIF2.GDP binary complex is released before the joining of 60S subunit to 48S initiation complex (Hershey, 1991). The recent studies (Ramaiah *et al,* 1992), however, suggest that some amount of eIF2.GDP is translocated from the 48S initiation complex to the 60S subunit of 80S initiation complex. From here, the release of eIF2.GDP appears to be affected by another heteropentameric protein called eIF2B (Gross *et al,* 1985; Thomas *et al,* 1985; Pavitt *et al.,* 1997) as has been depicted in a model (Ramaiah *et al,* 1992).

6) eIF2B protein is required for eIF2.GDP to enter into another round of initiation. In the presence of physiological Mg^{2+} concentration, eIF2 has higher affinity for GDP than for GTP. However, GDP inhibits the joining of eIF2 to Met-tRNA$_i$. Hence, it is important that GDP in eIF2.GDP binary complex has to be replaced by GTP so that eIF2.GDP, released at the end of initiation, can now enter the initiation cycle. This guanine nucleotide exchange is catalyzed by the largest subunit (Epsilon subunit in mammalian systems or GCD6 in yeast) of the pentameric eIF2B protein (Fabian *et al,* 1997; Pavitt *et al,* 1997).

1.2 *Elongation of protein synthesis;*

After the formation of 80S initiation complex, the ribosome moves by three bases towards the 3' end of mRNA inorder to read the information present in the template. Depending on the nucleotide sequence information, corresponding amino acids are brought by the respective tRNAs to be properly positioned on mRNA. The process requires EF1 and EF2 (Elongation Factors 1 and 2) in eukaryotes. EF1 is equivalent in its function to EF.Tu and EF.Ts present in prokaryotes. Afterwards, peptide bond formation occurs between adjacent amino acids probably due to the presence of an enzyme like peptidyl transferase (Watson *et al.,* 1987; Spirin 1986). EF2 hydrolyzes GTP and catalyzes translocation of aminoacylated tRNAs on ribosomes with a concomitant movement of the message.

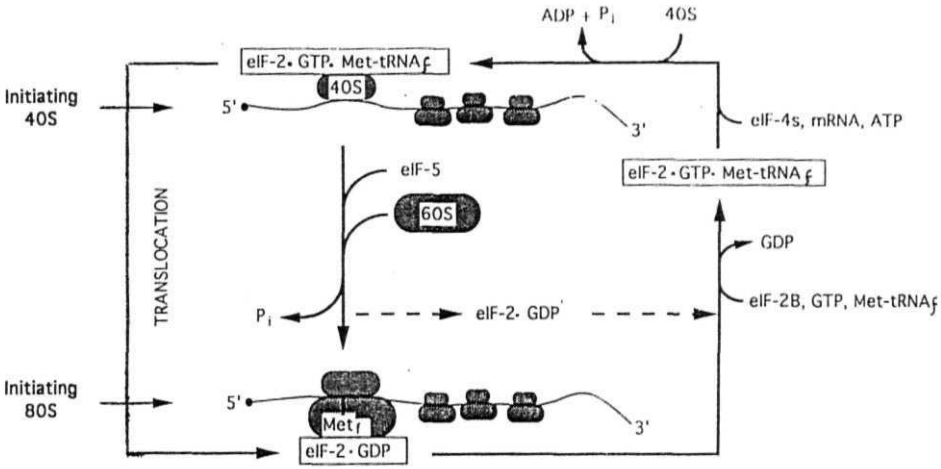
1.3 ***Termination:***

Termination of nascent polypeptide chain occurs when the 80S complex reaches the termination codon, like UAA, UAG or UGA. Termination of protein synthesis requires the assistance of a releasing factor, RF. While in prokaryotes, there are three RFs, in eukaryotes, it has been observed that a single RF recognizes all three termination codons.

2.0 REGULATION OF PROTEIN SYNTHESIS

Phosphorylation of protein factors is one of the principal means of regulating protein synthesis (Hershey, 1989). Enhanced phosphorylation of serine 51 residue in the small or alpha-subunit of eIF2 involved in the initiation step decreases protein synthesis globally as in reticulocytes (London *et al.*, 1987; Jackson, 1991), or specifically in yeast (Hinnebusch, 1988; 93). In contrast, phosphorylation of other initiation factors like eIF4E (serine-53 residue) and the cpsilon subunit of pentameric eIF2B protein increases the overall translational activity and the guanine nucleotide exchange activity of eIF2B protein respectively. Phosphorylation of threonine residues at 56 and 58 in the elongation factor 2 inhibits protein synthesis by causing a block in the elongation step of protein synthesis (Redpath and Proud, 1989; Price *et al.*, 1991a; Naresh Babu and Ramaiah, 1996). In addition, phosphorylation of the p-subunit of elongation factor 1(EF-1β), which can decrease the rate at which this factor catalyzes guanine nucleotide exchange on EF1a.GDP **(Janssen *et al,*** 1988), may provide a mechanism for regulating polypeptide chain elongation. Also, phosphorylation of S6, a protein component of 40S ribosomal subunit, is enhanced in cells and *in vitro* under a wide variety of stimuli which is correlated to enhanced translation (Blenis and Erickson, 1986). Aminoacyl tRNA synthetases which are required in coupling amino acids to the respective tRNAs have been shown to undergo phosphorylation by purified kinases *in vitro.* Some reports indicate that atleast valyl-tRNA synthetase may be regulated by phosphorylation

Fig. 2. Model for the recycling of eIF2 (Ramaiah et al., 1992).



(Venema *et al.,* 1991a, b). Accumulation of uncharged tRNAs due to alterations in the synthetase activity caused by phosphorylation can lead to changes in the translational rates. It is not known, however, if the kinase activity associated with tRNA synthetases is important to transfer directly or indirectly the signals generated by amino acid starvation (Clemens, 1990).

In addition to phosphorylation, other changes such as polyadenylation of mRNAs, changes in mRNA reading frame caused by slippage of tRNA derivative from one codon to an overlapping codon in the -1 or to +1 reading frame, structural features in 5' and 3' untranslated regions (UTRs) of mRNAs, intracellular pH, energy charges, calcium levels, redox potential, hormonal imbalance, sugar phosphates, small nuclear mRNAs, cytoskeletal interaction with polysomal mRNAs, glycosylated proteins can also alter translational efficiencies of mRNAs (Proud, 1992). Some of these agents are again shown to regulate the phosphorylation of protein factors involved in translation and thereby their activity.

Since the present thesis deals with regulation of protein synthesis that is mediated by changes in eIF2 activity, the current information available on the structure, function and regulation of eIF2 has been described here in detail.

2.1 *Regulation of protein synthesis by* ***initiation factor 2:***

This laboratory is working on the regulation of eIF2 activity in plant and animal systems (Janaki *et al.,* 1995; Naresh Babu and Ramaiah, 1996; Krishna *et al,* 1997; Thanuja *et al.,* 1997). eIF2 is a key factor involved in the initiation step of protein biosynthesis. It is a three subunit protein with different molecular masses. The subunits are designated as -a (38 kDa), -p (51 kDa) and -γ (52 kDa). This factor catalyzes GTP-dependent binding of $Met\text{-}tRNA_i$ to the 40S ribosomal subunit. At the end of initiation, hydrolysis of GTP bound to eIF2 occurs and the latter is released as eIF2.GDP binary complex (Hershey, 1991; Merrick, 1992). eIF2 has a 100-fold preference for GDP over GTP. Like many other 'G' binding proteins, when eIF2 is bound to GDP, it becomes inactive. It cannot join the initiator tRNA (Met-tRNAj) unless the GDP is exchanged for

GTP. The guanine nucleotide exchange on eIF2 is catalyzed by a rate-limiting pentameric protein called elF2B (Matts *et al,* 1983). eIF2 is also a phosphoprotein. Two of the three subunits, -a and -β, are known to be phosphorylated by cyclic AMP-independent kinases. Hence, the eIF2 regulation is dependent on eIF2 kinases, phosphatases and also on eIF2B protein.

2.1.1 *Guanine nucleotide binding domains in eIF2:*

The primary function of eIF2 is to bind initiator tRNA in a GTP-dependent manner. Some of the earlier biochemical studies using denatured eIF2 or affinity labelling of eIF2 with GTP derivatives indicated controversial results. An earlier report suggested that a-subunit of eIF2 was responsible for binding guanine nucleotide (Barrieux and Rosenfeld, 1997). Another report implicated β- and y-subunits (Anthony *et al,* 1990). When the cDNAs encoding all the three subunits of eIF2 (a, β and y) were cloned and sequenced (Ernst *et al,* 1987; Pathak *et al.,* 1988a; Gaspar *et al,* 1994), the consensus elements for GTP binding domains (DXXG and NKXD) were found in both the β and y subunits, reinforcing the possibility that these two subunits might be involved in guanine nucleotide binding. However, the recent studies involving mutations in asparagine residue in the NKXD consensus element of both subunits and the overexpression of these mutants to determine the translational efficiency of a reporter mRNA for dihydrofolate reductase in COS-1 cells reveal that mutations in the y-subunit can repress protein synthesis severely, whereas, those in the P-subunit are mildly inhibitory (Naranda *et al,* 1995). These findings support the view that GTP binds exclusively to the γ-subunit and contradicts the earlier view that GTP binding is 'shared' by β- and y-subunits.

These findings are supported by the following facts a) that yeast eIF2p lacks completely the NKXD consensus element, whereas, all the three consensus elements are present in the yeast eIF2y (Donahue *et al.,* 1988; Hannig *et al.,* 1993) and b) two of the three GTP binding domains in human eIF2 are greater on the p-subunit but these are separated by a far greater distance than is normally found in GTP-binding proteins (Pathak *et al,* 1988a).

However, structural analysis of eIF2 complex are required to provide an explanation to understand the differences in results obtained by affinity cross-linking with GTP analogues and the translational defects observed due to mutations in the GTP binding domain (NKXD sequence) of the γ-subunit.

2.1.2 *eIF2 interaction with mRNA:*

Binding and translation studies support the concept that eIF2 interacts specifically with mRNA during protein synthesis and that this interaction is important for translational control. Kaempfer and his group (1974, '79, '81, '82) have shown that in Satellite Tobacco Necrosis Virus RNA or Mengovirus RNA, eIF2 recognizes and protects specific nucleotide sequences that overlap the RNA binding sites. Their findings suggested that once bound to the 40S ribosomal subunit, eIF2 may interact with mRNA directly and thus guide the 40S subunit to its binding site in mRNA. Consistent with this idea, the recent results by Dasso et al. (1990) indicate that eIF2 promotes selection of the 5'-proximal translation initiation site by ribosomes. There is genetic evidence in yeast that elF2 recognizes the AUG initiation codon,, mutations permitting the utilization of an UUG initiation codon map into the a- and P-subunits of eIF2 (Cigan *et al,* 1989; Di Segni *et al,* 1979) with those in eIF2α mapping to a zinc finger motif that may function in mRNA recognition (Cigan *et al,* 1989).

The importance of the interaction of mRNA and eIF2 for translation control is supported particularly well by the close correlation between the affinity of an mRNA species for eIF2 and its ability to compete in translation (Kaempfer, 1984). Thus rabbit β-globin mRNA binds to eIF2 with higher efficiency than α-globin mRNA and competes more effectively during translation (DiSegni *et al,* 1979). Human β-globin mRNA out-competes the fetal γ-globin mRNA species both in translation and in binding to eIF2 (Marsh *et al,* 1990). Translational competition between other mRNAs could like-wise be alleviated by excess eIF2 (Kaempfer and Konijn, 1983; Scheper *et al,* 1991).

It is interesting to know how eIF2 binding to mRNA and Met-tRNA$_i$ / GTP are regulated. It appears that these are mutually exclusive in their binding to eIF2 (Kaempfer

et al, 1979; Rosen ***et*** *al,* 1981; Chaudhuri *et* ***al,*** 1981), yet their recognition involves distant epitopes in the protein (Harary and Kaempfer, 1990). eIF2 also binds with high affinity to ATP, yet does not hydrolyze it (Gonsky *et al,* 1990). Interaction of ATP with eIF2 in ternary complex with $Met\text{-}tRNA_f$ and GTP results in displacement of $Met\text{-}tRNA_f$, while promoting the binding of mRNA (Gonsky *et al,* 1990). ATP may than regulate the dual binding activities of eIF2 during translation switching from a $Met\text{-}tRNA_f$ binding mode into an mRNA binding mode. The eIF2P subunit, but not -a or -γ harbors binding sites for mRNA as well as for ATP (Gonsky *et al,* 1992). During initiation of protein synthesis, the mammalian eIF2p subunit thus may interact with three ligands important for translational control: $Met\text{-}tRNA_f$, mRNA and ATP. Some of the RNA structure that is recognized and bound by eIF2, while containing the AUG initiation codon, is generated through long-range intramolecular interactions between distant parts of the mRNA molecule (Kaempfer ***et*** *al,* unpublished observations).

2.1.3 *Phosphorylation* ***of eIF2β:***

Some of the cyclic AMP-independent kinases have been shown to phosphorylate the a- and p-subunits of eIF2 both ***in*** *vitro* and *in vivo*. Casein kinase II (CK II) and protein kinase C (PKC) are capable of phosphorylating eIF2p. While CK II phosphorylated Ser-2 and Ser-67, the rat brain PKC phosphorylates Ser-13 atleast *in vitro* (Clark *et al,* 1989; Welsh *et al,* 1994). cAMP-dependent protein kinase also has the ability to phosphorylate eIF2P *in vitro* on Ser-218 (Welsh *et al,* 1994). The physiological significance of the P-subunit phosphorylation of eIF2 is not however understood. A recent report (Singh *et al,* 1994) suggests that a modest increase in the GDP binding to eIF2 occurs when the p-subunit of eIF2 is phosphorylated.

2.1.4 *Physiological conditions* ***which promote eIF2α phosphorylation:***

It has been shown that several physiological stresses such as physical, chemical and biological agents which inhibit protein synthesis can stimulate eIF2α phosphorylation. These are hemin deprivation (reviewed in London *et al,* 1987),

chloroquine treatment (Surolia and Padmanabhan, 1991), nutrient deprivation (reviewed in Pain, 1994; Hinnebusch, 1994), heat shock (reviewed in Panniers, 1994), viral infection or double stranded RNA treatment (Farrell *et al,* 1977; Reichel *et al,* 1985), serum deprivation (Duncan and Hershey, 1985; Rowlands *et al,* 1988b), ethanol treatment (reviewed in Jackson, 1991), heavy metal ions (Matts *et al,* 1991), oxidized glutathione (Kan *et al,* 1988), absence of protein thiol reducing systems caused by absence of either a NADPH generating system, thioredoxin reductase, hexose sugar phosphates (reviewed in Jackson, 1991), redox cofactors like pyrroloquinoline quinone (Ramaiah *et al,* 1997), treatment with N-ethylmaleimide (Chen *et al,* 1989; Janaki *et al,* 1995), O-iodosobenzoate (Gross
and Rabinowitz, 1972) and calcium deprivation (Kimball and Jefferson, 1990; Prostko *et al,* 1992; Srivastava *et al.,* 1995).

2.1.5 *Importance of* ***eIF2α phosphorylation:***

Down regulation of protein synthesis caused by eIF2α phosphorylation apparently provides a means for conserving resources and limiting cell division under adverse growth conditions. Interestingly phosphorylation of eIF2a in yeast, unlike in reticulocyte lysates, does not decrease protein synthesis globally. Phosphorylation of yeast eIF2a that occurs under ami no acid starvation due to increased GCN2 kinase activity, stimulates the synthesis of a transcription factor called GCN4. The latter in turn activates transcription of atleast forty different genes encoding amino acid biosynthetic enzymes; therefore induction of GCN4 enables the cell to alleviate the nutrient starvation conditions that induce eIF2a phosphorylation (Hinnebusch, 1994). In addition it has been shown that eIF2α kinases like double stranded RNA induced inhibitor (PKR or dsI) which gets activated during viral infection can also phosphorylate the transcriptional regulatory factor I-κB, an inhibitor of members of the NF-κB/C-rel family. Activation of NF-κB stimulates transcription of a wide range of genes associated with growth regulation, differentiation, immune and inflammatory responses (Kumar *et al,* 1994). These studies emphasize the importance of eIF2a kinases in the regulation of transcription of certain

genes which are important in cell growth and other functions. Consistent with this idea, expression of a mutant PKR or mutant eIF2α, which cannot be phosphorylated has been shown to cause transformation in NIH 3T3 cells (Donze *et al,* 1995) and substantiates the earlier result that PKR may act as a tumor suppressor gene product (Koromilas *et al,* 1992; Clemens, 1992; Lengyel, 1993; Meurs *et al,* 1993).

2.2 ***eIF2α****kinases,* ***their activation and regulation:***

Although several physiological stimuli, as mentioned above, can activate eIF2a kinases which can phosphorylate the serine-51 residue in eIF2α and inhibit protein synthesis, three of these eIF2a kinases have been well characterized (Samuel, 1993; Chen and London, 1995; Wek, 1994).

i) Heme-regulated eIF2α kinases called as HRI or HCR (Chen and London, 1995). The protein has 626 amino acids and the native reticulocyte kinase protein migrates as a 90 kDa protein on a 10 % SDS-PAGE. The protein is shown activated in reticulocytes and their lysates during heme-deficiency (London *et* ***al,*** 1987; Jackson, 1991). Heme-deficiency inhibits protein synthesis, whereas addition of heme maintains protein synthesis linearly for 30-60 min in reticulocyte lysates (Hunt *et al,* 1972). Also delayed addition of hemin to inhibited heme-deficient lysates, restores protein synthesis. In the absence of heme, HRI kinase is activated, i.e., its ability for autophosphorylation and to phosphorylate the substrate, eIF2α, is enhanced. In the presence of hemin, HRI is inactivated. Based on several physical and chemical studies, it has been concluded that HRI exists as a dimer. Sulfhydryl reactive agents such as dithiothreitol, oxidized glutathione, N-ethylmaleimide, toxic heavy metals and absence of a functional thioredoxin/reductase system have been found to enhance eIF2a phosphorylation presumably due to the activation of HRI (Jackson, 1991). Presence of hemin is shown to promote disulfide bond fromation in HRI protein (Chen *et al,* 1989). Recently, it has been shown that a novel redox factor such as pyrroloquinoline quinone (PQQ) is shown to stimulate HRI activity in hemin-supplemented lysates and thereby enhances lysate eIF2α phosphorylation (Ramaiah *et al,* 1997). HRI inactivation may be occuring due **to**

the transfer of reducing equivalents to HRI by reduced PQQ. These observations are consistent with the suggestion that HRI is active in the monomeric form, [HRI-$(SH)_2$], but becomes inactive when dimer formation occurs (HRI -S-S-HRI) and that -SH groups in HRI protein play an important role in its activation. Recent studies suggest that heat shock proteins such as hsp90 or hsp70 also interact with HRI and regulate its activity (Mendez and de Haro, 1994; Matts *et al,* 1992; Rose *et al,* 1989; Gross *et al,* 1994) probably through the modification of -SH groups.

A recent study (unpublished observations of Chen and Ramaiah) suggests that purified tyrosine kinases such as src and lck phosphorylate tyrosine residues in HRI and down regulate its activity. This will be interesting since tyrosine kinases are involved in signal transduction pathways. This is perhaps a second physiological agent (the first one is heme) which is shown to directly decrease the HRI autophosphorylation and thereby the ability of HRI to phosphorylate eIF2a. In addition to these studies, a glycosylated protein, p67, is shown to inhibit the ability of autophosphorylated HRI to phosphorylate eIF2a, thereby suggesting that it some how protects eIF2a phosphorylation but not the activation of kinase (Datta *et al,* 1989; Gupta, 1993). It is suggested that during heme deficiency, p67, is deglycosylated and loses its ability to inhibit the eIF2a phosphorylation catalysed by HRI. However, it is not known if addition of hemin facilitates glycosylation of p67 in inhibited heme-deficient lysates treated with the delayed addition of hemin. This is because delayed addition of hemin restores protein synthesis in inhibited heme-deficient lysates. Further recent evidence indicates that p67 is a methionine peptidase (Li and Chang, 1996) and requires cobalt for its activity. The physiological significance of p67, however, is still not clear.

ii) Double stranded RNA induced inhibitor or regulated protein kinase (dsI or PKR) is another eIF2a kinase protein with 551 amino acids and an approximate molecular mass of 62 kDa has been well characterized (Meurs *et al,* 1990; Patel and Sen, 1992a). The protein is present in low levels in most mammalian cells and the expression of it is induced several fold by interferon treatment. Unlike HRI, which is mostly found in the non-ribosome fraction (Naresh Babu and Ramaiah, 1996), PKR is found associated with

ribosomes of the cytoplasm and also nucleolus (Levin *et al,* 1981; Jimenez-Gracia *et al,* 1993). The significance of nuclear PKR, which is not induced by interferons (Jeffrey *et al,* 1995), is not known. PKR binds double stranded RNA (dsRNA) with high affinity and low concentrations of viral double stranded RNA or synthetic dsRNA like poly (IC) (ng/ml) can cause activation of PKR in rabbit reticulocyte lysates and enhance eIF2a phosphorylation (London *et al,* 1987; Jackson, 1991). In addition to dsRNA, PKR is activated by polyanions such as heparin (Patel *et al,* 1994).

The mechanism by which dsRNA binding turns on PKR protein so as to act as an eIF2a kinase is not clear. It is likely that one PKR molecule can phosphorylate its neighbour, while both are bound to a single stretch of dsRNA. Once PKR is phosphorylated, its protein kinase activity becomes independent of dsRNA. High concentrations of dsRNA prevent PKR activation (Manche *et al,* 1992). This may be because the formation of monomeric (PKR-dsRNA) complex, rather than dimer formation (PKR-dsRNA-PKR) under those conditions. While homodimerization of HRI has been postulated to decrease its activation (Chen and London, 1995), the PKR monomers, formed probably in the presence of high concentrations of dsRNA, appear to down regulate the activation of PKR kinase (Clemens *et al,* 1996).

Interest in studying PKR activity and regulation has been increasing since a) interferon stimulates its expression and b) there is an exciting prospect that it may act as a tumor suppressor gene product (Clemens, 1992; Koromilas *et al,* 1992).

iii) A third protein kinase, GCN2 has also been identified which gets activated during amino acid starvation in yeast (Dever *et al,* 1993). GCN2 has 1590 amino acids and has an apparent molecular mass of 182 kDa. Like PKR, GCN2 is associated with ribosomes. Most likely GCN2 is activated by uncharged tRNA (Ramirez *et al,* 1992). Because of its similarities to histidyl tRNA synthetase, it may be involved in binding RNA like PKR. Since HRI and PKR can substitute functionally for GCN2 kinase in yeast, it is consistent with the information that there are several sequences in the cDNAs of these kinases which are homologous to each other. In addition, each of these kinases carry specific sequences such as the heme binding region in HRI, dsRNA binding region in PKR or

sequence related to aminoacyl tRNA synthetases as in GCN2 which may be involved in their regulation (Samuel, 1993; Wek, 1994; Chen and London, 1995;).

In addition to these kinases, recently another eIF2a kinase has been detected in Drosophila (Santoyo ***et al,*** 1997). Since this kinase resembles to GCN2 protein kinase, it has been named as DGCN2. This is consistent with the idea that insect cells (*Spodoptera frugiperda*) also regulate protein synthesis by eIF2a phosphorylation mechanism (Chefalo *et al.*, 1994).

Mammalian kinases have also been shown to phosphorylate plant eIF2a (Janaki *et al,* 1996; Krishna *et al,* 1997). A recent report indicates that a purified eIF2a kinase from plants can inhibit wheat germ translation probably through eIF2a phosphorylation (Langland ***et al,*** 1996). However, this kinase requires high concentrations of dsRNA (mg/ml) for its activation and appears to be unlike mammalian PKR that requires low concentrations of dsRNA (ng/ml).

3.0 MECHANISM OF PROTEIN SYNTHESIS INHIBITION BY eIF2α PHOSPHORYLATION

3.1 *Identification of eIF2B and its inhibition by eIF2α phosphorylation:*

It is now generally agreed that protein synthesis inhibition can be more than 90 % inhibited when only 35-40 % of eIF2a is phosphorylated (Leroux and London, 1982). Addition of purified eIF2 could rescue protein synthesis activity *in vitro* in translating lysates and this rescue was found to be less effective purer the preparation of eIF2. These findings suggested that there must be yet another rate-limiting factor which regulates eIF2 activity (London ***et al,*** 1987; Jackson, 1991). A protein factor was purified from the post-ribosomal supernatant by several laboratories during the period between 1979-'83, which was able to overcome the protein synthesis inhibition caused by heme-deficiency in reticulocyte lysates. This factor enjoyed several names in the literature such as anti-HRI (Amesz *et al.,* 1979), Co-eIF2C (Das *et al.,* 1979), reversing factor or RF (Siekierka *et al,* 1981., Grace ***et al.,*** 1982; Matts *et al,* 1983); guanine nucleotide exchange factor or

GEF (Panniers and Henshaw, 1983) and eIF2B (Konieczny and Safer, 1983). It is now referred as eIF2B. This protein is a pentamer with five different subunits. These are -a (34 kDa), -p (40 kDa), -γ (55 kDa), -5 (65 kDa) and -e (82 kDa). Quite frequently, the eIF2B protein comigrates with eIF2 during purification procedures (Reichel *et al,* 1985; Matts *et al.,* 1983).

Purified eIF2B protein rescues protein synthesis inhibition in heme-deficient lysates catalytically (Matts *et al.,* 1983). eIF2B protein has been shown to exchange the GDP bound to eIF2 for GTP (Siekierka ***et*** *al,* 1981). This is very critical, since, eIF2 binds GDP with much higher affinity than GTP in physiological conditions (in the presence of Mg^{2+}) and GDP inhibits the joining of eIF2 with Met-tRNA$_i$. Phosphorylated eIF2a has been shown to inhibit the ability of eIF2B to exchange guanine nucleotides on eIF2 ***in*** *vitro* (Clemens *et* ***al,*** 1982) and also in translating lysates (Matts and London, 1984). Further studies revealed that phosphorylated eIF2α forms a tight complex with eIF2B which migrates as a 15S complex on 10-30 % sucrose gradients, [eIF2(aP).eIF2B]. In this complex, eIF2B is non-functional (Thomas *et al,* 1985; Gross *et* ***al,*** 1985). This is because eIF2(αP).GDP is a competitive inhibitor, competing with eIF2.GDP to bind eIF2B. Since the magnitude of the differences in affinities of eIF2.GDP or eIF2(αP).GDP for eIF2B is so great, that, this type of competitive binding effectively sequesters all the available eIF2B with phosphorylated eIF2a (Rowlands *et* ***al,*** 1988). This is consistent with the idea that, a) eIF2B levels were found to be 15-20 % of total eIF2. Hence, 15-20 % phosphorylation of eIF2a decreases all the available activity **of eIF2B** and b) in Ehrlich ascites cell, higher levels of eIF2a phosphorylation (40-50 %) is required for inhibition of protein synthesis to occur. This is presumably due to higher levels of eIF2B activity in these cells (Rowlands *et al.,* 1988).

3.2 ***Dephosphorylation*** *of eJF2(aP) of* ***the 15S*** *complex* ***liberates functional eIF2B*** *activity:*

In the inhibited heme-deficient lysates, eIF2(aP) is phosphorylated and the latter sequesters eIF2B into a complex, [eIF2(aP).eIF2B], in which eIF2B becomes non-

functional. It has been shown that addition of hemin or MgGTP to inhibited lysates reduces eIF2α phosphorylation, rescues eIF2B activity and protein synthesis (Matts *et al,* 1986). Under these conditions, it is presumed that lysate eIF2(aP) is dephosphorylated by an endogenous phosphatase. Also, treatment of the eIF2(αP).eIF2B complex *in vitro* with alkaline phosphatase restored eIF2B activity (Thomas *et al,* 1984). Recent studies (Naresh Babu and Ramaiah, 1996) have shown, further, that the recovery of eIF2B activity that is observed by the delayed addition of hemin to inhibited heme-deficient lysates is reduced by type 1 phosphatase inhibitors such as heat stable inhibitor II and high concentrations of okadaic acid. These findings suggest eIF2a phosphorylation is the cause for inhibition of eIF2B activity and the involvement of a type 1 phosphatase in dephosphorylating eIF2(aP) in physiological conditions and in the restoration of eIF2B activity.

3.3 *Overexpression of wild type and* ***mutants of eIF2α and eIF2B subunits:***

To determine, further, the importance of eIF2a phosphorylation in growth and development, in localizing the defects caused by eIF2a phosphorylation and in determining the interaction between trimeric eIF2 and pentameric eIF2B proteins, wild type and mutants of eIF2α and the five subunits of eIF2B and different combinations of the subunits are overexpressed by various researchers. Generation of site-specific mutants of eIF2α like the 48A or 51A mutants, in which the serine residues in eIF2α have been replaced by **alanine,** suggest that 51 serine residue is the only phosphorylation site in eIF2cx **(Pathak *et al,*** 1988; Kaufman *et al,* 1989; Choi *et al,* 1992) and thus eliminating the possibility that serine-48 was also a potential site of phosphorylation as had been thought previously (Wettenhall *et al,* 1986). 51A mutant cannot be phosphorylated, whereas, 48A mutant of eIF2α can be phosphorylated on serine 51 residue. Expression of these mutant subunits of eIF2a, but not wild type, protects the inhibition of translation caused by adenoviral mRNAs, plasmid derived mRNAs, heat shock or calcium sequestration (Davies ***et*** *al,* 1989; Kaufman *et al,* 1989; Choi ***et*** *al,* 1992; Murtha-Riel *et al,* 1993; **Srivastava** *et al,* 1995). These findings suggest clearly that mutants of

eIF2a, 48 or 51A, can be used to detect translation inhibition caused by eIF2a phosphorylation. This is possible, because, the expressed subunit of eIF2a (wt or mutant) readily exchanges into the native trimeric eIF2 complexes (Choi *et al,* 1992) and the native complex can then behave like a mutant eIF2.

In a recent study (Ramaiah *et al,* 1994), it has been shown that inhibition of eIF2B activity via phosphorylation of eIF2α either by addition of purified reticulocyte HRI to Chinese hamster ovary (CHO) cell extracts, or by CHO cell eIF2α kinase activated by heat shock, is reduced by the overexpression of 48A or 51A mutants of eIF2a, but not the wild type eIF2a. These findings point out that 48A mutation, although does not affect eIF2α phosphorylation, may affect the interaction between eIF2(αP) and eIF2B. In addition, the above study (Ramaiah *et al,* 1994) raises a possibility that 48A mutation in eIF2α can lead to eIF2B nucleotide exchange on eIF2 as a secondary effect. Also genetic studies in yeast indicated that mutations within 40 amino acids of the phosphorylation site can overcome the inhibitory effects caused by eIF2a phosphorylation (Vazquez de Aldana *et al,* 1993).

In addition to localizing the defects caused by eIF2α phosphorylation and in understanding the mechanism of interaction between eIF2(αP) and eIF2B, the availability of these mutants also helped to express recombinant heme-regulated rabbit eIF2a kinase (HRI) in insect cells (Chefalo *et al,* 1994). In the absence of any mutant eIF2α, the expression of recombinant HRI is poor, since, the insect cell protein synthesis is diminished due to eIF2α phosphorylation. Overexpression of the 51A mutant eIF2α, which cannot be phosphorylated, helped to bypass the protein synthesis inhibition caused by the expression of recombinant mammalian eIF2α kinase. Mutants of eIF2a have also been used to determine the mechanism of regulation of protein synthesis by K3L, the vaccinia viral gene product which is shown to potentiate translation by inhibiting PKR, the double stranded RNA-dependent eIF2a kinase (Davies *et al,* 1992). Abrogation of eIF2a phosphorylation achieved by expressing a mutant form of eIF2a, which cannot be phosphorylated on serine 51, has been shown to cause malignant transformation of NIH 3T3 cells (Donze *et al.,* 1995).

Also, the availability of the cDNAs for various subunits of mammalian (-a, -β, -y, -5 and -e) and yeast eIF2B (called GCN3, GCD7, GCDl, GCD2 and GCD6 equivalent to a-E subunits of mammalian eIF2B respectively) facilitated their expression recently in insect cells using baculovirus expression system (Fabian *et ah,* 1997) and in yeast system (Pavitt *et* ***ai,*** 1997) respectively. The findings of these studies indicate that the epsilon subunit of yeast eIF2B carries the ability to exchange guanine nucleotides on eIF2 *in vitro* and it forms a catalytic complex in yeast with GCDl subunit. The latter, enhances the catalytic ability of GCD6 atleast 8-10 fold *in vitro* and the subcomplex is insensitive to inhibition by eIF2(αP) *in vitro.* Further, using histidine-tagged eIF2, it has been shown that an eIF2B complex of the GCD3, GCD7 and GCDl (equivalent to a, β and y) binds to eIF2 and has a higher affinity for eIF2(αP). This subcomplex lacks nucleotide exchange activity. Based on these findings, it is suggested that the five subunits in yeast eIF2B form two subcomplexes. The GCD6 and GCDl form a catalytic complex and may be interacting **with** the GDP bound to the y-subunit of eIF2. In contrast, GCN3, GCD7 and GCD2 form a regulatory complex that interacts with eIF2(aP). Further, the four subunit yeast cIF2B lacking GCN3 or five subunit eIF2B with mutations in GCN3 or GCD7 subunits catalyzed GDP exchange using eIF2(αP) or eIF2.GDP as substrate.

These studies with the overexpressed subunits of eIF2 and eIF2B indicate that eIF2a phosphorylation on serine 51 residue is the cause for inhibition in eIF2B activity. eIF2(aP).GDP is an inhibitor for eIF2B activity. Phosphorylated eIF2a has higher affinity **for eIF2B** than unphosphorylated eIF2. Mutations in eIF2a may weaken the interaction between eIF2(αP) and eIF2B. In contrast, regulatory mutations in eIF2B does not weaken the interaction between eIF2(αP) and eIF2B and the mutated eIF2B can exchange guanine nucleotides both from eIF2.GDP and eIF2(αP).GDP (Pavitt *et ah,* 1997).

3.4 *Physiological site of* ***eIF2αphosphorylation and eIF2 recycling:***

Anaysis of eIF2 phosphorylation in translating reticulocyte lysates indicated that phosphorylation of eIF2a is enhanced in cycloheximide-treated heme-deficient

reticulocyte lysates in which polysomes are maintained (Ramaiah *et al,* 1992). Immunoblot analysis of eIF2 distribution in sucrose gradients of actively protein synthesizing lysates indicates that eIF2 is distributed at low levels throughout the polysomes profiles (Ramaiah *et al,* 1992). Also, the heme-regulated eIF2a kinases is found at low levels on ribosomes, although majority of it is present in the non-ribosome fraction (Naresh Babu, Ph. D. thesis). However, a major fraction of other eIF2a kinases like PKR or dsl and GCN2 are attached to ribosomes (Clemens *et al,* 1997; Ramirez *et al.,* 1991). In addition, Clemens and co-workers (1987) have shown that a diminished tRNA synthetase activity is associated with increased phophorylation of eIF2a but with no change in eIF2α kinase or phosphatase activity. These findings suggest that eIF2.GDP formed at the end of initiation is somehow translocated from the 48S initiation complex to the 60S subunits of SOS initiation complexes and the eIF2 of 60S subunits of 80S initiation complex is the substrate for eIF2α phosphorylation in physiological conditions **(Ramaiah *et al.,* 1992).**

Earlier studies have indicated that a) eIF2 and eIF2B comigrate together during several steps of purification (Matts *et al,* 1983; Reichel *et al,* 1985) b) that eIF2a in the eIF2.eIF2B complex is not readily phosphorylated *in vitro* by HRI (Matts et al, 1983; Grace *et al.,* 1982), and c) that the polysomal bound eIF2.GDP can be released in the presence of **puriifed** eIF2B (Thomas *et al,* 1985; Gross *et al,* 1985). It has been suggested that elF2.GDP present on 80S monosomes and polysomes is not complexed with eIF2B and is in the binary complex, eIF2.GDP, which is a good substrate for eIF2a kinases. This interpretation is consistent with limited availablility of eIF2B even in normal reticulocyte lysates. These findings and interpretation raise the possibility that eIF2B protein, which exchanges guanine nucleotides on eIF2 is also involved in releasing eIF2.GDP from ribosomes. This second function of eIF2B, that has been invoked here, is found consistent **with** some recent results in yeast (Pavitt *et al,* 1997). These authors have found **that** GCD1/GCD6 subcomplex of yeast eIF2B has high-level of nucleotide exchange activity in cell extracts, but is not sufficient to provide the essential function of eIF2B *in vivo,* which is interesting. Overexpression of the four essential subunits of

eIF2B (without GCN3) was sufficient to overcome the growth inhibitory effects of eIF2α ftyperphosphorylation (a sensitive, but indirect measurement of eIF2B activity), whereas co-overexpression of just GCD1 and GCD6 had no such effect. In cell extracts, however, both complexes (4 subunits of eIF2B and GCD1/GCD6 showed identical high level eIF2B activity **that** was insensitive to eIF2 phosphorylation. Based on these and other findings, it is suggested that GCD1/GCD6 subcomplex is competent for nucleotide exchange on free eIF2.GDP *in vitro*, GCD2 and GCD7 (other subunits of yeast eIF2B) are additionally required ***in*** *vivo* to localize eIF2 bound to the ribosome or release eIF2.GDP from ribosome subunits.

3.5 *Regulatory mechanisms which affect the functioning* ***of eIF2B:***

eIF2B **is** guanine nucleotide exchange protein that specifically exchanges guanine nucleotides from cIF2 protein and is critically required for eIF2 recycling. As mentioned above, phosphorylation of one of the subunits of the substrate (that is eIF2a) impairs eIF2B activity. In addition, it has been shown that phopshorylation of one of the subunits of mammalina elF2B (82 kDa, or epsilon subunit) by CK I and II, stimulates its ability to exchange guanine nucleotides on eIF2 (Dholakia *et ai,* 1988; Singh *et ai,* 1994; Aroor *et al.,* 1994; Singh *et* ***ai,*** 1996). In contrast, phosphorylation of the same subunit of mammalian eIF2B by glycogen synthase kinase-3 (Welsh *et ai,* 1996) decreases its guanine nucleotide exchange activity. Further, eIF2B activity is also regulated by the redox state of **pyridine** dinucleotides (NAD/NADP) (Dholakia *et ai,* 1986; Akkaraju, 1991; Oldficld and Proud, 1992), by changes in adenylate energy (Kimball *et ai,* 1994; Kimball and Jefferson, 1995), by polyamines (Gross *et al.,* 1988; Aroor *et al.,* 1995) and by a novel redox factor like pyrroloquninoline qunine (Ramaiah *et ai,* 1997).

Inorder to further understand the importance of these various subunits of eIF2B in the regulation **of eIF2B** and eIF2 activities, the wild type and mutant subunits of these factors are overproduced in mammalian (Kaufman *et ai,* 1989; Choi *et ai,* 1992; Naranda *et* ***ai,***] 995), yeast (Pavitt *et ai,* 1997) and in insect cells (Fabian *et ai,* **1997).** Here, for the first time, we could overexpress human eIF2a, one of the subunits of eIF2

and a mutant in which serine-48 residue in eIF2α is replaced by alanine (48A mutant) which can still be phosphorylated on its 51 serine residue in insect cells using baculovirus expression system. These stuides are carried out inorder to understand the importance of eIF2α phosphorylation in the regulation of eIF2B activity. Our findings support the hypothesis (Ramaiah *et al.,* 1994) that the 48A mutant of eIF2a reduces the affinity for eIF2B when eIF2α is phosphorylated.

4.0 BACULOVIRUSES

The unique features of baculoviruses have made them the most powerful and versatile eukaryotic expression vectors whose immense potential has become truly legion. Numerous genes from a variety of sources ranging from eukaryotes, fungi, plants, bacteria and viruses have been cloned and expressed using the baculovirus expression vector system **(BEVS),** thus making it the most popular and efficient system for expressing heterologous genes.

Baculoviruses [family Baculoviridae (Mathews, 1982)] are a diverse group of large double stranded (between 80 Kbp and 200 Kbp) and circular DNA viruses that infect insects as their natural hosts. The most extensively studied baculovirus strain is the *Autographa californica* Nuclear Polyhedrosis Virus (AcNPV) whose entire genome has been fully sequenced (O'Reilly ***et*** *al,* 1992; 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 army worm *(Spodoptera frugiperda)* and the cabbage looper *(Trichoplusia ni).* Though most of the expression vectors harness AcNPV infection of *Spodoptera frugiperda* cells, heterologous proteins are also produced in silkworm larvae (*Bombyx mori,* Bm) 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 the viral genome.

4.1 *The infection process:*

Baculoviridae is further classified into two subfamilies, the Eubacteriovirinae (occluded baculoviruses) that infect the larvae of lepidoptera, coleoptera, diptera, etc. and the Nudibaculovirinae (non-occluded baculoviruses). Baculovirus infection is characterized by two biochemically and morphologically distinct virus forms: extracellular or budded virion (BV) and occluded virion (OV) or the polyhedra derived virion (PDV). In the latter form, the occlusion bodies are embedded in crystalline polyhedrin protein matrix and are responsible for primary infection.

Insects get infected with baculovirus when they ingest food contaminated with PDVs. The insects' midgut alkaline environment releases the embedded virions which invade the columnar epithelium cells of the microvilli by receptor-mediated membrane fusion (Horton and Burand, 1993). Secondary infection and systemic spread of the virus within the insect is set off by the budded virions released from the infected columnar epithelium cells through the insect hemolymph. They enter the cells by adsorptive endocytosis (Volkman and Goldsmith, 1985, Charlton and Volkman, 1993).

Following entry into the cell, the nucleocapsids migrate to the nucleus to get uncoated which requires formation of actin micro filaments (Charlton and Volkman, 1993). DNA is released, nucleus gets enlarged and distinct electron dense granular structure, the virogenic stroma (Fraser, 1986) is formed. This is thought to be the site for nucleocapsid assembly, viral transcription and replication. By 12 h p.i. progeny BVs are produced and released into extracellular compartment. 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.

Polyhedra protein accounts for nearly 30-50 % of the total insect cell protein but it is dispensable for the baculovirus life cycle under tissue culture conditions but essential for *in vivo* infection of larvae. Most BEVS take advantage of this phenomenon by replacing the polyhedrin coding sequence with a foreign gene of choice.

4.2 *The myriad advantages of baculovirus expression system:*

The myriad advantages of baculovirus expression system renders it as the system of choice. There are atleast two non-essential gene, the p10 and the polyhedrin genes, which are under **the** control of powerful late promoters. These promoters along with their flanking regions have been harnessed to express foreign genes. This eukaryotic system is excellent to obtain biologically active eukaryotic recombinant proteins. The following are few of the attributes **of BEVS:**

1. Functional recombinant protein. The baculovirus expression system provides a conducive ambience **for** proper folding, disulphide bond formation and oligomerization for the overexpressed recombinant proteins besides correct post-translational modifications. As a **result,** the product resemble its authentic counterpart structurally and functionally. in cases, where the product requires to be a heterotrimer or relies on tissue or species-specific modification, the binding partner or the modifying enzyme should be co-expressed.

2. Post-translational modifications. Most of the post-translational modifications like N- and O-linked glycosylation, phosphorylation, acylation, amidation, carboxymethylation, prenylation, signal peptide cleavage and proteolytic cleavage at the correct sites in the overexpressed protein have been reported to occur. However, the high level of expression of the product in this system overwhelms the ability of the cell to modify the product to the required level.

3. Exceptionally high expression. Compared to other higher eukaryotic systems, the impressive feature of the baculovirus expression system is its potential to achieve high expression levels of a cloned gene.

4. Capacity **of large** insertions. The flexibility in the expandability of the capsid structure of baculovirus allows **for** the insertion of very large genes.

5. Capacity to express unspliced genes. Baculoviruses have the ability to perform intron/exon **splicing** provided the required splicing factors are present in the insect cell recombinant machinery.

6. Simplicity of technology. With the rapid strides made in the BEVS technology, kits are available which are easy to use, reliable and fast. Moreover, the recombinant protein is not required to be expressed as a fusion protein.

7. Targeting of proteins. Correct targeting of the expressed protein occurs e.g. membrane proteins will be anchored into the cell membrane, secreted proteins will be secreted by insect cells.

8. Simultaneous expression of multiple genes. For dimer or multimer formation of proteins for activity, the proteins can be simultaneously expressed using the baculovirus expression system.

9. Ecologically acceptable. As baculovirus infect only arthropods, their high host-specificity renders them ecologically acceptable and effective alternative to chemicals in the control of forest and agricultural insect pests.

4.3 *Development of baculoviruses as expression vectors:*

The two major advantages of baculoviruses as expression vectors are firstly the nonessential regions present in the genome and secondly most of the nonessential genes especially the late genes being under very powerful promoters. The polyhedrin and p10 genes are non-essential for viral infection and replication under tissue culture conditions and have very powerful promoters thereby making these promoters along with their associated flanking regions as most exploited for expressing foreign genes using BEVS.

Initially, foreign genes were directly inserted into the baculovirus genome but this proved to be cumbersome due to the large size of the viral genome and not easy to manipulate. The easier and most common way to construct a recombinant baculovirus is by introducing the foreign gene into a transfer or transplacement vector. This vector is designed by having a bacterial plasmid as the backbone and with a portion of the baculovirus genome spanning the gene promoter and transcription terminator to be used for the heterologous gene expression. The vector must also have a suitable restriction site after the promoter gene for inserting the foreign gene. The foreign genes are finally inserted into the viral genome by co-transfecting the insect cells with the infectious virus DNA and the plasmid transfer vector. By homologous recombination, the foreign gene gets expressed.

Various strategies for the improvement in the screening of recombinant virus is based on the polh promoter making it the workhorse of BEVS. Infection of AcNPV wt

virus results in the production of opaque light refractile occlusion bodies in the insect cells. This phenomenon was used in BEVS to identify the polyhedra negative recombinant baculovirus but it was tedious and the recombinants were at a low frequency. Reporter genes like luciferase (Hasnain *et al,* 1994; Palhan *et al,* 1995), β-gal (Vialard *et al.,* 1990; Weyer ***et*** *al,* 1990) have been coexpressed for a better and **a more** efficient screening of recombinant virus besides using techniques like antibody screening and PCR screening (Reilly *et al,* 1992).

The screening methods were recently revolutionized by Kitts et al. (1990, '93). This group have used linearized and a lethally deleted baculovirus DNA which gives practically 100 % recombinants. A recombinant baculovirus was constructed by introducing two sites for the restriction enzyme *Bsu36* I in the flanking sequences upstream of promoter and within the downstream of ORF 1629 which codes for the viral replicase, an essential gene. This linearized recombinant baculovirus named as BacPAK6 is co-transfected **with** a polyhedrin based transfer vector carrying the missing portion of the virus genome besides the target gene. The linearized virus genome recircularizes but is unable to produce viable viruses until it undergoes homologous recombination with the transfer vector **which** ensures the generation of recombinant viruses. Clontech markets this system under the trade name 'BacPAK' while Pharmingen sells it as 'Baculogold'.

5.0 OBJECTIVES

In **the** present study, we have determined the effects of vanadium complexes, which differ **in their** oxidation states, on protein synthesis in reticulocyte lysates and on eIF2 activity. It is known that redox conditions such as oxidized glutathione, sugar phosphates and heavy metals can modulate reticulocyte protein synthesis and eIF2 activity. Vanadium compounds are being used as insulin mimetic although vanadium does not mimic all the actions of insluin (Bosch *et al,* 1987; Clarke *et al,* 1985). Also, a

previous study by Ranu (1983) and the only one we are aware of, has observed that vanadate impairs protein synthesis initiation in reticulocyte lysates but the study does not reveal the mechanism.

Hence, the present study has taken up with the following objectives:

1. How docs vanadium influence protein synthesis in heme-deficient and hemin-supplemented rabbit reticulocyte lysates?
2. How do the oxidation states of vanadium differ in their effect on protein synthesis?
3. Can the effects of vanadium on protein synthesis be mitigated?
4. What effect vanadium has on eIF2 and eIF2B activities of reticulocyte lysates?

Since, this laboaratory is working on the regulation of translation in eukaryotes, we realized the advantages of having a system which can overproduce the initiation factors and their mutants in order to trace the defects in protein synthesis more precisely and characterize the involvement of the protein factors whose regulation is affected. As we are mainly focusing on the regulation of initiation factor 2 activity, the present study has also taken up the following:

5. Cloning and Expression of the wild type and 48A mutant eIF2a (in which serine-48 is replaced by alanine) in the ovarian cells of *Spodoptera frugiperda* insect using the Baculovirus Expression Vector System (BEVS).
6. Characterization of the expressed proteins.
7. Studied the mechanism by which a mutant eIF2a, which can be phosphorylated, can bypass the inhibition in eIF2B activity caused by eIF2a phosphorylation.

MATERIALS AND METHODS

MATERIALS

The following materials were used to carry out this work:-

Gifts: eIF2α monoclonal antibody and HRI were given by Prof. I. M. London and Dr. Jane Jane Chen, MIT, as a kind gift. eIF2a wild type and eIF2α 48A mutant DNA were generously provided by Prof. Randall Kaufman, University of Michigan Medical Centre, Ann Arbor, Michigan. Vadex and vasc were prepared by A. Sreedhara and Dr. Pulla Rao, IIT Powai, Mumbai.

Amersham plc., UK: [C]leucine (315 Ci/mmol, 50 μCi/ml), multiprime DNA labelling kit and hybond N' membranes.

Biological E. Ltd., India: Heparin and New Zealand White rabbits.

Biorad, USA: acrylamide, BIS-acrylamide, protein molecular weight markers and protein assay reagent,

Boehringer and Mannheim GmbH, Germany: DTT, creatine phosphate, creatine phosphokinase. GTP, GDP and T4 DNA ligase.

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

Calbiochem. USA: Poly (1C).

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

DIFCO, USA: bactoagar, bacto-tryptone and yeastolate.

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

Flow laboratories, Scotland: neutral red staining solution.

GIBCO BRL, USA: Graces' basal insect cell culture medium, 1 Kb DNA molecular size ladder, competent E. coli cells DH5a and FCS.

Indian **Immunologicals,** India: New Zealand White rabbits.

Indu, India: X-ray films, developer and fixer.

Loba-Chemie. India: TEMED, P-mercaptoethanol and vanadyl sulfate.

Merck, India: Glycine.

Millipore, USA: 0.45 μm filter discs.

NESTLE, India: non-fat dry milk.

New England Biolabs, USA: DNA polymerase I, klenow fragment and restriction enzymes.

Pharmacia, Sweden: Sephadex G-50 and CM-Sephadex.

Promega Corporation Inc., USA: Restriction enzymes, anti-mouse IgG raised in rabbit - AP conjugate, NBT and BC1P.

Qiagen, USA: Qiagen kit for DNA purification.

Qualigens, India: KCl, NaCl, glucose, HC1,. EDTA, magnesium chloride, acetone, toluene, glycerol, ammonium sulphate, TCA, isoamyl alcohol, isopropanol, potassium dihydrogen phosphate, disodium hydrogen phosphate, sodium flouride acetic acid, ammonium acetate, ammonium carbonate, silver nitrate, methanol and ammonium persulphate.

Sarabhai Chemicals, India: hydrogen peroxide.

Sartorius, Germany: filter units, 0.45 μm and 0.22 μm filter discs.

Sigma, St. Louis, USA: acetyl phenylhydrazine, pNADPH, ATP, HEPES, magnesium acetate, sucrose, SDS, TNM-FH medium, bromophenol blue, coomassie R250, BSA, antimycotic-antibiotic solution, ficoll, low gelling temperature agarose, agarose, sodium ortho vanadate, sodium bicarbonate, PMSF, PVP, sonicated salmon sperm DNA and trypan blue.

SISCO, India: phenol.

Spectrochem, India: Tris, glycine, POP and POPOP.

Whatman, UK: filter papers, DEAE-cellulose, phosphocellulose and nitrocellulose membranes.

METHODS

1.0 CELL FREE TRANSLATION SYSTEM

1.1 *Heme-deficient rabbit reticulocyte lysate preparation:*

New Zealand white male rabbits (2-3 months old) were made anemic by injecting them sub-cutaneously with 1% acetyl phenylhydrazine in water for four consecutive days (Hunt *et al.*, 1972; Ernst *et al.*, 1978). Five days later, blood was collected into heparin (300 IU for 30-50 ml blood) containing 30 ml precooled corex tubes. The blood was centrifuged at 3000 rpm for 5 min at 4°C in a Remi high speed centrifuge. The supernatant was discarded. The cell pellet was washed three times (3000 rpm for 5 min) in the presence of isotonic buffered saline (7.5 mM $MgCl_2$, 5 mM KC1, 130 mM NaCl, 5 mM glucose and 10 mM HEPES pH 7.2). The supernatant and the white buffy coat present over the cell pellet was carefully aspirated off. The cell pack volume of the pellet (RBCs) was noted and an equal volume of ice cold deionised water was added to lyse the RBCs. Lysed cells were centrifuged at 10,000 rpm for 20 min at 4°C. Part of the 10K supernatant (lysate) was collected, aliquoted and stored under liquid nitrogen for protein synthesis assays. Rest of the lysate was further diluted by addition of an equal volume of deionised water and saved at -70°C for purification of eIF2 and also for other factors.

1.2 *Reticulocyte lysate protein synthesis:*

Protein synthesis was carried out at 30°C. The protein synthesis cocktail (25, 20 or 15 µl) contains 60 % lysate, 10 mM Tris-HCl pH 7.6, 80 mM KCl, 1 mM $Mg(OAc)_2$, 0.2 mM GTP, 33 µM amino acid mix-leucine, 27 µM [^{14}C]leucine, 6 µM leucine, 0.004 M creatine phosphate and 0.1 mg/ml creatine phosphokinase (Ernst *et al.*, 1980). 20 uM hemin was added to the cocktail wherever mentioned. Other modifications to the cocktail are mentioned in the legends to the figures. Protein synthesis was monitored at various time points by taking an aliquot (5 µl) from the protein synthesis reaction mixtures and spotting on a Whatman No. 1 filter paper. The filter papers were dipped in 10 % cold

TCA for 20 min followed by 5 min in boiling TCA (5 %) and 5 **min** in RT TCA (5 %). The filters were thereafter washed in ethanol and in acetone. The acetone washed filters were dried and soaked in diluted H_2O_2 (30 % H_2O_2:H_2O, 1:1) for 10 min to bleach the pink colour of the **filters** followed by washing in ethanol and later in acetone. The filters were dried. Radioactivity of the dried filters was determined in a liquid scintillation counter.

As commercially available rabbit reticulocyte lysates are not heme-sensitive and also very expensive, efficient heme-sensitive lysates were routinely prepared. Protein synthesis in different batches of lysates is shown in Fig. 3. An efficient heme-sensitive lysate responds to added hemin and protein synthesis is linear for nearly an hour. In **the** absence of added hemin, protein synthesis is linear for only 10-15 min and then shuts off. In heme-insensitive lysates (Fig. 4), protein synthesis is linear for about an hour in the absence as well as in the presence of hemin. Also the efficiency of protein synthesis differs from lysate to lysate (compare Fig. 3 with Fig. 4). Hence, in all experiments dealing with reticulocyte lysates, importance is given to the trend of protein synthesis.

1.3 ***Ribosomal*** *profile on* ***10-50*** *% sucrose* ***gradient:***

Ribosomes of reticulocyte lysates were separated on 10-50 % sucrose gradients and analyzed by l SCO density gradient fractionator as described (Ramaiah and Davies 1985; Ramaiah *et al.*, 1992).

10-50 % sucrose solutions were made in TKM buffer containing Tris-HCl pH 7.8, 80 mM KCl and 1 mM magnesium acetate. 2.4 ml of 50 % sucrose was poured into a 5 ml SW 50.1 rotor tube. 2.4 ml of 10 % sucrose solution was gently layered over the 50 % sucrose solution. The gradients were capped and kept in a horizontal position for 3 **h at** RT after which they were carefully lifted and kept on ice.

Protein synthesis was carried out in rabbit reticulocyte lysate at 30°C in the presence of a cocktail that is devoid of labelled leucine. The assays were terminated by addition of an equal volume of ice cold TKM buffer and were layered on the 10-50 % preformed sucrose gradients and spun at 45,000 rpm for 45 min in a SW 50.1 rotor in **a**

Fig. 3. Protein synthesis in heme-deficient reticulocyte lysates.

Protein synthesis was carried out in different batches of reticulocyte lysates at 30°C under two conditions: i) -heme and ii) +heme, 20 μM, as described in 'Materials and Methods'. Protein synthesis was measured [(^{1}C)leucine incorporated in cpm] in 5 μl aliquots at 30 and 60 min.

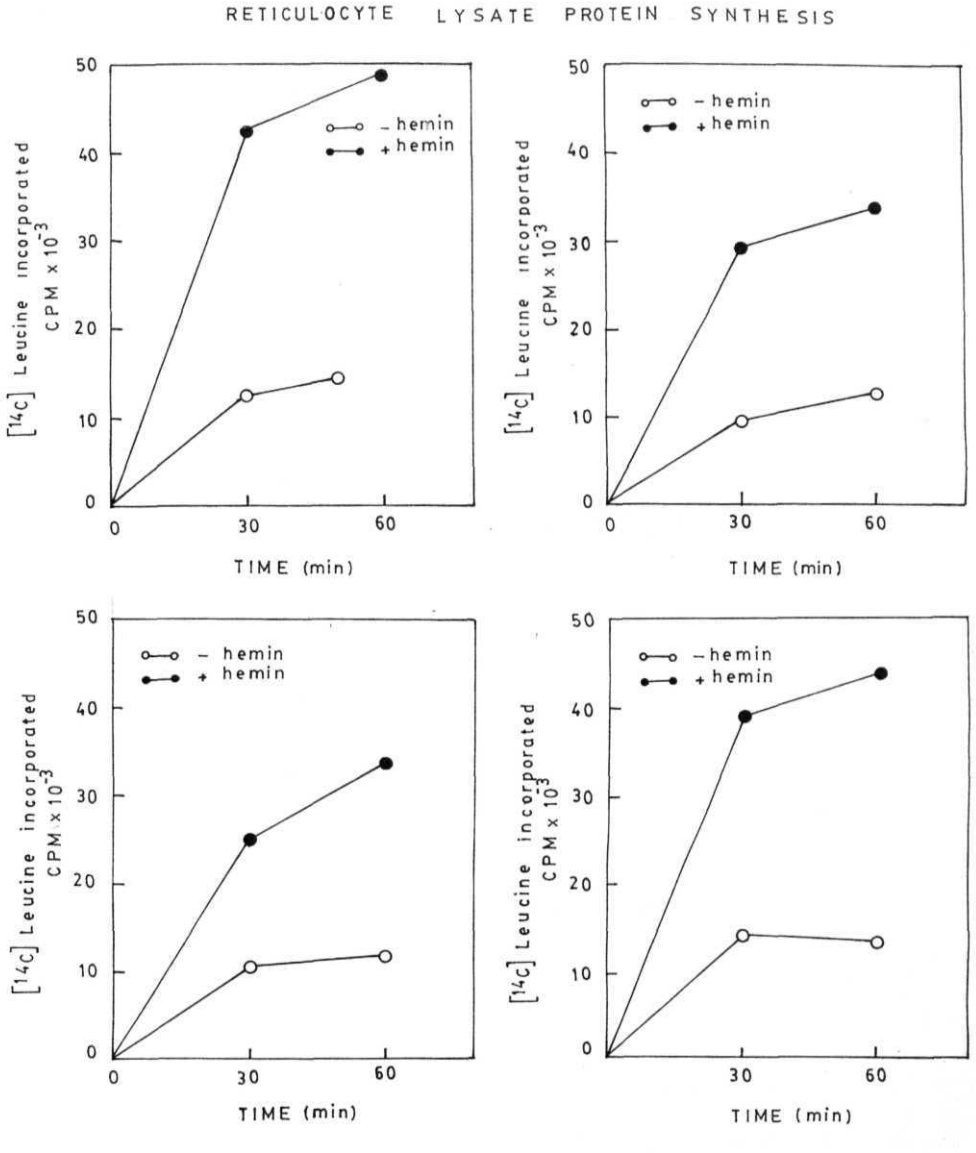
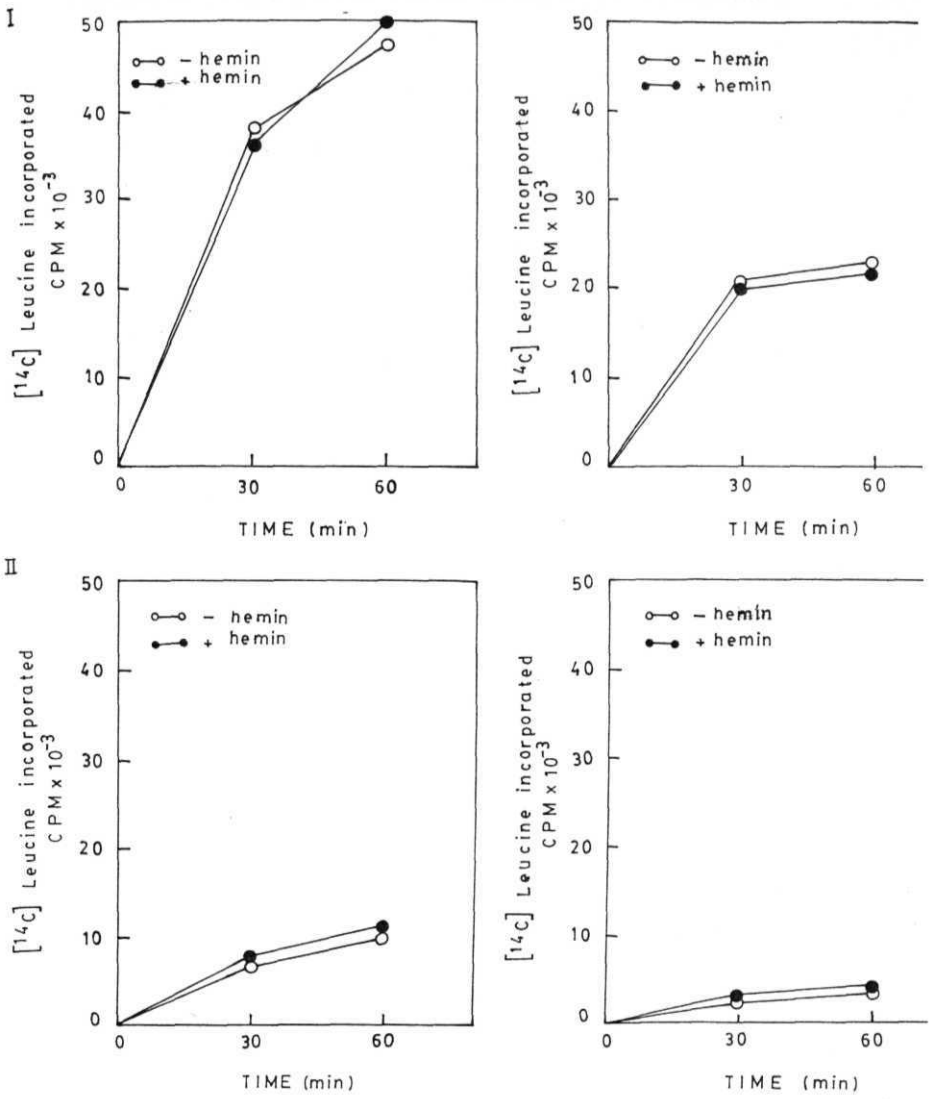

RETICULOCYTE LYSATE PROTEIN SYNTHESIS
[14C] Leucine incorporated CPM x 10-3
50
40
30
20
10
0
0
30
60
TIME (min)
– hemin
+ hemin

Fig. 4. Protein synthesis in heme insensitive and in dead lysates.

Protein synthesis was carried out as descibed in the legend to Fig. 3. in different batches of reticulocyte lysates. Protein synthesis was measured at 30 and 60 min.


I
II
– hemin
+ hemin
[14C] Leucine incorporated
CPM x 10-3
TIME (min)
0
10
20
30
40
50
0
30
60

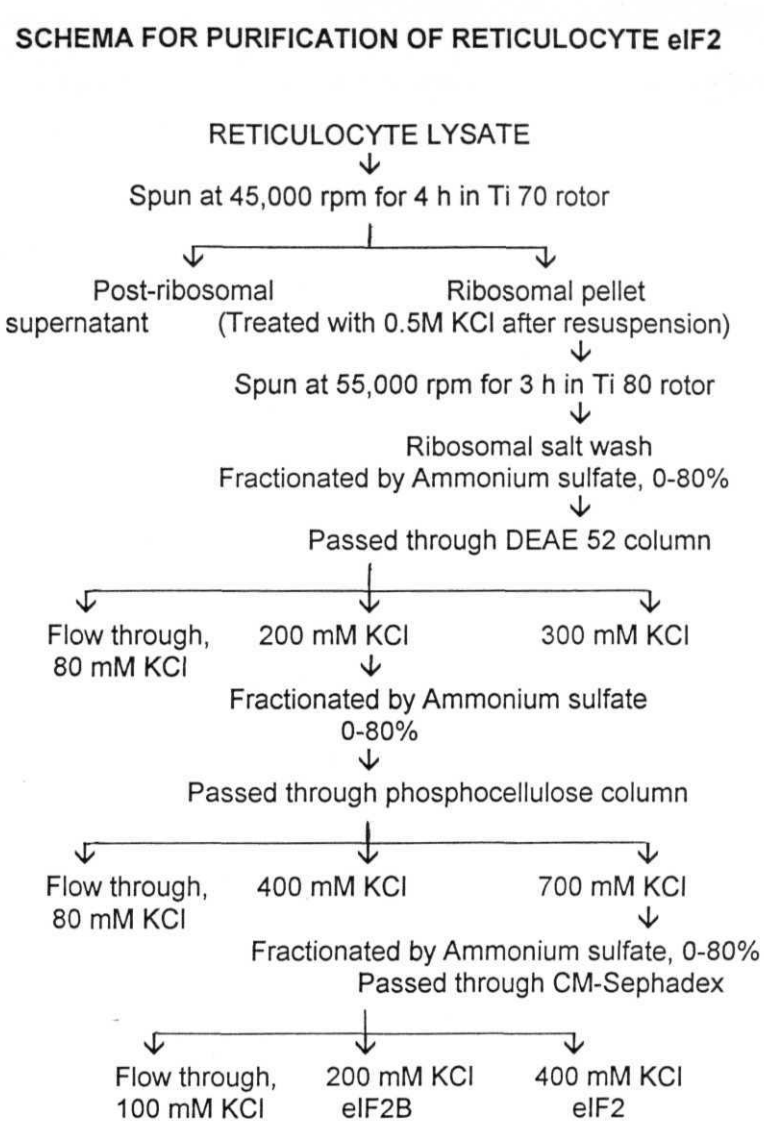


Fig. 5. Schema for rabbit reticulocyte eIF2 purification.

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

Beckman Ultracentrifuge. The gradients were analyzed at A_{254} nm using an Isco (model 185) density gradient fractionator.

2.0 PURIFICATION OF RETICULOCYTE eIF2

2.1 *Purification of eIF2 from ribosomal salt wash:*

eIF2 was purified from ribosomal salt wash (Andrews *et al.*, 1985; Naresh Babu and Ramaiah, 1996) and the various steps involved is shown schematically in Fig. 5. Lysate was layered over a glycerol cushion [50 % glycerol, 10 mM Tris-HCl pH 7.8, 5 mM NaCl, 25 mM KCl and 2 mM $Mg(OAc)_2$] and ultracentrifuged at 45,000 rpm for 4 h at 4°C in a Ti 70 rotor in a Beckman Ultracentrifuge. The post-ribosomal supernatant was removed and stored at -70°C. The ribosomal pellet obtained was resuspended in buffer A [20 mM Tris-HCl pH 7.8, 2 mM $Mg(OAc)_2$, 80 mM KCl, 5 % glycerol and 0.1 mM 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 dialyzed against buffer B /80 mM KCl. The dialyzed ribosomal salt wash was chromatographed on DEAE-cellulose equilibrated in buffer B /80 mM KCl. The column was washed with buffer B /80 mM KCl and elution was done with buffer B /200 mM KCl. The peak fractions eluted with buffer B /200 mM KCl were pooled and concentrated (0-80 % ammonium sulphate) and dialyzed against buffer B /80 mM KCl. After dialysis, the fraction was applied to a phosphocellulose column equilibrated in buffer B /80 mM KCl. The column was washed with buffer B /80 and 400 mM KCl prior to elution with buffer B /700 mM KCl. The peak fraction with buffer B /700 mM KCl elution contained eIF2.

The various fractions obtained during the purification of eIF2 were separated on a 10 % polyacrylamide - 0.1 % sodium dodecyl sulphate gel to show the various stages during purification and also to check the purity of eIF2 (Fig. 6A). The various fractions were probed with an eIF2α (38 kDa) monoclonal antibody to determine the eIF2<x subunit in the purified eIF2 (Fig. 6B). The purification profile of a second batch of

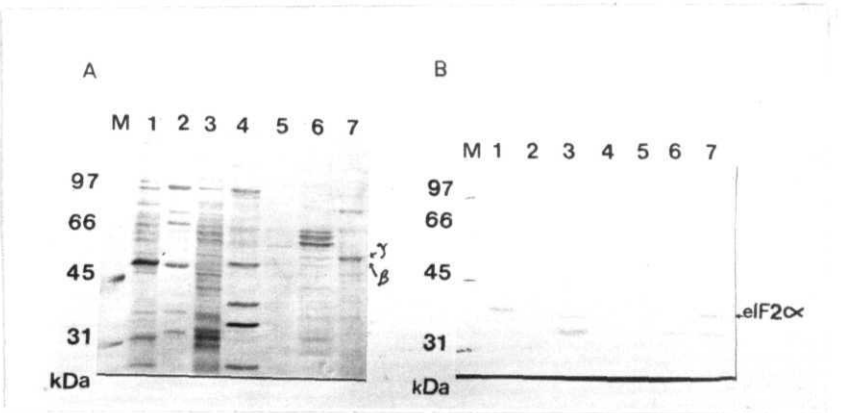


Fig. 6. Purification of reticulocyte eIF2 from ribosomal salt wash (first batch).

Panel A is a coomassie stained gel showing the purification profile of eIF2 from ribosomal salt wash (RSW). Lane 1 contains RSW. Lanes 2, 3 and 4 represent 0.1, 0.2 and 0.3 M KCl fractions of DEAE-52 column. Lanes 5, 6 and 7 represent 0.2, 0.4 and 0.7 M KC1 peak fractions obtained from phosphocellulose column.

Panel B is an immunoblot of the fractions shown in panel A cross-reacting with an eIF2 monoclonal antibody. A 10 % SDS-PAGE was run showing the same profile as in panel A, transferred onto a nitrocellulose membrane and probed with an eIF2 monoclonal antibody to detect and identify the alpha subunit (38 kDa) of eIF2.

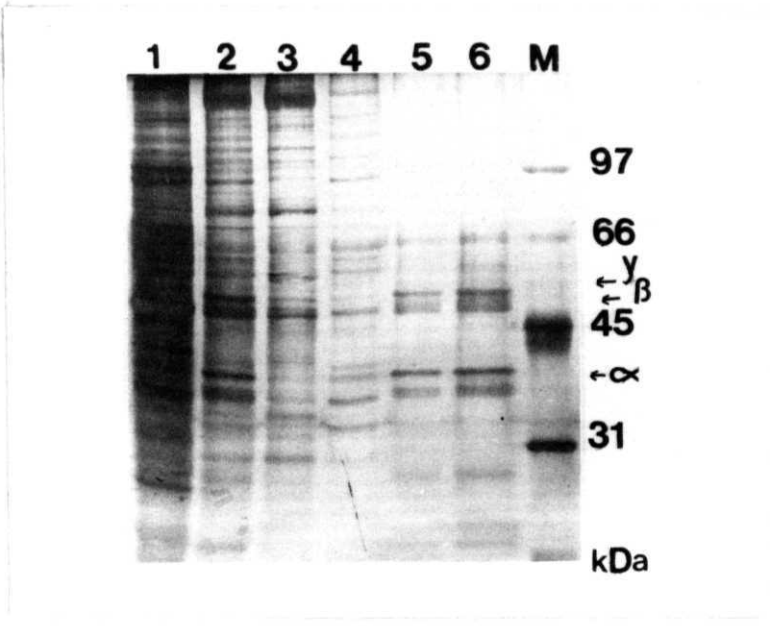


Fig. 7. Purification of reticulocyte eIF2 from ribosomal salt wash (second batch).

The various fractions obtained during purification by ion-exchange chromatography were loaded on a 10 % SDS-PAGE and silver stained. Lanes 1 and 2 represent 0.1 and 0.2 M KCl fractions of DEAE-52 column. Lanes 3, 4 and 5 represent 0.2, 0.4 and 0.7 M KCl fractions obtained during chromatography on the phosphocellulose column. A concentrated 0.7 M KCl fraction from phosphocellulose was loaded in lane 6.

endogenous reticulocyte eIF2 from ribosomal salt wash is shown in Fig. 7. Binary complex formation using different concentrations of the purified eIF2 and labelled GDP was carried out as described (please see section 3.0) and is shown in Fig. 9.

2.2 *Purification of eIF2 from post-ribosomal supernatant:*

Post-ribosomal supernatant was chromatographed on DEAE-cellulose equilibrated in buffer B /100 mM KCl. The column was washed with buffer B /100 mM KCl and elution was done with buffer B /250 mM KCl. The peak fractions were pooled and applied to a phosphocellulose column equilibrated with buffer B /250 mM KCl. The column was washed with buffer B /400 mM KCl prior to elution with buffer B /700 mM KCl. The peak fractions were pooled, concentrated (0-40 %, 40-80 % ammonium sulphate) and the 40-80 % fraction was dialyzed against buffer B /100 mM KCl. The dialyzed fraction was further chromatographed on a CM-Sephadex column equilibrated with buffer B /100 mM KCl. The column was washed with buffer B /400 mM KCl. The peak fractions were pooled, concentrated (0-80 %) and dialyzed against buffer B /100 mM KCl.

The purity of the eIF2 fraction was ascertained by loading the various eIF2 fractions on a 10 % polyacrylamide - 0.1 % sodium dodecyl gel and probing with an eIF2a (38 kDa) monoclonal antibody as shown in Fig. 8.

The results indicate that the CM-Sephadex 0.4 M KCl fraction appears to be rich in eIF2 (a- 38 kDa, β- 51 kDa and y- 52 kDa) compared to other fractions. There is also a prominent 67 kDa protein band (p67) which is known to copurify with eIF2 and protect eIF2a subunit from phosphorylation by HRI (Datta *et al.*, 1988a, b, 1989; Gupta, 1993).

3.0 FORMATION AND **DISSOCIATION OF LABELLED BINARY COMPLEX, eIF2.[^{3}H]GDP**

3.1 *Formation of eIF2.[^{3}H]GDP binary complex-An assay for eIF2 activity:*

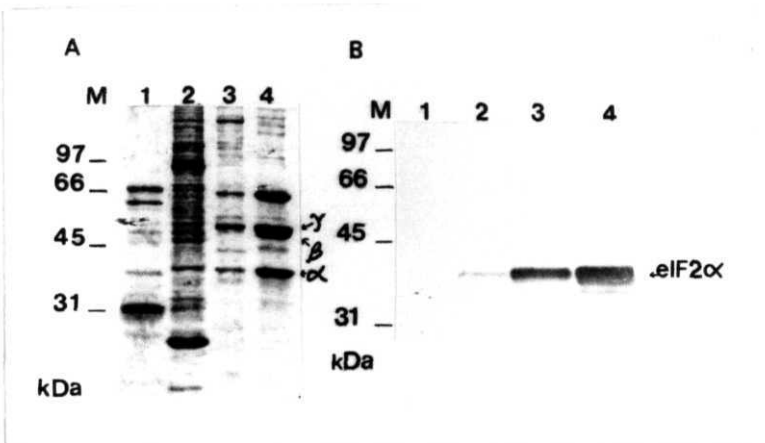


Fig. 8. Purification of eIF2 from postribosomal supernatant (PRS).

Panel A is a coomassie stained gel showing the purity of eIF2 at different stages of purification. Lanes 1- PRS; 2- DEAE-52 0.2 M KC1 fraction; 3- Phosphocellulose 0.7 M KC1 fraction; 4- CM-Sephadex 0.4 M KC1 fraction.

Panel B is an immunoblot of the gel shown in panel A. After the proteins were separated on a 10 % SDS-PAGE gel, they were transferred onto a nitrocellulose membrane and immunoblotted with an eIF2α monoclonal antibody to identify the alpha subunit (38 kDa) of eIF2.

TABLE 1

EFFECT OF PHOSPHORYLATION OF eIF2.[^{3}H]GDP COMPLEX ON ITS DISSOCIATION *IN VITRO-TO* CHECK FOR THE PRESENCE OF eIF2B

Experimental conditions	**pmols of eIF2.[^{3}H]GDP dissociated 5 min**
-HRI -GDP	**3.82**
-HRI +GDP	**5.26**
+HRI -GDP	**2.29**
+HRI +GDP	**2.42**

*** 15.55 pmols (0.5 μg) of eIF2.[^{3}H]GDP added per** assay

CM-Sephadex purified eIF2 from PRS was incubated with [^{3}H]GDP (2 μM, 1100 cpm/pmol) in a 20 μl cocktail as described in 'Materials and Methods' to form a binary complex, eIF2.[^{3}H]GDP. A phosphorylation cocktail with or without HRI was incubated for 10 min at 30^{o}C before adding to the binary complex (15.55 pmols of eIF2.[^{3}H] GDP in 20 μl). To detect for the presence of eIF2B activity in the purified eIF2 protein, the labeled GDP in the binary complex was chased with cold GDP (40 μM) for 5 min at 30°C. The reactions were terminated by addition of cold wash buffer. Values represent net picomoles of labeled eIF2.[^{3}H]GDP dissociated at the end of 5 min after the addition of cold GDP.

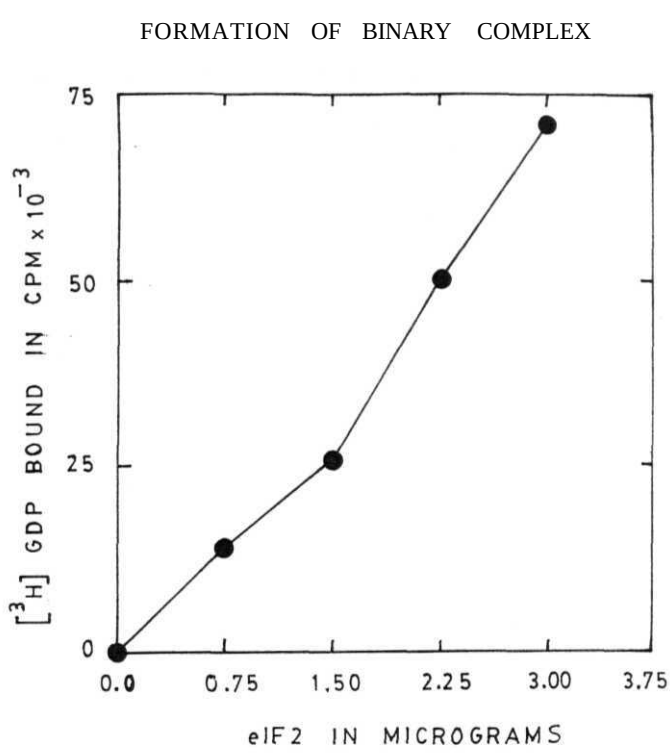


Fig. 9. Binary complex formation.

Different concentrations of purified eIF2 were incubated with [^{3}H]GDP (2 uM, 1100cpm/pmol) in a cocktail to form eIF2.[^{3}H]GDP binary complex as described in 'Materials and Methods'. Once the formation was completed, the reaction mixtures were filtered through a 0.45 μm nitrocellulose membrane and the amount of [^{3}H]GDP retained on the nitrocellulose filters was taken as the amount complexed to eIF2 and used to plot this Fig. This Fig. depicts the binding ability of the purified eIF2 to GDP, which is one of the assays for characterizing eIF2 activity.

The innate ability of eIF2 to bind to GDP in the presence of 1 mM Mg^{2+} is used as an assay system for the presence and activity of eIF2. Purified eIF2 from ribosomal salt wash and from post ribosomal supernatant was incubated with labelled 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 oi' labelled binary complex [eIF2.(H)GDP] formed was assessed by terminating the reactions with 3 ml cold wash buffer (20 mM Tris-HCl pH 7.8, 80 mM KC1 and 1 mM Mg^{2+}). The reactions were then filtered through 0.45 μm nitrocellulose filter membranes and the filters were thereafter washed with another 6 ml of cold wash buffer. The filters were dried and the amount of labelled GDP that bound to eIF2 and retained **on** the millipore filter was determined by liquid scintillation counter.

Different concentrations of eIF2 purified from ribosomal salt wash were tested for their GDP binding ability as shown in the Fig. 9. The results indicate that the 0.7 M KCl phosphocellulose fraction appears to be rich in eIF2.

It is known that cIF2 is often purified from ribosomal salt wash. We have also purified it from post ribosomal supernatant which forms a source for eIF2B as well. To assess the amount **of eIF2B** present in the CM-Sephadex purified eIF2, the dissociation of labelled binary complex was measured in the presence and absence of excess cold or unlabelled GDP (40 μM) at 30°C for the specified time points. This assay is also referred to as the GDP exchange assay or GDP chase assay. The reactions were terminated by the addition **of cold** wash buffer as described above, and the pmol of eIF2.[^{3}H]GDP retained on the **filter** in the absence and presence of excess cold GDP was measured.

The amount of [H]GDP bound to eIF2 in the presence and absence of unlabelled GDP is shown in table 1. It is always observed that the amount of labelled GDP bound to **eIF2** decreases when it is chased with cold GDP. This is due to two reasons: 1) mass exchange which is usually very minimal and 2) presence of eIF2B (not only exchanges **out** GDP for GTP but also GDP for GDP).

To confirm the dissociation of eIF2.GDP occurs specifically by contaminant eIF2B, the preformed labelled binary complex was subjected to phosphorylation by activated HR1 (HRI incubated with 100 uM ATP, 20 mM Tris-HCl pH 7.8, 80 mM KC1, 2 mM Mg^{2+} for 10 min at 30°C for activation) and incubated for 5 min at 30°C before the addition of cold GDP. The reactions were terminated at the required time points by addition of cold wash buffer as described above and filtered. If there is comparatively more labelled binary complex being retained on the filter in the presence of excess cold GDP when eIF2 is in the phosphorylated state than in the unphosphorylated state, then it argues **for** the presence of eIF2B activity as eIF2B activity is inhibited upon eIF2a phosphorylation by HRI and hence, it is unable to exchange the labelled GDP in eIF2.GDP binary complex in the presence of unlabelled GDP (Table 1).

The results in **the** Table 1, indicate that 1.44 pmol of labelled eIF2.GDP is dissociated in the presence of excess unlabelled GDP. In contrast, when eIF2a is phosphorylated by addition of HRI and ATP, only 0.13 pmol of labelled binary complex is dissociated, suggesting that the eIF2 preparation of 15.5 pmol approximately contain a small amount of contaminant eIF2B activity which can dissociate approximately 1.3 pmol of labelled binary complex in 5 min. Since, eIF2 and eIF2B comigrate together during several steps of purification, a small amount of eIF2B contamination is expected to occur with highly purified eIF2 preparations.

3.2 *Assay for endogenous **eIF2B** activity in protein synthesizing lysates / insect cell extracts:*

Dissociation of preformed binary complex was also studied in protein synthesizing lysates (Matts ***et al.***, 1984, Naresh Babu and Ramaiah, 1996) under different conditions as described in the legends to the Tables and Figs. Lysate protein synthesis reactions (20-25 μl) were carried out at 30°C as described above except that in the place of labelled leucine, unlabelled leucine was used. Protein synthesis was carried out for 10-15 min prior to the addition of preformed labelled binary complex (20 ul), 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.[H]GDP binary complex bound to the filters were measured. The difference between the pmol of labelled binary complex added initially to the lysates and that left on the filters after the exchange assay is the pmol of eIF2.[^{3}H]GDP dissociated which is supposed to reflect the eIF2B activity.

The preparation of insect cell extracts is described later (section 9.2). Dissociation of preformed binary complex in reticulocyte lysates supplemented with insect cell extract was assessed as described above. Insect cell extract was added to the reticulocyte lysate and incubated for 10 min at 30°C prior to the addition of preformed labelled binary complex.

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.

4.0 PHOSPHORYLATION ASSAYS

4.1 *In vitro phosphorylation assay;*

In vitro phosphorylation assays were carried out by incubating eIF2 with HRI and [γ^{-32}P]ATP (3000 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 ATP. The protein kinase assays were terminated by the addition of 2X SDS sample buffer (Tris-HCl pH 6.8, 10 % SDS, 10 % glycerol, β-mercaptoethanol and bromophenol blue). The samples were heated for 2-3 min in boiling water and analyzed on 10 % polyacrylamide - 0.1 % sodium dodecyl gel and analyzed by autoradiography.

4.2 *In situ phosphorylation assay:*

Lysates (15 µl) were pulsed with [γ^{32}P]ATP at 10-15 min of protein synthesis. Protein synthesis was carried out at 30 C with cold leucine. The reactions were terminated 5 min after the pulse by taking out 10 ul of the reaction mixture and adding it to 800 µl of cold NaF and 5 mM EDTA (to inhibit the phosphatase activity besides diluting and lowering the temperature of the reaction mixture). 10 µl of 0.5 M glacial

acetic acid was added to the terminated reactions to concentrate the proteins by pH 5 precipitation. The samples were incubated on ice for 1 h and then spun at 12,000 rpm in a Remi high speed centrifuge at 4°C. The radioactive supernatant was carefully aspirated off. The light pink coloured pellet was dissolved in 2X sample buffer and heated for 2-3 min in boiling water. The proteins were separated on a 10 % polyacrylamide - 0.1 % sodium dodecyl gel and the phosphoproteins were analyzed by autoradiography.

5.0 SEPARATION **OF IeIF2(aP).eIF2B] 15S COMPLEX ON 10-30 % SUCROSE GRADIENTS:**

Hemin supplemented (20 µM) reticulocyte lysate protein synthesis reactions (100 µl) were carried out in the presence of insect cell extract (25 µg) at 30°C for 15 min as detailed in the legends to the Figs. Protein synthesis reactions were arrested by addition of an equal volume of ice cold TKM buffer [20 mM Tris-HCl, pH 7.6; 80 mM KC1 and 1 mM $Mg(OAc)_2$] and layered over a 4.8 ml chilled 10-30 % sucrose gradient, prepared in TKM buffer. Samples were ultracentrifuged in a Beckman model for 6 h at 40,000 rpm at 4°C in SW 50.1 rotor to separate out the free eIF2 from [eIF2(aP).eIF2B] complex. With the help of an ISCO gradient fractionator, fractions (400 µl) were collected by upward displacement of the gradient. The fractions were concentrated by pH 5 precipitation as described (Ramaiah *et al,* 1992; Krishna *et al,* 1997) and resolved on a 10 % SDS-PAGE. The proteins were transferred onto a nitrocellulose membrane and probed with a human eIF2α monoclonal antibody and later by a secondary rabbit anti-mouse IgG-alkaline phosphatase conjugate antibody.

6.0 INSECT CELL TISSUE **CULTURE**

6.1 *Cell line and virus:*

Sf9 cell line (Vaughn *et al.,* 1977), which serves as a host for AcNPV was used for the expression study. Sf9 cells were maintained in complete medium (TNM-FH

medium supplemented with 10 % fetal bovine serum, and antimycotic-antibiotic solution as described by Summers and Smith, 1987).

6.2 ***TNM-FH medium:***

TNM-FH medium (Mink, 1970) is Grace's basal insect cell culture medium (Grace, 1962) 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 litre of TNM-FH medium, 46.3 g of Grace's medium was dissolved in 700 ml of distilled water, 0.35 g of $NaHCO_3$ was also added and the medium was **adjusted** to pH 6.2 with KOH. Lactalbumin hydrolysate (3.33 g) as well as yeastolate (3.33 g) were added before the volume was made to one litre. The medium was filter sterilized (0.22 μm) in the hood. 10 % fetal bovine serum and antibiotics were later added to make **complete** medium.

Cells were maintained at 27°C in complete medium and grown as a monolayer or in suspension. **Sf9** cells double every 18-24 h at 27°C and hence they are subcultured twice a week once the cells reach > 90 % confluency.

6.3 *Monolayer culture:*

Sf9 cells were maintained in 25 cm^2 or in 75 cm^2 tissue culture flasks for monolayer cultures. Cells were dislodged by washing the surface by gentle pipetting (O'Reilly *el al.*, 1992). For each subculture, 1 X 10^6 cells or 3 X 10^6 cells were approximately seeded in 25 cm^2 or 75 cm^2 flasks or in 10 ml of complete medium **respectively.** The viability of the cells was checked by staining with 10 % v/v trypan blue (non-viable cells stain blue). Only cells with greater than 95 % viability were used for experiments.

6.4 *Freezing and thawing of cells:*

Cell line stocks were made from healthy Sf9 log-phase cultures. The cells were harvested and the cell pellet was suspended in complete medium containing DMSO (10

%). The final cell density was kept at 4 X 10^6 cells/ml. The cell suspension was aliquoted (1 ml) and IVcezed slowly. The cells were initially placed at -20°C for one h, then at -70°C o/n and later transferred to under liquid nitrogen the next day.

The above mentioned stocks were removed from liquid nitrogen when required and thawed by gentle agitation in a 37°C water bath. Once the stock was thawed, the vial was wiped with 70 % ethanol before taking it to the hood. The cells were transferred to a centrifuge tube and further diluted with TNM-FH medium. The cells were harvested and to the cell pellet, 10 ml of TNM-FH complete medium was added and seeded in a 75 cm^2 flask. The medium was changed once the cells were seeded.

7.0 MOLECULAR CLONING

7.1 *Transformation and Amplification:*

Competent DH5α cells (100 µl) were transformed with DNA by incubating the cells for 30 min on ice (Sambrook *et al.*, 1989). The cells were heat-shocked for 2 min at 42°C. 500 µl of LB (for one litre of LB: 10 g bacto-tryptone, 5 g yeast extract, 10 g NaCl, pH adjusted to 7.5 with NaOH) was added and incubated for 30 min at 37°C. The transformed cells were plated on LB agar (LB + 15 % bacto-agar) plates containing ampicillin (50 µg/ml). The plates were incubated for 10-12 h at 37°C to allow the growth of transformed ampicillin resistant bacterial colonies.

Larger cultures LB + ampicillin (50 µg/ml) were inoculated with a single transformed colony with the help of loop/tooth pick. The cultures were left at 37°C for 18-20 h.

7.2 *Isolation of plasmid DNA:*

The plasmid DNA was isolated from small cultures by following the alkaline-SDS lysis protocol (Sambrook *et al.*, 1989).

A 1.5 ml culture of transformed E. coli cells were centrifuged in an eppendorf tube and resuspended in 100 µl of chilled solution I (50 mM glucose, 25 mM Tris-HCl

pH 8.0, 10 mM EDTA). The cell suspension was incubated on ice for 10 min. To this, 200 ul of freshly prepared ice cold solution II (0.2 N NaOH, 1 % SDS) was added and mixed by inversion. It was incubated thereafter on ice for 10 min. 150 µl of solution III (3 M NaOAc pH 4.8) was added and gently mixed and incubated on ice for 20 min.

The eppendorfs were centrifuged at full speed in a microfuge for 20 min. The supernatant was transferred to a fresh tube and an equal volume of buffer (Tris-HCl pH 8.0, EDTA pH 8.0)-saturated phenol/chloroform/isoamyalcohol (25:24:1) was added and vortexed for 30 to 60 seconds before centrifuging at top speed for 10 min. The aqueous phase was taken to a fresh tube and the earlier step was repeated. The aqueous phase was once again taken to a fresh tube and isopropanol (0.8 vol) was added and incubated for 5 min at RT before centrifuging at 10K for 20 min. The pellet thus obtained was washed in 70 % ethanol at RT. The precipitate was centrifuged down and the ethanol was removed. The plasmid DNA was allowed to air dry at 37°C for 10 min. The plasmid DNA was resuspended in TE buffer.

Plasmid DNA from larger cultures was isolated using the commercially available Qiagen column. The instructions of the company were followed and for DNA elution from the agarose gel piece, the Qiaquick spin column was used.

7.3 *Restriction digestion:*

All DNA manipulations were carried out according to Sambrook et al. (1989). Plasmid DNA (1 µg) was incubated at 37°C for 2-4 h with appropriate restriction endonuclease enzyme and buffer. The volume was made up to the required volume with deionised water. For bulk DNA digestion, the incubation was carried out for o/n.

7.4 ***Ligation:***

All ligation reactions were carried out using T4 DNA ligase in 1X ligase buffer (20 mM Tris-HCl pH 7.6, 5 mM $MgCl_2$, 5 mM DTT, 1 mM ATP).Then ligation reaction was carried out at 16°C for ~16 h in a total volume of 20 ul. The ligation mix was used for transforming competent DH5α cells. The positive colonies carrying the insert was

used for transforming competent DH5α cells. The positive colonies carrying the insert were identified by colony hybridization.

7.5 *Electrophoresis of DNA:*

Isolated plasmid DNA as well as DNA treated with various restriction enzymes were separated on 1 % agarose gel, prepared in TAE buffer (0.04 M Tris-acetate, 1 mM EDTA pH 8.0 and 250 µg/ml of ethidium bromide was also added). DNA samples were prepared in gel loading dye (0.025 % ficoll, 0.025 % bromophenol blue and 0.025 % xylene cyanol) and loaded onto the slots. Electrophoresis was carried out at 50 volts in TAE buffer. The DNA bands were viewed under UV light.

7.6 *Colony hybridization:*

Colony hybridization was done as described (Anjali, 1996). The bacterial colonies obtained after transformation with the ligation mix were transferred onto a nylon membrane (nylon N^+, Amersham plc, UK) and grown o/n at 37^oC. The following day, the membrane was placed on a polythene sheet with the colonies facing up and treated as follows: DNA was 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 2 h before proceeding for pre-hybridization and hybridization with the radioactive probe.

The membrane was briefly soaked in 4X SSC buffer (for 20X SSC, 175.3 g of NaCl and 88.2 g of sodium citrate was dissolved in 800 ml of H_2O and pH adjusted to 7.0 with 10 N NaOH and the volume made upto 1 litre) and gently cleaned with a cotton swab. The membrane was pre-hybridized with a buffer containing 6X SSC buffer pH 7.0, 5X Denhardt's solution (10 g each of Ficoll, PVP and BSA were dissolved in 500 ml of H_2O, to prepare 50X Denhardt's solution), 200 µg/ml sonicated salmon sperm DNA and 0.05 % SDS at 55°C for 5 h in a rotary shaker as described (Sambrook *et al.*, 1989).

After pre-hybridization, the blot was hybridized at 55°C for 16 h. The membrane was washed as follows:

In 2X SSC, twice for 10 min each at RT.

In 0.2X SSC with 0.1 % SDS, twice for 10 min each at 55°C.

In 0.1 X SSC with 0.1 % SDS, 10 min at 65°C.

The membrane was then dried, covered with saran wrap and subjected to autoradiography to identify the positive colonies.

7.7 *Radiolabelling of cDNA:*

75-100 ng of DNA fragment was labelled by random priming using a multiprime DNA labelling kit (Amersham, UK) as described (Anjali, 1996). DNA was denatured by boiling for 5 min and chilled on ice for 10 min. Random hexanucleotide primers, dNTPs (-dCTP) in a concentration buffer solution (Tris-HCl pH 7.8, $MgCl_2$ and |3-mercaptoethanol), [α^{-32}P]dCTP (30 μCi) and klenow fragment of E. coli DNA polymerase I (2 units per reaction) were added and the reaction volume made up with water to 50 ul and incubated at 37°C for 30 min. The enzyme was inactivated by heating at 65°C for 5 min and the probe was purified by gravity.

For gravity purification, a 1 ml syringe was packed with a slurry of sephadex G-50 equilibrated with TE buffer. The labelling reaction was diluted to a total volume of 80 ul and loaded onto a column. Once the sample entered the column, 80 μl of TE buffer was added each time to ensure that the column does not go dry. Fractions of two drops (~80 μl) were collected into microcentrifuge tubes and scanned using a hand-held mini monitor (Morgan, series 900, UK). Two clear peaks were obtained, the first representing the labelled DNA while the second peak of the unincorporated nucleotides. The fractions containing the labelled DNA were pooled, heated at 95°C for 10 min for denaturation to occur and added to the prehybridization solution.

7.8 ***In-gel*** *hybridization:*

The agarose gel containing the DNA to be probed was dried and denatured for 30 min in a denaturation buffer (0.5 N NaOH, 0.15 M NaCl) at RT. This was followed by incubation in neutralization buffer (0.5 M Tris-HCl pH 8.0 0.15 M NaCl) for 30 min at

RT. The gel was washed in water and then incubated with the probe (labelled DNA in pre-hybridization solution) at 55°C o/n. Pre-hybridization is not required. The gel was later taken out, wrapped in saran wrap and subjected to autoradiography to identify the DNA band of interest.

8.0 PROPAGATION AND MAINTENANCE OF AcNPV - DERIVED BACULOVIRUSES

8.1 *Co-transfection:*

The Clontech kit was used for the expression of recombinant transfer vector in Sf9 cells to obtain recombinant viruses and the procedure followed was as detailed by the suppliers. Sf9 cells were co-transfected with pBacPAK8 viral transfer vector containing the cDNA of interest and with *Bsu36* I digested BacPAK6 viral DNA inorder to obtain an infectious complete virus genome with the required cDNA. The transfection was carried out using lipofectin. Sf9 cells were seeded in 35 mm tissue-culture dishes in complete medium. Just before transfection, the complete medium was replaced by 2 ml of incomplete medium and incubated for 20-30 min. Meanwhile the DNA-lipofectin complex was prepared in polystyrene tubes in 100 ul volume. To the recombinant plasmid DNA (500 ng, 100 ng/ml), 5 ul of BacPAK6 viral DNA (*Bsu36* I digest, supplied by Clontech) and 40 ul of sterile water was added in polystyrene tubes. To this, 50 µl of lipofectin solution (0.1 mg/ml) was added, gently mixed and incubated at RT for 15 min for the DNA-lipofectin complex to take place. A negative control, without the BacPAK6 viral DNA and a mock without any DNA were also carried out.

The medium of cells was once again replaced by fresh incomplete medium of 1.5 ml. To this, lipofectin-DNA complex was added dropwise and the cells were incubated for 5 h at 27°C. Afterwards the transfectant supernatant was removed and stored in polystyrene tubes. To the cells, 1.5 ml of incomplete medium was added and incubated at 27°C for 60-72 h with a moist paper towel. The recombinant viral supernatant was later used in the plaque assay as described below.

8.2 ***Plaque*** *assay:*

This step was carried out to obtain active recombinant virus **from** lysed transfected cells. The procedure involves the following steps.

1. Two million cells were seeded in a 35 mm tissue-culture dishes with complete medium and were left for a while.

2. Meanwhile, the viral supernatant from the virus infected cells (described under Co-transfection section) were taken and filtered through a 0.22 urn ministart filter unit with the help of a 1 ml syringe.

3. From each of the transfection experiment described as in previous section, serial dilution of the virus stock was carried out as mentioned below in duplicates.

i) neat or 100 % ii) 10^{-1} diluted and iii) 10^{-2} diluted. Dilutions were done with medium.

4. The viral cultures were then used to infect freshly seeded cells as mentioned in 1. Before adding the viral inoculum, the medium was removed and then 150 µl of the viral inoculum (neat or diluted) were added dropwise. The tissue dishes were left for 1 h with gentle rocking every 15 min.

5. 3% low gelling temperature agarose (LGTA) was prepared in 50 % water and in 50 % medium taking all the necessary precautions. 1 ml of this LGTA was added dropwise to one side of the tissue-culture dishes containing the virus infected cells slowly without any bubbles. Tissue-culture dishes were left undisturbed. Once the LGTA soldifies, 2 ml of complete medium was added slowly from one side of the tissue dish over the agarose layer. The dishes were left at 27°C for 96 h. 1.2 ml of neutral red stain (1 mg/ml in PBS) was added to 20 ml of plaque assay buffer (0.82 g of NaCl, 0.2 g of KCl, 0.114 g of Na_2HPO_4 and 0.02 g of KH_2PO_4 in 100 ml of H_2O, pH 7.3) 2 ml of the diluted stain was added to each tissue dish containing the cells. After 1 h, the stain was removed and the plates were left overturned at 27°C. After 24 h, the plaques became visible and **were** picked up and released into eppendorfs containing 100 ul of complete medium. The eppendorfs were left at 4°C for the virus to be released into the medium. This virus was used for infection studies.

The plaques were counted in each case and the virus titre (plaque 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 I/dilution factor

8.3 *Amplification of the recombinant viral stock:*

A step wise amplification of the recombinant viral titre (determined by dot-blot) was done from a 96 well plate to a 35 mm tissue dish through a 24 well and 6 well plates and finally to a 25 cm flask.

96 well plate: 0.1 X 10^6 Sf9 cells in 100 ul of medium were seeded in each well of a 96 well plate. After 2 h, the medium was carefully removed without disturbing the monolayer. 50 ul of the viral stock was added to each well and left for 1 h with gentle rocking every 15 min. 50 ul of complete medium was added to each well. The plate was wrapped in parafilm and left at 27°C in an incubator with a moist paper towel in a box for 48-60 h. Later the viral supernatant was removed and stored at 4°C for further amplification.

24 and 6 well plates: As mentioned above, cells were seeded and made to attach to the surface as an even monolayer. The viral stock obtained from the 96 well plate was used for infecting the cells. The medium was carefully aspirated off before adding the virus and the plates were gently rocked every 15 min for 1 h. Later medium was added such that the final volume was 500 ul and 2 ml in each well of a 24 well and 6 well plates respectively. The plates were left at 27°C until all the cells get infected. The viral supernatant was stored at 4°C for further amplification.

35 mm tissue-culture dishes: 2 X 10^6 cells in 2 ml of complete medium were seeded in each 35 mm tissue-culture dish. 1.5 ml of fresh medium was also added and the dishes were left undisturbed for the cells to get attached for 1 h. After the cells were seeded, the medium was carefully aspirated off and 150 ul of the recombinant virus obtained from the 6 well plate was added to each dish and left for 1 h with gentle rocking every 15 min. At the end of 1 h, the supernatant was removed and stored at +4°C. 2 ml of complete medium was carefully added without disturbing the monolayer and the plate was covered

with a parafilm. They were left at 27°C with a moist paper towel for the multiplication of the virus.

25 cm^2 flask: The procedure is same as above except that confluent Sf9 cell culture flask were taken and the virus inoculum was not removed and the medium was directly added after 1 h period. As usual, 5 ml of complete media was added and the flasks left for about one week until all the cells become well infected.

In all the above amplification steps, a mock-infected as well as a wild type AcNPV virus-infected controls were used. The cells were observed at intervals under the inverted light microscope to monitor the course of infection. A plaque assay was done to determine the virus titre. High viral titre stocks were aliquoted and stored at -70°C for long term storage.

9.0 PRODUCTION AND ANALYSIS OF THE EXPRESSED RECOMBINANT PROTEIN

9.1 *Dot-Blot hybridization:*

To check whether the recombinant vector harboring the insert cDNA has been incorporated into the viral genome, a dot-blot hybridization has been performed.

A radioactive probe against the insert cDNA was prepared as mentioned earlier and was used to detect the insert in the recombinant infectious virus and thereby identify the positive plaques. 0.1 X 10^6 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) was also used. 50 µl of the virus (virus released from the plaques obtained with different dilutions of the virus stock) was added to each well after the medium was carefully removed. The plate was left for 1 h at 27°C after which 50 µl of complete medium was added and the plates were kept in box containing a 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°C) and in dot blot solution (0.2 N NaOH, 1 M ammonium acetate). After the transfer, the membrane was baked for 2 h, treated with pre-hybridization and hybridization solution as mentioned before and then exposed to X-ray film to identify the positive plaques.

9.2 *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.

2 million cells were seeded in 35 mm tissue-culture dishes. One dish was needed for one time point for each virus as well as one dish for a mock-infected control. Cells were infected as described earlier. At each time point, the dishes were placed on ice and the cells were suspended in the medium and the cell suspension was centrifuged at 3000 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) and cells were collected each time by centrifuging at 3000 rpm for 5 min. The cells were lysed in lysis buffer (20 mM Tris-HCl pH 7.8, 1 mM Mg^{2+}, 1 mM DTT, 1X pepstatin A (0.7 mg/ml methanol = 1000X), leupeptin (0.5 µg/ml triple distilled water, final) and 1X aprotinin (5000X = 5 mg/ml) and centrifuged at 10,000 rpm for 10 min. To the lysate supernatant, PMSF (prepared in isopropanol, 1 mM final) and KC1 (80 mM final) were added and immediately aliquoted and stored under liquid nitrogen.

Protein estimation was carried out in all the cell extracts using the Bio-rad protein assay kit.

9.3 *Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PA GE):*

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 blotting:***

The proteins separated on SDS-PAGE were transferred onto a nitrocellulose membrane electrophoretically at 70 volts. The transfer was done for 3 h in transfer buffer (25 mM Tris, 195 mM Glycine in 20 % methanol). After the transfer the membrane was stained with ponceau S red 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 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 1 h, the blocking solution was replaced with TBST (TBS with 0.05 % Tween-20) containing the primary antibody in the required dilution for 1 h. The membrane was later washed thrice with TBST for 5-10 min each time to remove the unbound antibody. Later the membrane was incubated in TBST containing the appropriate anti-IgG-AP conjugate for 45 min. The membrane was once again washed thrice with TBST, 5-10 min each time. Then the membrane was developed with a colour development solution with NBT (66 ul) and BCIP (33 µl) as substrates in 10 ml of AP buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$). The colour development was arrested by washing the membranes in distilled water, The membrane was air-dried and stored between filter papers and kept away from light.

CHAPTER I

EFFECTS OF VANADIUM COMPOUNDS ON PROTEIN SYNTHESIS

INTRODUCTION

Vanadium is known as an essential nutrient required by living organisms. High levels of vanadium are toxic as it causes bronchitis and pneumonia. The major sources of vanadium are from burning fossil fuels and from mining (Ramasarma and Crane, 1981). Intracellular concentrations of vanadium, e.g., in erythrocytes, may occur in the micromolar range (Benabe *et al,* 1987). Vanadate (+V) is a diuretic, a natriuretic, and a vasoconstrictor (Nechay *et al,* 1986; Rehder *et al,* 1991; Kramer *et al,* 1995; Balfour *et al,* 1978; Simons, **1979).** Vanadium ions are known to be potent inhibitors of Na^+, K^+ ATPase, Ca^{2+} ATPase, Mg^{2+} ATPase, and adenylate kinase (Simons, 1979; Cantley *et al.,* 1977).

Vanadate inhibits a number of enzymes of the glycolytic pathway (Benabe *et al,* 1987; Simons, 1979). Vanadate compounds are being used as an oral insulin mimetic, although vanadium does not mimic all the actions of insulin (Bosch *et al,* 1987; Clarke *et al,* 1985). Vanadium therapy is given to diabetic patients to normalize blood glucose levels and also to alleviate many of the abnormalities associated with hyperglycemia (Elberg *et al,* 1994; Heyliger *et al,* 1985; Meyerovitch *et al,* 1987; Gil *et al,* 1988). Vanadate is a potent protein tyrosine phosphatase inhibitor (Liao and Lane, 1995). It is also known that vanadyl ions (+IV) inhibit receptor tyrosine kinases like insulin receptor while the vanadate form stimulates cytoplasmic protein tyrosine kinases in rat adipocytes (Elberg *et al,* 1994).

Vanadate that enters the erythrocytes via the phosphate channel is reduced to its vanadyl state by cytoplasmic glutathione, in a nonenzymatic process (Benabe *et al,* 1987; Macara *et al,* 1980; Chasteen, 1983). Earlier studies indicate that vanadyl binds to ATP as well as to a variety of metalloproteins (Benabe *et al,* 1987; Macara *et al,* 1980; Parra-Diaz *et al,* 1995). Recently we have demonstrated that vanadyl (+IV) saccharide complexes introduce nicks in pUC18 DNA and cause lipid peroxidation in isolated rat hepatocytes (Sreedhara *et al,* 1996). Protein synthesis in reticulocyte lysates is known to be regulated by phosphorylation-dephosphorylation of protein factors and also by redox levels (Hershey, 1989; Jackson, 1991; London *et al,* 1987). A previous study (Ranu,

1981) suggests that vanadate impairs protein synthesis initiation but does not reveal its mechanism. In this study, the effect of vanadium compounds in +IV and +V oxidation states on protein synthesis has been investigated. In addition, the ability of reducing agents to counter the vanadium mediated inhibition of protein synthesis has been investigated. This is the first report to our knowledge which suggests that protein synthesis inhibition mediated by vanadium is decreased by reducing agents such as GSH, NADPH and DTT and that vanadyl ions are less inhibitory than vanadate.

Both the species of vanadium do not increase the phosphorylation of the alpha subunit of initiation factor 2 (eIF2) or decrease the guanine nucleotide exchange activity of eIF2B protein, which is required in the recycling of eIF2.GDP binary complex. These negative findings suggest that the mechanism of protein synthesis inhibition in the presence of vanadium species is different than that of other oxidizing agents such as heavy metals or oxidized glutathione or pyrroloquinoline quinone (Hurst *et al.*, 1987; Matts *et al*, 1991; Kosower *et al*, 1972; Palomo *et al*, 1985; Ernst *et al*, 1978; Kan *et al*, 1988; Ramaiah *et al*, 1997). The findings are consistent with the result that GSH can prevent significantly the inhibition of protein synthesis in vanadate-treated hemin-supplemented lysates.

RESULTS

1.0 EFFECT OF VANADIUM ON PROTEIN SYNTHESIS

1.1 *Vanadate and Vanadyl Ions Inhibit Protein Synthesis:*

An earlier study described vanadate inhibition of protein synthesis initiation in reticulocyte lysates (Ranu, 1981). Most of the earlier studies involving vanadium effects on various biological processes or enzyme reactions suffer from a lack of knowledge about the oxidation states of vanadium. In contrast to the earlier studies on protein synthesis in the presence of vanadate, we have evaluated here, the effects of the following four compounds: sodium orthovanadate (+V), vanadyl D-glucose (+IV, vadex), vanadyl diascorbate **(+IV,** vasc), and vanadyl sulfate (+IV). Vadex is a novel anionic saccharide complex which is very stable (Sreedhara *et al.*, 1994). The structures of vadex and vasc are shown in Fig. 10.

All vanadium compounds mentioned above inhibited protein synthesis of both heme-deficient as well as of hemin-supplemented rabbit reticulocyte lysates (Tables 2 to 5). The inhibition was concentration dependent. Among the vanadium compounds tested, sodium orthovanadate (+V) was the most potent and produced maximum inhibition of 70% at 10 μM concentration (Fig. 11). Vanadyl diascorbate and vanadyl sulfate (+IV) were less inhibitory while vadex (+IV) was relatively more inhibitory (Fig. 11).

1.2 *Vanadium Impairs Polysome Formation:*

Addition of one of the compounds, vanadyl D-glucose, to hemin-supplemented lysates decreased the polysomes and increased monosomes (Fig. 12). This finding suggests that protein synthesis inhibition occurs at the initiation step and is in accordance with the previous results (Ranu, 1981) that vanadate inhibits the protein chain initiation.

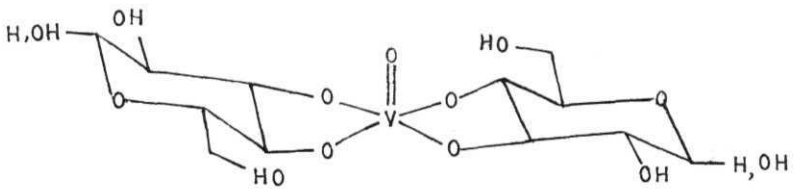
2.0 EFFECT OF VANADIUM ON eIF2α PHOSPHORYLATION

Previous studies have shown that the inhibition of protein synthesis in reticulocyte lysates occurs under a variety of conditions which include addition of oxidants or

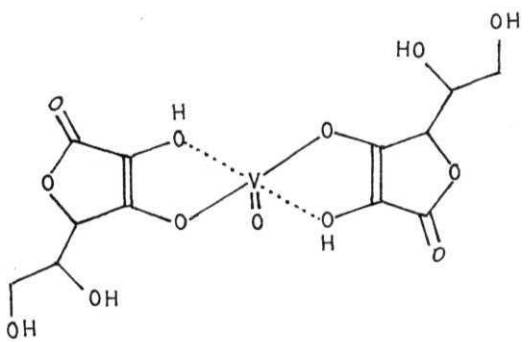
Fig. 10. Vanadium complexes.

Panel A showing the proposed structure of vanadyl- D-glucose (Rajiv and Rao, 1997).

Panel B showing the structure of vanadyl diascorbate (Kramer et al., 1995).



VANADYL D-GLUCOSE



VANADYL DIASCORBATE

Tables 2 to 5

Effect of different vanadium compounds on protein synthesis in reticuocyte lysates. Protein synthesizing lysates (15 μl) were incubated at 30°C under two different conditions: (I) minus hemin (-h), and (ii) plus hemin (+h, 20 μM). Increasing concentrations of different of different vanadium compounds were added at 0 min to separate assays as indicated in the tables. Protein synthesis was measured by using [C]leucine incorporation in 5 μl aliquots at 30 and 60 min as described under Materials and Methods.

TABLE 2
PROTEIN SYNTHESIS IN THE PRESENCE OF SODIUM ORTHOVANADATE

Experimental conditions	[^{14}C]Leu incorporated, cpm		Synthesis %	
	30'	60'	30'	60'
-h	13,301	14,247	100	100
-h+10μM	8,806	10,534	66	74
-h+20μM	4,091	5,241	31	37
-h+50μM	2,387	2,542	18	18
+h	29,795	37,181	100	100
+h+10μM	11,002	10,130	37	27
+h+20μM	5,788	7,126	19	19
+h+50μM	3,029	3,041	10	8

TABLE 3
PROTEIN SYNTHESIS IN THE PRESENCE OF VANADYL D-GLUCOSE

Experimental conditions	[^{14}C]Leu incorporated, cpm		Synthesis %	
	30'	60'	30'	60'
-h	15,050	19,074	100	100
-h+10μM	12,662	16,412	84	86
-h+20μM	13,366	14,228	89	75
-h+50μM	6,428	7,596	43	40
+h	37,546	48,696	100	100
+h+10μM	25,904	34,042	69	70
+h+20μM	12,446	16,128	33	33
+h+50μM	6,170	7,828	16	16

TABLE 4
PROTEIN SYNTHESIS IN THE PRESENCE OF VANADYL DIASCORBATE

Experimental conditions	[^{14}C]Leu incorporated, cpm		Synthesis %	
	30'	60'	30'	60'
-h	12,322	15,826	100	100
-h+10μM	12,366	14,826	100	94
-h+20μM	13,100	15,792	106	98
-h+50μM	11,516	15,260	94	96
+h	26,222	43,670	100	100
+h+10μM	20,826	31,804	79	73
+h+20μM	15,462	20,138	59	46
+h+50μM	11,418	14,708	44	34

TABLE 5
PROTEIN SYNTHESIS IN THE PRESENCE OF VANADYL SULFATE

Experimental conditions	[^{14}C]Leu incorporated, cpm		Synthesis %	
	30'	60'	30'	60'
-h	14,605	17,394	100	100
-h+10μM	16,327	18,599	112	107
-h+20μM	13,614	15,368	93	88
-h+50μM	12,755	14,712	87	85
+h	25,450	38,772	100	100
+h+10μM	20,158	29,225	80	75
+h+20μM	15,099	19,717	59	51
+h+50μM	12,313	14,028	48	36

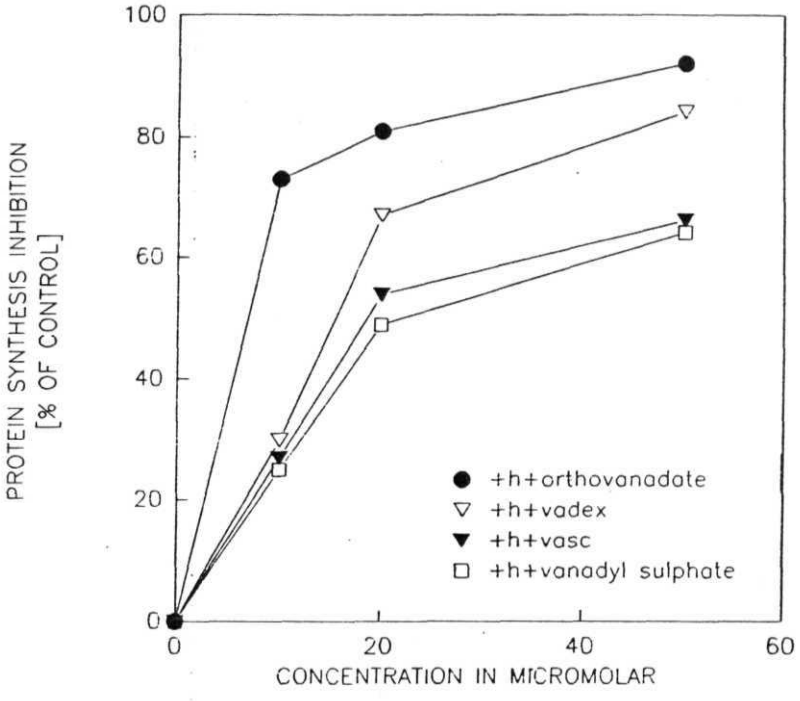


Fig. 11. Percent inhibition in protein synthesis by vanadium compounds at different concentrations in hemin-supplemented reticulocyte lysate at 60 min.

Using the data shown in tables 2 to 5, the percent inhibition was calculated taking hemin-supplemented lysates as the control.

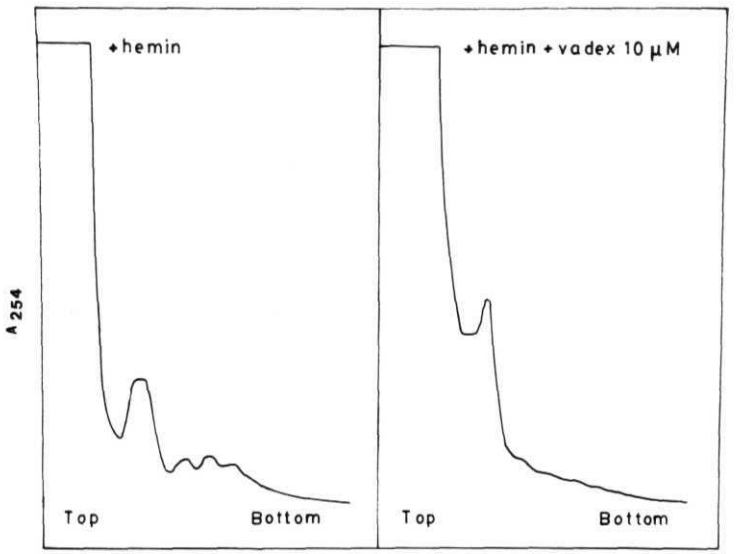


Fig. 12. Effect of vadex on lysate polysomes.

Standard reticulocyte lysate protein synthesis mixtures were incubated for 10 min. at 30°C with 20 μM hemin (A) or 20 μM hemin with 10 μM vadex. The mixtures were then diluted, loaded on the sucrose gradient, centrifuged and analyzed as described in Materials and Methods. At 254 nm, the OD was taken with a chart speed of 300 cm/h, absorption of 1.0, and a flow rate of 3 ml/min.

sulfhydryl reactive agents, heavy metals and heat stress (reviewed in Jackson, **1991;** London *et al.,* 1987). Under all these conditions, the protein synthesis inhibition **occurs** apparently due to the activation of a kinase with properties similar to heme-regulated inhibitor (HRI) which stimulates phosphorylation of the alpha or small subunit of initiation factor 2 (eIF2α) (reviewed in Chen and London, 1995).

2.1 ***Vanadyl D-glucose does not affect eIF2α phosphorylation in vitro:***

Phosphorylation of eIF2α was studied both *in vitro* as well as in situ in lysates treated with vanadium. *In vitro* phosphorylation of eIF2a by HRI is unaffected by vadex (Fig. 13) There is no enhancement in the phosphorylation of eIF2α in the presence of vadex (10 μM) over the control, eIF2 + HRI (compare lanes 3 and 4 in Fig. 13). Phosphorylation of eIF2(3 is observed, indicating that some amount of CK II is present in the eIF2 preparations (lane 5 in Fig. 13).

2.2 ***Vanadyl D-glucose has no effect on eIF2α phosphorylation in situ:***

In the in situ phosphorylation assays, protein synthesis was carried out in reticulocyte lysates under a variety of conditions as described in the legend to Fig. 14. *[y~* ^{32}P]ATP (3000 Ci/mmol) pulse was given at 7-12 min and at 15-20 min. In heme-deficient lysates, a 38 kDa protein (eIF2α) gets phosphorylated which is prominently absent in hemin-supplemented lysates (lanes 1 and 2 of Fig. 14). Vadex, at 10 and 20 uM concentration, did not affect the eIF2α phosphorylation profile of hemin-supplemented lysates (lanes 3, 4, 6 and 7). DTT, 1 mM, was added to vadex-treated lysates to check if DTT has any effect on vadex-mediated eIF2α phopshorylation status, if any. As evident from the Fig. 14, there is no change in eIF2a phosphorylation in vadex and DTT-treated hemin-supplemented lysates.

2.3 ***Effect of different vanadium compounds on eIF2 phosphorylation in situ:***

To see if there is any difference among the vanadium compounds on their effect on eIF2α phosphorylation, phosphoprotein profiles of protein synthesizing hemin-

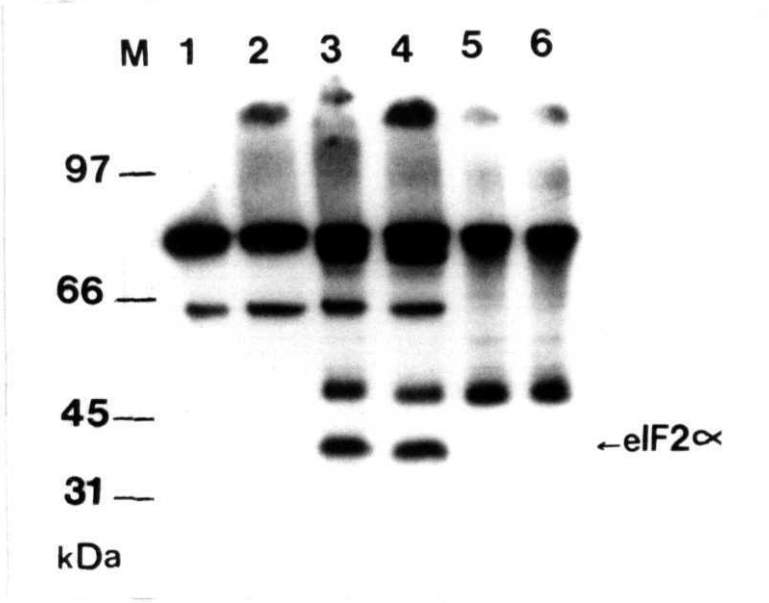


Fig. 13. Effect of vanadyl D-glucose on HRI and eIF2 phosphoryiation *in* *vitro*.

An autoradiogram of eIF2 phosphorylated by HRI kinase ***in*** *vitro*. eIF2 was incubated with 5 μCi of [γ^{-32}P]ATP in a standard reaction mixture (20 μl) as described in 'Materials and Methods' before separating them on 10 % SDS-PAGE gel. 10 μM of vadex was used wherever mentioned. Lane 1, HRI; lane 2, HRI + vadex; lane 3, HRI + vadex + eIF2; lane 4, HRI + eIF2; lane 5, eIF2; lane 6, eIF2 + vadex.

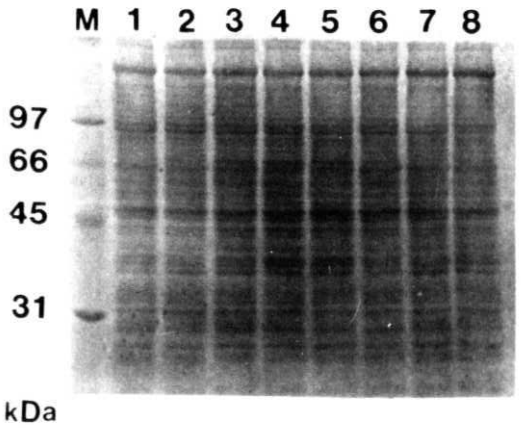
Fig. 14. *In situ* **phosphorylation of eIF2 in the presence of vadex.**

Protein synthesizing lysates (15 µl) containing unlabeled leucine were pulsed with [γ^{-32}P]ATP (5 µCi) at 7-12 or 15-20 min. The reactions were terminated 5 min after the pulse and lysate proteins were pH 5.0 precipitated. The samples were processed as described in the 'Materials and Methods' and were analyzed on 10 % SDS-PAGE gel and later by autoradiography.

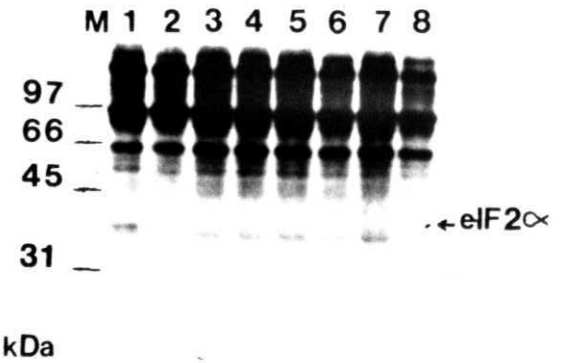
Panel A is the coomassie stained gel having in lane 1, -heme lysate; lane 2, hemin supplemented lysate, -h (20 µM); lane 3, +h +vadex, 10 µM; lane 4, +h +vadex, 20 µM; lane 5, +h +vadex. 10 uM +DTT, 1 mM; lane 6, +h +vadex, 10 uM; lane 7, +h +vadex, 20 µM; lane 8. +h -vadex, 10 µM +DTT, 1 mM. Lysates of lanes 1 to 5 were pulsed with [y* P]ATP at 7-12 min while for lysates in lanes 6 to 8, the pulse was given at 15-20 min.

Panel B is an autoradiogram of the above gel. It shows the phosphoprotein profile of pH 5 precipitated lysate proteins.

A



B



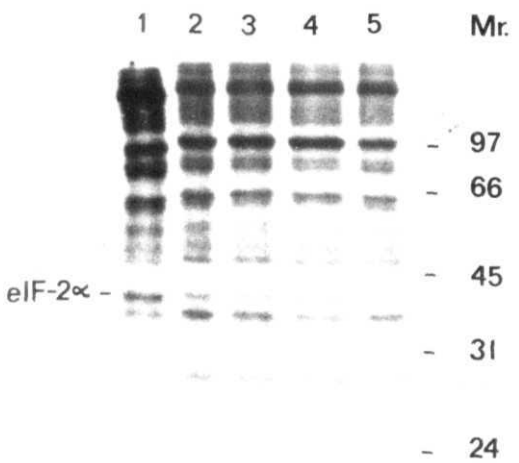


Fig. 15. Effect of different vanadium compounds on [^{32}P] phosphoprotein profiles of protein synthesizing lysates.

This is an autoradiogram of the phosphorylation profile of reticulocyte lysates treated as follows: Lane 1, heme-deficient lysate, -h; lane 2, hemin-supplemented lysate, +h; lane 3, +h +20 uM vadex; lane 4, +h +20 uM vasc; lane 5, +h + 20 μM orthovanadate. Assays were pulsed with [γ^{-32}P]ATP at 10-15 min. of protein synthesis. Samples of each assay were pI I 5.0 precipitated and then separated on 10 % SDS-PAGE gels. 20 uM hemin was used wherever mentioned.

supplemented reticulocyte lysates were checked both in the presence as well as in the absence of vanadium compounds (Fig. 15). None of the vanadium compounds affected eIF2α (38 kDa) phosphorylation.

2.4 *Reducing Agents Like DTT, βNADPH and GSH Mitigate Vanadium Mediated Inhibition:*

Vanadate is hypothesized to interact with protein -SH groups of the enzymes like glyceraldehyde 3-phosphate dehydrogenase and consequently inhibit their activity (Benabe *et at,* 1987). Also there are reports that vanadate is converted to its vanadyl state in erythrocytes by intracellular glutathione (GSH) (Benabe *et at,* 1987; Macara *et at,* 1980; Chasteen, 1983). Hence, we have studied here the effects of DTT, βNADPH, and GSH, as well as low and high concentrations of sugar phosphates on protein synthesis in vanadium-treated lysates. DTT and βNADPH mitigated significantly the inhibition caused by all vanadium compounds at a low concentration (10 uM, Tables 6A and 6B) but were unable to act effectively to counter the inhibition caused by high concentrations of vanadate on protein synthesis (Table 6C). We have also studied the effect of GSH and Glu-6-P in vanadate-treated lysates. It was observed that while GSH could decrease the inhibitory effect of vanadate on protein synthesis, a similar effect could not be obtained with the addition of low and high concentrations of Glu-6-P (Table 6B) suggesting that vanadate does not inhibit protein synthesis simply by generating an oxidizing stress which depletes the Glu-6-P of the lysate. However, the results do not reveal if vanadate can affect the enzyme, Glu-6-P dehydrogenase. The effect of reducing agents to overcome the inhibition of protein synthesis caused by low concentrations of vanadate or vanadyl compounds is so impressive that it compels us to suggest that the mechanism of action is similar for both oxidation states of vanadium. Since vanadate is converted to vanadyl state intracellularly, an experiment was conducted to follow the fate of one of the vanadyl compounds, vadex, in lysates by electron paramagnetic spectra (EPR). The results of this experiment indicated that vadex remained in the +IV oxidation state (data not shown).

TABLE 6A
EFFECT OF pNADPH AND DTT ON PROTEIN SYNTHESIS IN VANADIUM- TREATED LYSATES

Addition	Incorporation (% of control)		
Reducing agent present:	None	NADPH	DTT
None	100	80	105
Vanadate	36	69	77
Vadex	50	88	87
Vasc	73	87	82
$VOSO_4$	85	95	103

Protein synthesis was carried out as described in the legend to Fig. 1 under the following conditions: (i) plus hemin (+h, 20 μM), (ii) + h +10 μM vanadium compound, (iii) +h +1 mM DTT, (iv) +h +1 mM pNADPH, (v) +h +10 μM vanadium compound +1 mM DTT, and (vi) +h +10 μM vanadium compound +1 mM PNADPH. Protein synthesis was measured in 5 μl aliquots at 60 min. as described under Materials and Methods.

TABLE 6B
EFFECT OF GSH AND GLUCOSE 6-PHOSPHATE ON PROTEIN SYNTHESIS IN 15 μM VANADIUM-TREATED LYSATES

Vanadate concentration:	0	15 μM
Addition		
None	100	56
DTT, 1 mM	113	83
GSH, 1 mM	118	85
Glu-6P, 50 μM	111	57
Glu-6P, 1 mM	97	55

Separate protein synthesizing lysates (20 μl) were carried out at 30°C in the presence of hemin, 20 μM. 15 uM sodium orthovanadate and 1 mM each DTT and GSH were used wherever indicated in the table. Two different concentrations of Glu-6-P, 50 and 1 μM, were used as shown. All additions were done at 0 min. Protein synthesis was monitored in 5 μl aliquots at 60 min.

TABLE 6C
EFFECT OF DTT AND (3NADPH ON PROTEIN SYNTHESIS IN 10-50 μM VANADATE-TREATED LYSATES

Vanadate concentration:	Incorporation (% of minus vanadate)			
Addition	0	10	20	50
None	18800(0)	12500(0)	7200(0)	3160(0)
DTT	23700 (+26)	19500 (+56)	11000 (+57)	4200 (+35)
NADPH	20000 (+7)	15700 (+25)	9500 (+33)	3700 (+19)

Protein synthesis was carried out in 15 μl volume in the presence of hemin or hemin and different concentrations of vanadate at 30°C as described in the legends to tables 6 A and 6B. The extent of protection in protein synthesis offered by reducing agents (1 mM DTT or pNADPH) in lysates treated with different concentrations of vanadate (10, 20, and 50 uM) has been determined.

2.5 ***eIF-2B Activity is Not Affected in Lysates Treated With Low Concentration of Vanadate:***

Phosphorylated eIF2α inhibits the guanine nucleotide exchange activity of eIF2B protein, which is required in the exchange of GTP for GDP in eIF2 . GDP binary complex, and thereby inhibits the recycling of eIF2 and initiation of protein synthesis (Matts and London, 1984; Naresh Babu and Ramaiah, 1996; Ramaiah *el al,* 1994). In addition to eIF2a phosphorylation, eIF2B activity is regulated by changes in redox levels (Ramaiah *et al,* 1997; Dholakia *et al,* 1986; Akkaraju *et al,* 1991; Oldfield and **Proud,** 1992) and the phosphorylation of the largest subunit (82 kDa) in eIF2B (Dholakia and Wahba, 1988; Welsh *et al.,* 1996).

To find out if vanadium has any effect on eIF2B activity independent of eIF2a phosphorylation, we have also studied the eIF2B activity of vanadium-treated lysates by monitoring the release of labeled GDP from a preformed eIF2.[^{3}H[GDP binary complex as has been described (Matts and London, 1984; Naresh Babu and Ramaiah, 1996; Ramaiah *et al,* 1994). As DTT is able to mitigate the inhibition of protein synthesis caused by vanadium compounds and DTT is also used generally in the preparation of binary complex *in vitro,* a control experiment was conducted in the presence and absence of DTT, to determine the changes in lysate eIF2B activity under standard protein synthesizing conditions, i.e., a) hemin-supplemented lysates which can carry active protein synthesis and b) hemin and poly (IC)-treated lysates where protein synthesis is inhibited due to eIF2a phosphorylation and inhibition in eIF2B activity (Table 7). The assay was carried out using a binary complex, eIF2.[^{3}H]GDP, which was prepared with or without DTT. The dissociation of the labeled GDP was monitored for a period of 5 min. at 30°C. The dissociation of eIF2.[^{3}H]GDP binary complex (prepared in the presence or absence of DTT) was typically inhibited in poly (IC)-treated lysates as expected due to enhanced eIF2α phosphorylation. However, the levels of dissociation of preformed binary complex in lysates differ depending on the presence or absence of DTT. The above preformed binary complex was more rapidly dissociated in +heme lysates and

TABLE 7
EFFECT OF DTT ON eIF2.[^{3}H]GDP DISSOCIATION IN HEME OR HEME AND POLY (IC)-TREATED LYSATES

Protein synthesis conditions	eIF2.[^{3}H]GDP bound, cpm x 10^-	eIF2.[^{3}H]GDP dissociated in pmols (5 min)
-DTT +h, 0'	20.7	
-DTT+h	7.9	11.61
-DTT +h +Poly (IC)	15.2	5.0
+DTT+h,0'	21.8	
+DTT+h	3.5	16.6
+DTT +h +Poly (IC)	12.6	8.4

Protein synthesizing lysates (25 µl) were incubated at 30 C in the presence of hemin or hemin and poly (IC) [20 uM hemin, 300 ng/ml poly (IC)] for 10 min. Labeled binary complex, eIF2.[^{3}H]GDP was prepared in the presence and absence of 1 mM DTT and added to the above translating lysates at 10 min. of protein synthesis. The reactions were incubated for a period of 5 min. at 30°C to determine the dissociation of labeled GDP from the binary complex.

TABLE 8
EFFECT OF DIFFERENT CONCENTRATIONS OF ORTHOVANADATE ON THE DISSOCIATION OF eIF2.[^{3}H]GDP COMPLEX AND ON PROTEIN SYNTHESIS IN HEME OR HEME AND POLY (IC)-TREATED LYSATES

Protein synthesis incorporation conditions	eIF2.[^{3}H]GDP dissociated in pmols (15 min)	[^{14}C]Leu cpm x 10^{-3} (30 min)
+h	16.88	13.7
+h +10μM Na_3VO_4	15.76	9.2
+h +20μM Na_3VO_4	16.24	3.7
+h +50μM Na_3VO_4	16.66	2.5
+h+Poly(IC)	0.0	10.1
+h +Poly (IC) +10μM Na_3VO_4	1.91	5.8
+h +Poly (IC) +20μM Na_3VO_4	2.37	3.0
+h +Poly (IC) +50μM Na_3VO_4	3.76	2.6
+h +DTT +10μMNa_3VO_4	16.98	
+h +DTT +20μM Na_3VO_4	17.07	
+h +DTT +50μM Na_3VO_4	17.76	

Hemin-supplemented (20 μM) protein synthesizing lysates (25 μl) were incubated at 30°C in the presence of various agents as shown in the table. DTT (1 mM) and 300 ng/ml of poly (IC) were used where indicated. At 15 min. of protein synthesis, 20 ul of preformed binary complex, formed in the absence of DTT [21 pmols, eIF2.(^{3}H)GDP] was added and its dissociation was assayed for 5 min. at 30°C as described under Materials and Methods. In another set of protein synthesizing lysates, using [^{14}C]leucine cocktail, protein synthesis was carried out in hemin-supplemented lysates in the presence of poly (IC) or poly (IC) and orthovanadate. Protein synthesis was carried out in 25 ul under the following conditions: (i) plus hemin (+h); (ii) +h + poly (IC), 300 ng/ml; (iii) +h +orthovanadate; and (iv) +h +orthovanadate +poly (IC). Protein synthesis was carried out at 30°C and monitored in 5 ul aliquots at 30 min. as described under Materials and Methods.

strongly inhibited in +heme and poly (IC)-treated lysates in the presence of DTT than without DTT (Table 7). These findings are consistent with the idea that reducing conditions stimulate eIF2B activity (Ramaiah *et al,* 1997; Akkaraju *et al,* 1991; Oldfield and Proud, 1992; Dholakia and Wahba, 1988) and also eIF2a kinase activity (reviewed in Jackson, 1991; London *et al,* 1987; Chen and London, 1995) of heme-deficient reticulocyte lysates.

Since omission of DTT in the preparation of binary complex does not alter the trend in the inhibition in eIF2B activity of lysates caused by poly (IC) treatment, a similar experiment was carried out to determine the eIF2B activity of lysates treated with different concentrations of vanadate or vanadate and DTT (Table 8). Presence of low concentrations (10 and 20 μM) of vanadate inhibited marginally the dissociation of binary complex, whereas poly (IC) treatment caused a maximum inhibition in the dissociation of binary complex. This comparison reveals that the minimal inhibition in eIF2B activity by vanadate is unlike the inhibition in eIF2B activity caused by poly (IC). Addition of 1 mM DTT to vanadate-treated lysates (10 or 20 μM) could overcome this marginal inhibition in eIF2B activity as the dissociation of binary complex increased and was on par with +heme lysates. The ability of DTT to reverse the small decrease in eIF2B activity observed in the presence of vanadate (Table 8) can be best explained by the ability of DTT to stimulate eIF2B activity (Table 7) and protein synthesis (Tables 6A-6C) in untreated lysates. This may be a consequence of the natural depletion of the lysate reducing ability during prolonged incubation of the lysate. Hence the findings suggest that low concentrations of vanadate which inhibits protein synthesis significantly do not appear to affect eIF2B activity.

In contrast, higher concentrations (50 uM) of vanadate which inhibited protein synthesis more severely (Fig. 11) were also found to enhance the dissociation of eIF2 . GDP binary complex in lysates (Table 8). This latter finding suggests that the dissociation of eIF2.GDP binary complex does not truly represent the functional eIF2B activity. To confirm this artifactual situation, the dissociation of preformed eIF2.[H]GDP binary complex and protein synthesis were monitored in poly (IC)-treated lysates which

were supplemented with the increasing concentrations of vanadate. The results suggest that the dissociation of the above binary complex increases with increasing concentrations of vanadate in poly (IC)-treated lysates, whereas the protein synthesis is further reduced in poly (IC)-treated lysates (Table 8). Hence the dissociation of binary complex in the presence of higher concentrations of vanadate does not appear to be mediated by eIF2B protein. The binary complex, eIF2.["H]GDP dissociation varies with higher vanadate concentrations in lysates and the variation is also contributed by lysates in which eIF2α is unphosphorylated or phosphorylated [+heme or +heme +poly (IC)]. This apparent discrepancy may be due to the fact that vanadate at higher concentrations may be competing with a phosphate for the enzyme binding site (Simons, 1979; Ranu, 1981).

DISCUSSION

Earlier studies indicate that cytoplasmic vanadate is reduced to the +IV oxidation state (Benabe *et al.,* 1987; Balfour *et al,* 1978; Chasteen, 1983). Effects of such vanadyl complexes on various cellular activities have not been well documented. In an earlier communication (Sreedhara *et al,* 1994), reduction of vanadate by multihydroxy molecules such as saccharides and ascorbic acid was reported. The vanadyl complexes thus formed were able to produce nicks in pUC18 DNA and also exhibit lipid peroxidation in isolated rat hepatocytes (Sreedhara *et al.,* 1996). These saccharide and ascorbate complexes of vanadium were found to be hydrolytically and oxidatively stable in lysates (data not shown). The availability of these vanadyl complexes allowed us for the first time to evaluate the effects of vanadate and vanadyl compounds directly on total protein synthesis in hemin-supplemented rabbit reticulocyte lysates. In addition, reticulocyte lysate is a good translational model system to investigate the mechanism of action of various agents such as heavy metals, oxidizing/reducing agents, antibiotics, and lectins, on protein synthesis *in vitro.* The reticulocyte extracts contain a number of reducing agents common to cells like L-ascorbic acid, glutathione, and cysteine, which can convert vanadate (+V) to vanadyl (+IV) state. A very early study (Ranu, 1981) is the only report that we are aware of, suggesting that vanadate (10-40 μM) inhibits protein chain initiation in reticulocyte lysates and produces defective 80S initiation complexes. This study also points out that higher concentrations of vanadate (above 50 uM) inhibit polypeptide chain elongation to some extent. Consistent with this observation, our results also indicate that vanadium inhibits protein synthesis initiation (Tables 2-5, Figs. 11 and 12). In addition, we observed that vanadate inhibits protein synthesis more strongly than vanadyl complexes. Also the protein synthesis inhibition caused by low concentrations of vanadium (10 and 20 μM) can be easily mitigated by reducing compounds like PNADPH, DTT, or GSH but not by glucose 6-phosphate (Tables 6A and 6B).

These findings suggest that both species of vanadium can deplete the reducing power of the lysate. Consistent with the idea that vanadate can be reduced to vanadyl state in the presence of GSH or NADPH (Macara *et al,* 1980), we find here that the

inhibition of protein synthesis is more severe in the presence of vanadate ions (Fig. 11). This suggests that vanadate can more effectively deplete the lysate GSH or NADPH for its reduction than vanadyl ions. Also, we observed that vanadyl compounds are not reduced any further in the lysate during the reaction period (data not shown).

Previous studies have shown that maintenance of protein synthesis initiation in rabbit reticulocyte lysates requires the presence of hemin, a sugar phosphate (e.g. Glu-6-P) and a reducing system which is capable of reducing disulfide bonds. Sugar phosphates are required as a stimulating 'cofactor' affecting protein chain initiation and for NADPH generation by way of metabolism through pentose phosphate shunt (reviewed in Jackson, 1991; London *et ai,* 1987). The requirement for reducing power is thought to be met by this NADPH generation, together with an active thioredoxin/thioredoxin reductase system. A variety of conditions which include oxidants or sulfhydryl reactive agents, heat stress, and heavy metals (reviewed in Jackson, 1991; Chen and London, 1995; Hunt, 1979) have been shown to inhibit protein synthesis in hemin-supplemented reticulocyte lysates due to the activation of heme-regulated eIF2α kinase which subsequently stimulates eIF2α phosphorylation. As a consequence, the guanine nucleotide exchange activity of eIF2B, which is required in the recycling of eIF2 . GDP binary complex, is inhibited. In addition to oxidative stress, the activation of heme-regulated eIF2a kinase is also dependent on its association with heat shock proteins and is influenced by hemin, ATP, and GTP (reviewed in Chen and London, 1995).

The inhibition of protein synthesis which is accompanied by enhanced eIF2α phosphorylation in lysates treated with heavy metals (Hurst *et al.,* 1987; Matts *et ai,* 1991) or with GSSG (Kosower *et ai,* 1972; Palomo *et ai,* 1985; Ernst *et ai,* 1978; Lan *et ai,* 1988) can be reversed by DTT but not by GSH. These findings suggest that sulfhydryl groups present in some critical protein(s), possibly dithiols present in thioredoxin reductase, thioredoxin, and or heat shock proteins, are modified in the presence of the above treatments, which lead to the activation of HRI and enhanced eIF2α phosphorylation.

In our studies here, vanadate or vanadyl species have not been able to stimulate eIF2a phosphorylation (Figs. 13-15) or to decrease significantly the lysate eIF2B activity (Table 8). Moreover, GSH, a monothiol, which cannot prevent or restore protein synthesis caused by oxidizing metals or GSSG (Matts *et al.,* 1991; Palomo *et al.,* 1985), has been found here to prevent significantly protein synthesis inhibition caused by vanadate. These findings emphasize that the mechanism of protein synthesis inhibition in vanadate-treated lysate appears to be different from the inhibition caused by other oxidizing metal ions or GSSG. The reducing agents which are found here to prevent significantly the protein synthesis inhibition in vanadium-treated lysates not only are the reductive type of agents but also can complex with metal ions. Hence, it is likely that vanadyl species generated due to reduction of vanadate or added vanadyl compounds are able to deplete the lysate GSH or NADPH by complexing with them and thereby causing inhibition of protein synthesis in reticulocyte lysates. Other possibilities which need to be tested include binding of vanadyl ions to some critical proteins which are required for protein synthesis and thereby modifying their activities.

CHAPTER II

CLONING AND CHARACTERIZATION OF THE BACULOVIRUS-EXPRESSED HUMAN eIF2 ALPHA SUBUNIT

INTRODUCTION

The availability of site-specific mutants of human eIF2a like the 48A or 51A in which the serine residues in the respective positions of eIF2a have been replaced by alanine has advanced our understanding in identifying that a) serine 51 residue is the only phosphorylation site in mammalian eIF2a (Pathak *et al,* 1988), b) translation block caused by adenoviral mRNAs, plasmid-derived mRNAs, heat shock or calcium sequestration is due to increased eIF2α phosphorylation (Kaufman *et al,* 1989; Choi *et al,* 1992; Murtha-Riel *et al,* 1993) or in localizing the translational inhibition caused by eIF2α phopshorylation and c) phosphorylation of eIF2α plays a critical role in cell proliferation (Donze *et al,* 1995). Also, the co-expression of a mutant eIF2α which cannot be phosphorylated has facilitated the expression of mammalian eIF2α kinases like heme-regulated inhibitor in insect cells (Chefalo *et al,* 1994).

In order to understand the mechanism of regulation of eIF2B activity by phosphorylated eIF2α, we have used the baculovirus system to overproduce human eIF2α wt and eIF2α 48A mutant. eIF2a wt represents wild type human eIF2a cDNA (1.6 kb). This subunit can be phosphorylated on its serine-51 residue. This is the only site of phosphorylation in human eIF2a. Phosphorylation of eIF2a inhibits the guanine nucleotide exchange activity of eIF2B and thereby inhibits protein synthesis. In eIF2a 48A mutant, the serine-48 residue has been replaced by alanine by site specific mutagenesis (Kaufman et al., 1989). Both the cDNAs were kind gifts from Prof. Randal Kaufman, Department of Biological Chemistry, Howard Hughes Medical Institute, University of Michigan, USA.

eIF2α 48A can be phosphorylated on its 51 serine residue and has been shown to rescue protein synthesis inhibition caused by PKR (Kaufman *et al,* 1989; Srivastava *et al,* 1995). Consistent with these earlier observations, our findings indicate that baculovirus-expressed 48A mutant eIF2a mitigates the inhibition of eIF2B activity of reticulocyte lysates caused by eIF2α phosphorylation via PKR kinase activity and reduces the affinity for eIF2B when eIF2α is phosphorylated.

Fig 16. Schema for expression of proteins.

This is a flow chart depicting the various steps involved in cloning and expression of human eIF2α wild type and 48A mutant in *Spodoptera frugiperda* (Sf9) insect cells using the baculovirus expression system.

CLONING AND EXPRESSION OF HUMAN WT AND MUTANT eIF2a IN INSECT CELLS USING BACULOVIRUS EXPRESSION VECTOR SYSTEM

A FLOW CHART

Transformation, Amplification, Purification and Excision of human eIF2a cDNA (wild type and a mutant) from the parent vector, **pETFVA**

↓

Ligation of eIF2a cDNA insert into baculovirus transfer vector, pBacPAK8

Transformation of DH5a cells by **recombinant** vector pBacPAK8 and purification

↓

Preparation of a radioactive probe of the insert to identify colonies 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 Spodoptera frugiperda (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 eIF2a insert

↓

Plaque assay and amplification of recombinant virus

↓

Identification of positive plaques by Dot-blot hybridization, analysis of the recombinant protein by SDS-PAGE, Immunoblot analysis and functional characterization by Phosphorylation assay

We have used the Baculovirus Expression Vector System to express the human **eIF2a** wt and 48A mutant in Sf9 cells. A flow chart of the various steps followed in the cloning and expression of these two cDNAs is shown in Fig. 16. The cDNAs were present in parent vectors, pETFVA˙. The target gene had to be recloned into a transfer vector, pBacPAK8 (Clontech). pBacPAK8 has a plasmid orign of replication and an ampicillin resistance gene for propagation in *E. coli* cells, but they are unable to replicate in insect cells. It also has the powerful polyhedrin locus and the flanking sequences, **but** the polyhedrin coding sequence has been replaced by multiple restriction sites. The modified transfer vector harboring the target gene is co-transfected with a viral expression vector, BacPAK6 (*Bsu*36 I digest) into insect cells. A double recombination between the transfer vector and the viral expression vector will yield a viable recombinant virus, capable of expressing the target gene.

RESULTS

1.0 CLONING

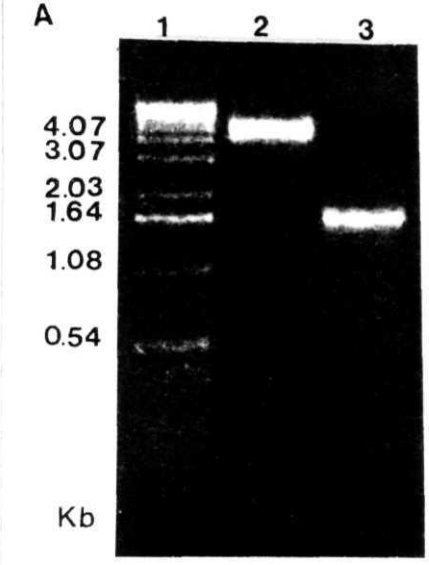
1.1 *Preparation of the recombinant baculovirus transfer vector:*

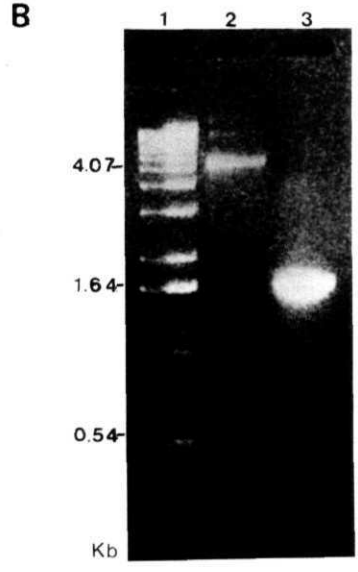
DH5α cells were transformed with the parent vector harboring wt or 48A mutant eIF2a cDNA to amplify the parent vector. Vector DNA was isolated using the Qiagen column and digested with *Eco*R I to separate the 1.6 Kb eIF2a insert. The insert was electroeluted from 1% agarose gel and purified by Qiaquick spin column. Figs. 17A **and** 17B show the linearized pBacPAK8 *(EcoR* I digested) vector (5.5 Kb) and both the eIF2a cDNAs. The eIF2a inserts were ligated to the transfer vector, pBacPAK8 *(EcoR* I digested) adjacent to the powerful polyhedrin promoter. An in-gel hybridization was done to check if a probe prepared from the 48A mutant could identify the eIF2α wt (Fig. 18). It was observed that the mutant probe can be used to identify the eIF2a wild type as well. DH5α cells were transformed with the recombinant pBacPAK8 vector and the positive colonies containing the vector with eIF2a insert were identified by colony hybridization (Anjali, 1996). Figs. 19A and 19B show the positive colonies obtained after colony hybridization. The insert orientation in the vector was checked by double restriction

Fig. 17A and B. 1% agarose gel showing the linearised vector DNA, pBacPAK8 and 1.6 Kb fragment of eIF2α cDNA (wt or 48A mutant).

Panel A: Lane 1, marker DNA; lane 2, pBacPAK8 viral DNA treated with *EcoR* I; lane 3, 1.6 Kb fragment of eIF2a cDNA wild type.

Panel B: Lanes 1 and 2 are similar as in Panel A. Lane 3, 1.6 Kb fragment of eIF2a cDNA 48A mutant.


A
1
2
3
4.07
3.07
2.03
1.64
1.08
0.54
Kb


B
1
2
3
4.07
1.64
0.54
Kb

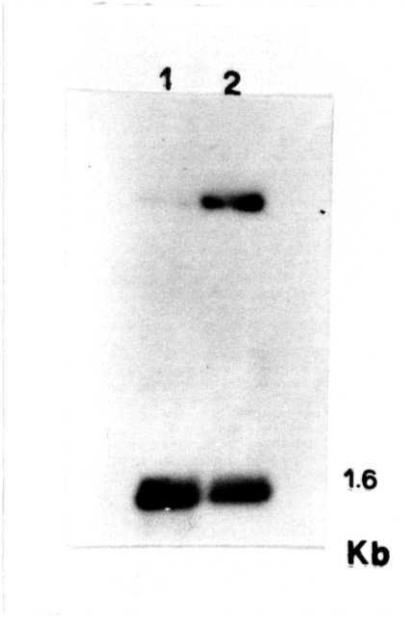


Fig. 18. In gel hybridization.

A [α^{-32}P] dCTP labelled eIF2α probe was prepared by random primer labelling using eIF2α 48A mutant as the template. This probe was used to identify the eIF2α cDNAs. The 1 % agarose gel on which the cDNAs were run was directly incubated with the probe as described under Materials and Methods. Lane 1, *EcoR* I digested parent vector containing the eIF2α wt; lane 2, *EcoR* I digested parent vector containing eIF2a 48A mutant.

Fig. 19. Colony hybridization to identify DH5α cell colonies containing pBacPAK8 harboring *wt* or mutant eIF2a insert.

Panel A: Positive colonies containing pBacPAK8 transfer vector with wild type eIF2a insert. A total of 200 transformed colonies were screened using a radioactive probe against eIF2α cDNA and the positive colonies were identified by autoradiography. The film was exposed to the membrane for 6 h at -70°C.

Panel B: Positive colonies containing pBacPAK8 tranfer vector with eIF2a 48A mutant. A total of 200 transformed colonies were screened and the positive colonies were identified by autoradiography. The film was exposed to the membrane for 2 1/2 **h** at -70°C.

A

B

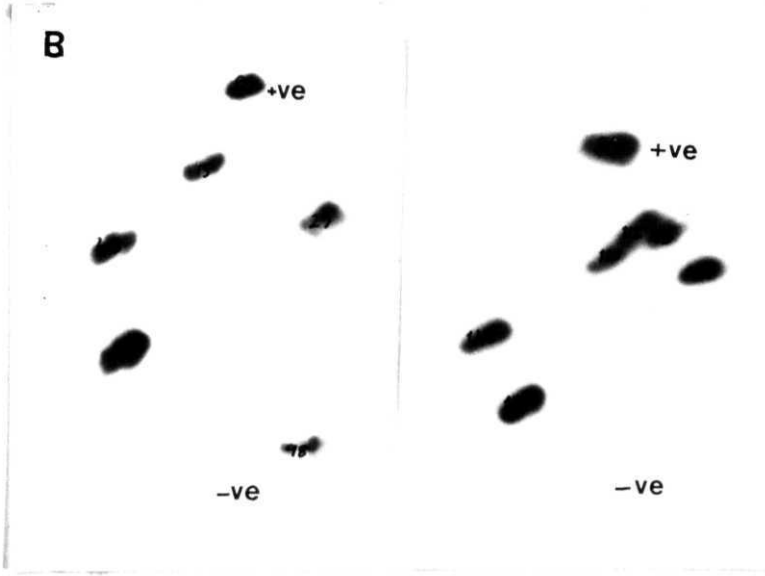
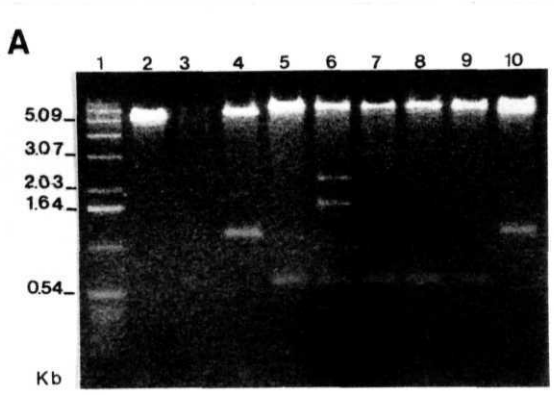
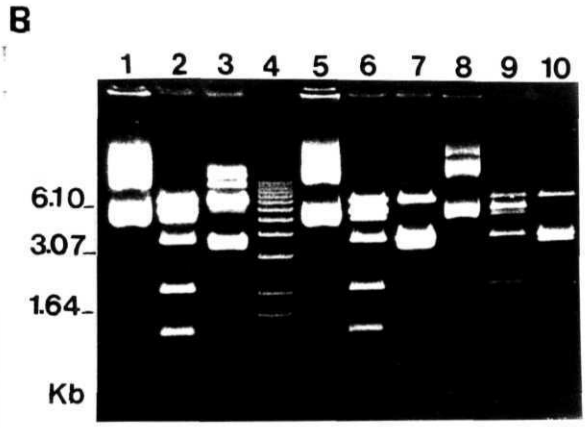


Fig. 20. Identification of the orientation of the wild type eIF2a insert in the vector, pBacPAK8.

Panel A: Six positive colonies identified by colony hybridization were randomly picked and their plasmid DNA digested with *Mlu* I and *Sca* 1 and then loaded on a 1 % agarose gel. eIF2a wt insert in lanes 1, 3 and 7 are found to be in the right orientation since the size of the fragments match with the expected values. Lanes 2, 5 and 6 contain plasmid DNA from colonies which have the insert in the wrong orientation. Lane 4 is the marker lane, 1 Kb DNA ladder.

Panel B: DNA from the right oriented colonies were cut with *Sca* I and *Mlu* I or with *Sca* 1 alone and loaded on a 1 % agarose gel. Lane 4 is the DNA marker lane. Lanes 1, 5 and 8 contain uncut DNA. Lanes 2, 6 and 9 show the DNA upon double digestion with *Sca* I and *Mlu* I from three different colonies. Plasmid DNA from the three different colonies were cut with *Sca* I alone and is seen in lanes 3, 7 and 10.


A
1 2 3 4 5 6 7 8 9 10
5.09
3.07
2.03
1.64
0.54
Kb


B
1 2 3 4 5 6 7 8 9 10
6.10
3.07
1.64
Kb

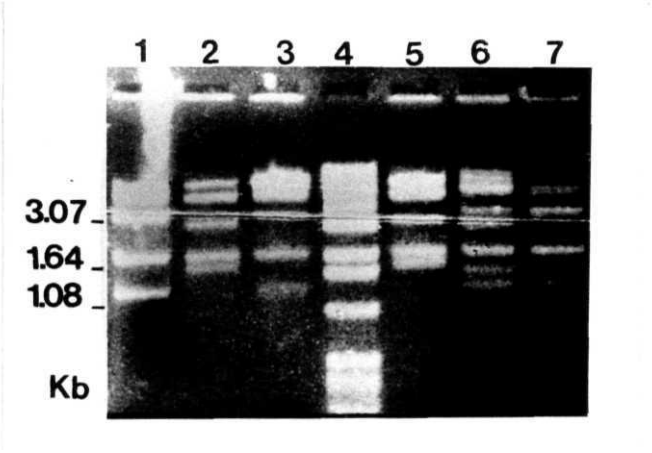


Fig. 21. Identification of the orientation of eIF2α 48A insert in pBacPAK8.

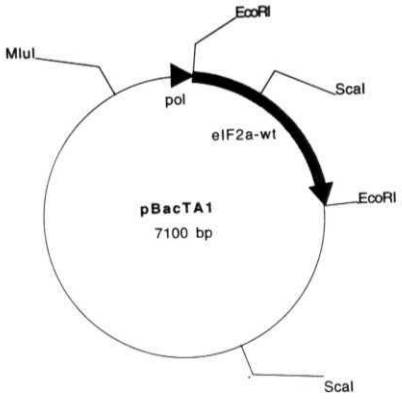
Eight colonies, identified by colony hybridization technique were randomly picked and their plasmid DNA treated with *BamH* I and *Sph* I. Lane 1 DNA ladder; lane 2, vector, pBacPAK8 cut with *BamH* I and *Sph* I; lanes 5, 7, 8 and 9 contain the insert in the right orientation. Lanes 3, 4, 6 and 10 show the plasmid DNA from colonies which have the insert in the wrong orientation.

Fig. 22. Schematic representation of the baculovirus constructs.

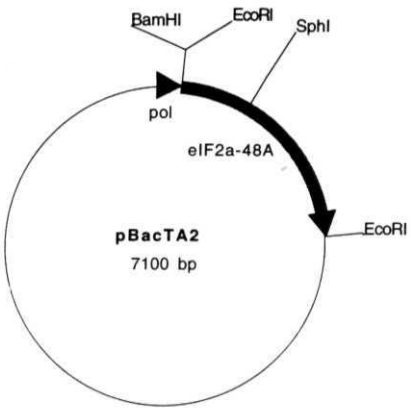
Panel A: pBacTAl having the insert, human eIF2α wt in the right orientation. The constructs also show the restriction sites that were used to check the orientation of the inserts.

Panel B: pBacTA2 having the insert, human eIF2α 48A mutant in the right orientation.

A


EcoRI
MluI
ScaI
pol
eIF2a-wt
EcoRI
pBacTA1
7100 bp
ScaI

B


BamHI
EcoRI
SphI
pol
eIF2a-48A
EcoRI
pBacTA2
7100 bp

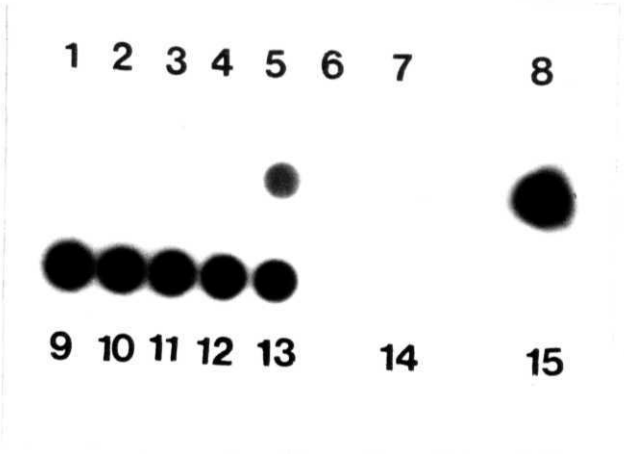


Fig. 23. Dot blot hybridization.

A radioactive probe against eIF2α cDNA was used to identify plaques containing recombinant virus expressing eIF2α wt or 48A mutant. The supernatant from the plaques were used to infect Sf9 cells **in** a 96 well plate as described under Materials and Methods (9.1). Out of the six plaques used for wt eIF2α (wells 1-6), two of them (5 and 6) were found to be positive. For the eIF2α 48A,all plaques that were screened here were found to be positive (wells 9 to 13). Wells 7 and 14 represent uninfected cells. Well 15 represent cells infected with non-recombinant AcNPV virus. Well 8 contains a positive control, i.e., eIF2a insert.

digestion using *Mlu* I and *Sea* I or *Sph* I and *BamH* I enzymes for pBacPAK8 vector carrying wt or 48A mutant eIF2a cDNA. Six positive colonies, identified by colony hybridization, carrying the eIF2α wt were picked up randomly and their plamid DNA was digested with *Mlu* I and *Sca* I (Figs. 20A and B). *Mlu* I cuts the vector pBacPAK8 (5.5 Kb) at 546 bp. *Sea* I cuts the vector at 3990 bp while it cuts the eIF2α wt insert at 561 bp. If the eIF2α insert is in the right orientation in the vector, the above restriction digestion yields three fragments of the following sizes: 1308 bp, 2094 bp, 3736 bp. Three colonies had the insert in the right orientation (lanes 1, 3 and 7 in Fig. 20A and lanes 2, 6 and 9 in Fig. 20B). A similar analysis was done in the case of 48A eIF2α using *Sph* I and *BamH* I enzymes. The insert has a single *Sph* I site at 493 bp while the vector has none. *BamH* I cuts the vector at 1303 bp. Right orientation of the insert is expected to yield 543 and 6557 bp fragments (lanes 5, 7, 8 and 9 in Fig. 21). The reeombinant constructs with the insert in the right orientation are shown in Figs. 22A and 22B.

1.2 ***Recombinant*** *baculoviruses:*

Sf9 cells were co-transfected with the reeombinant pBacPAK8 viral transfer vector with *Bsu36* I digested BacPAK6 viral DNA in order to obtain an infectious complete virus genome with the eIF2α wt or mutant DNA. The transfection was carried out as described under Materials and Methods (section 8.1) in the presence of lipofectin. Plaque assays were carried out to obtain reeombinant viruses from a single clone and the positive plaques were identified by dot blot hybridization. Amplification of the reeombinant viruses were carried out to increase the titre of the reeombinant viruses in a step-wise manner. Results (Fig. 23), suggest, that out of six plaques picked up in the case of eIF2a wt, two (wells 5 and 6) were found to be positive. For the 48A eIF2α, all the plaques (wells 9 to 13) that were screened were found to be positive. No signal was detected in uninfected cells (wells 7 and 14) or in cells infected with wild type or non-recombinant virus (well 15). Well 8 in Fig. 23 contains a positive control, i.e., eIF2a insert from which the probe was prepared.

2.0 EXPRESSION

2.1 *Time course of expressionof wild type and 48A mutant human eIF2α:*

Extracts of cells from uninfected (72 h) and infected cells (infected with **non-**recombinant AcNPV or with recombinant, pBacTA1, wild type eIF2a, or pBacTA2, 48A eIF2α mutant, were prepared at different time points post-infection (12, 24, 36, 48, 60 and 72 h). Protein estimation was done and equal amount of protein extract was used for resolving on 10% SDS-PAGE. Results show that a protein with a molecular weight **of** 38 kDa was expressed in the cells infected with the recombinant virus from 24 h p.i. onwards upto 72 h (Fig. 24B). This protein is not found in uninfected cells or cells infected with non-recombinant wild type AcNPV virus (Fig. 24A).

2.2 *Identification of human eIF2αby a monoclonal antibody:*

Immunoreactivity **of** the expressed protein was tested by western blot analysis using anti-eIF2a monoclonal antibody (Fig. 25).Both the wild type (Fig. 25, lanes 3-7) and 48A mutant (Fig. 25, lanes 8-12) react equally well with the antibody and the signal **is** proportional to the expression of eIF2a protein. At 24 h p.i., the extracts contain low levels of eIF2α and accordingly the reactivity of the antibody is poor in these lanes (Fig. 25, lanes 3 and 8). In contrast,a strong signal appeared between 36-72 h (lanes 4-7 and 9-12) p.i. and is consistent with the previous result that expression starts around 24 h and increases with time upto 72 h. Neither control Sf9 cell extracts (lane 1) or wild type non-recombinant AcNPV infected cell extracts (lane 2) contain any polypeptide that is immunoreactive to eIF2a monoclonal antibody.

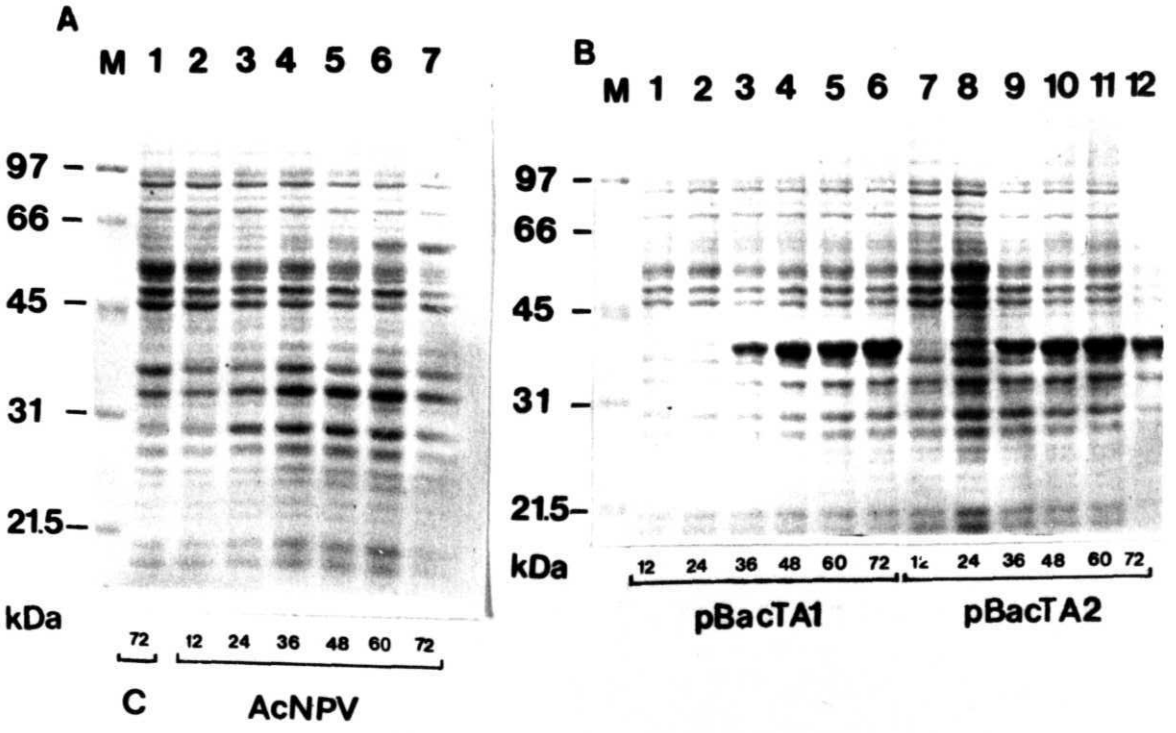
3.0 FUNCTIONAL CHARACTERIZATION OF THE EXPRESSED PROTEINS

3.1 *Phosphorylation of theexpressed protein by eIF2α kinase, HRI:*

Since the wild type eIF2a and 48A mutant eIF2α expressed in mammalian systems are shown to be substrates for eIF2a kinases, the ability of baculovirus-expressed eIF2a subunits have been tested to serve as substrates for phosphorylation in the presence of a purified heme-regulated eIF2α kinase (HRI) (Fig. 26). The results of this experiment

Fig. 24. Time course of protein expression.

Extracts were prepared as described in Materials and Methods from Sf9 cells infected with recombinant viruses (panel B) or with non-recombinant AcNPV (wt, panel A) at different time points as shown in the figure. Extract from uninfected cells (control, C, panel A) was prepared for only one time point (72 h). Each extract was prepared from 2 X 10^6 Sf9 cells. The viruses had an MOI of 10 in each case. Equal amount of protein extract (25 μg) was loaded in each well of a 10 % SDS-PAGE gel for the proteins to be resolved. The figure is a coomassie stained gel.


A
M 1 2 3 4 5 6 7
97
66
45
31
21.5
kDa
72
12 24 36 48 60 72
C
AcNPV
B
M 1 2 3 4 5 6 7 8 9 10 11 12
97
66
45
31
21.5
kDa
12 24 36 48 60 72
12 24 36 48 60 72
pBacTA1
pBacTA2

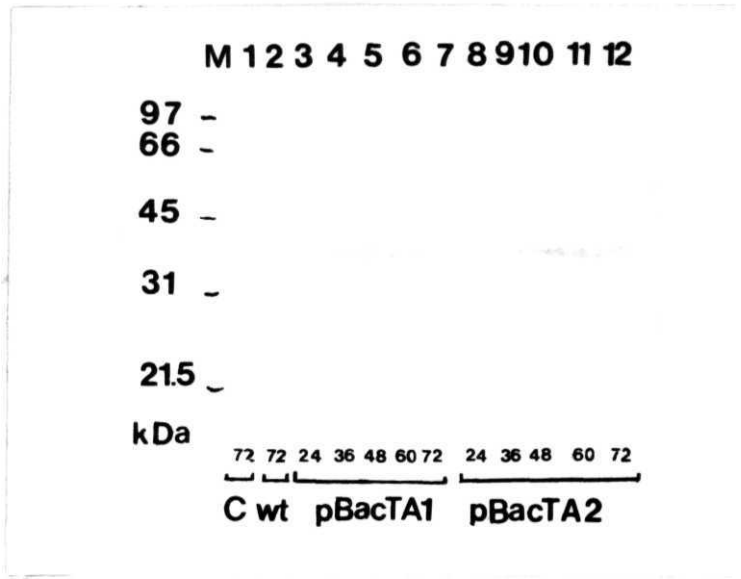


Fig. 25. Detection of recombinant eIF2a wild type and 48A mutant subunits by a human eIF2a monoclonal antibody.

Cell extracts were prepared as described in the legend to Fig. 24. The protein extracts (20 μg) were separated on a 10 % SDS-PAGE and transferred to a nitrocellulose membrane and probed with an eIF2α monoclonal antibody. The signal was detected with the help of anti-mouse alkaline phosphatase-conjugated secondary antibody raised in rabbit (Promega). In case of uninfected cells (C) and of cells infected with AcNPV (wt), only one time point extract, 72 h, was used.

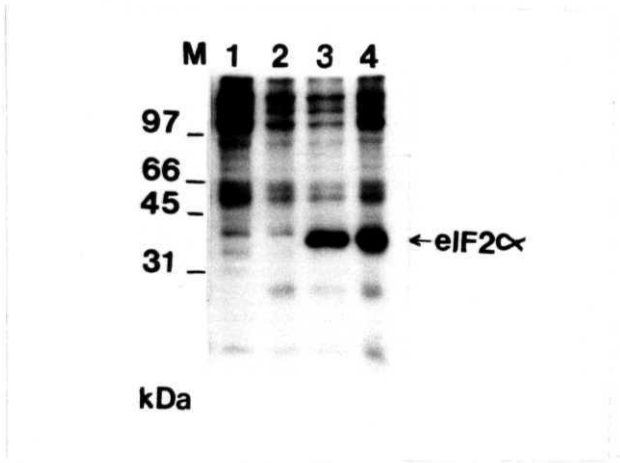


Fig. 26. Phosphorylation of recombinant human eIF2a wt and 48A mutant protein by reticulocyte HRI.

Insect cell extracts (25 μg) from uninfected and infected cells (infected with wild type or recombinant virus) prepared 48 p.i. were incubated at 30°C for 5 min prior to the addition of HRI cocktail (HRI was preincubated in a cocktail containing 20 mM Tris-HCl pH 7.6, 2 mM Mg^{2+}, 80 mM KCl and 30 uM ATP at 30°C for 5 min). The extracts were incubated for another 5 min before they were pulsed with [γ~ ˆP]ATP (10 μCi). Reactions were terminated 5 min after the pulse by the addition of 2X SDS-sample buffer and heated for 3 min in boiling water. The samples were resolved on a 10% SDS-PAGE gel and analyzed later by autoradiography. The figure is an autoradiogram. The various lanes represent the following: lane 1, uninfected cell extract, mock; lane 2, wild type AcNPV-infected cell extract; lane 3, recombinant virus-infected cell extract expressing eIF2α wild type and lane 4, recombinant virus-infected cell extract expressing eIF2a 48A mutant.

indicate that both wt and 48A mutant eIF2α can be phosphorylated (lanes 3 and 4). A similar signal corresponding to human eIF2a is lacking in the control and AcNPV infected cell extracts (lanes 1 and 2). These findings suggest that the baculovirus expressed eIF2α truly represents human eIF2a.

3.2 *48A mutant decreases* ***the inhibition*** *of* ***eIF2B*** *activity* ***in poly (IC)-treated*** *reticulocyte lysates:*

Recent studies have shown that the 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 the overexpression of mutant eIF2a and not by wt eIF2α in Chinese hamster ovary cells and in their extracts (Ramaiah *et ah,* 1994). To check the functional characteristics of baculovirus-expressed human wt and 48A mutant eIF2a, we have studied their effects on the inhibition of eIF2B activity of poly (IC)-treated reticulocyte lysates (Fig. 27 and Table 9). The kinetics of poly (IC)-induced inhibition in hemin-supplemented lysates are shown in (Fig. 27A). These results are consistent with the earlier findings that poly (IC) treatment induces eIF2α phosphorylation via double stranded RNA-dependent kinase (PKR) and causes inhibition of eIF2B activity in reticulocyte lysates (Matts and London, 1984; Naresh Babu and Ramaiah, 1996; Krishna *et al.,* 1997). When such poly (IC)-treated reticulocyte lysates were supplemented with 50 μg of insect cell extracts prepared from control Sf9 cells, wild type AcNPV-infected cells or recombinant virus-infected cell extracts expressing the wild type or mutant human eIF2a, it was observed that the inhibition of eIF2B activity caused by the presence of poly (IC) was readily decreased in the presence of extracts expressing mutant eIF2a (Fig. 27B). While carrying out this experiment, not only have we used equal amount of extract protein in each of the reactions but even the recombinant extracts had fairly equal amount of the expressed protein (wt or 48A mutant eIF2a) (Fig. 28). In another independent experiment (Table 9), the inhibition of eIF2B activity in poly (IC)-treated reticulocyte lysate was assessed in the presence of two concentrations (50 and 100 μg) of insect cell extracts expressing wild type or mutant eIF2a. The activity of

Figs. 27. Kinetics of eIF2.[H]GDP dissociation in reticulocyte lysates in the absence (panel A) and presence of insect cell extracts overproducing eIF2a wt or 48A mutant (panel B).

Protein synthesis (70 |il) was carried out at 30°C for 10 min in reticulocyte lysates under the following two conditions:

Panel A. 1) +heme (20 μM, •-•) and

2) +heme +poly (IC) (400 ng/ml, o-o).

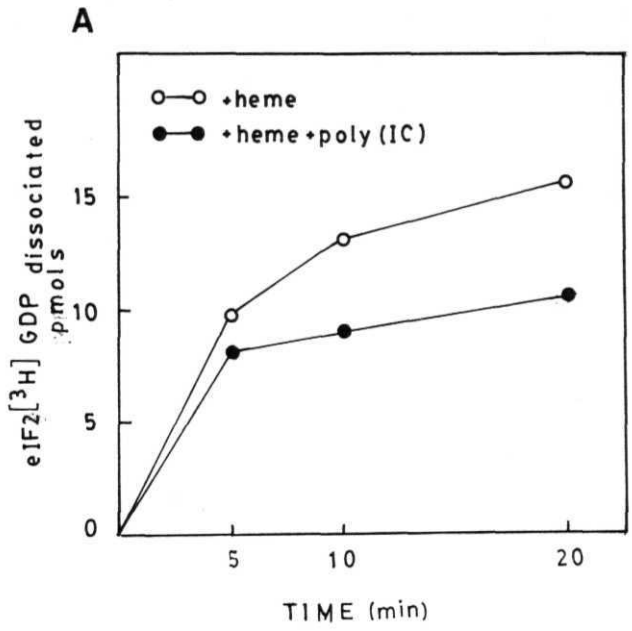
Panel B. 1) +mock-treated Sf9 cell extract (■-■)

2) +AcNPV-infected cell extract (D-D)

3) + recombinant virus-infected cell extract expressing eIF2a wild type (A-A) and

4) +recombinant virus-infected cell extract expressing eIF2a 48A mutant (o-o).

The Sf9 extracts (175 μg in 35 μl) were prepared 48 p.i. 70 μl (75.15 pmol) of the preformed binary complex was added to the above reticulocyte lysate reactions (panel A and B) and incubated at 30°C. At various time intervals. 50 μl aliquots were taken to determine the amount of eIF2.["H]GDP dissociated as described in the Materials and Methods.


A
+heme
+heme+poly (IC)
eIF2[3H] GDP dissociated pmols
0
5
10
15
5
10
20
TIME (min)

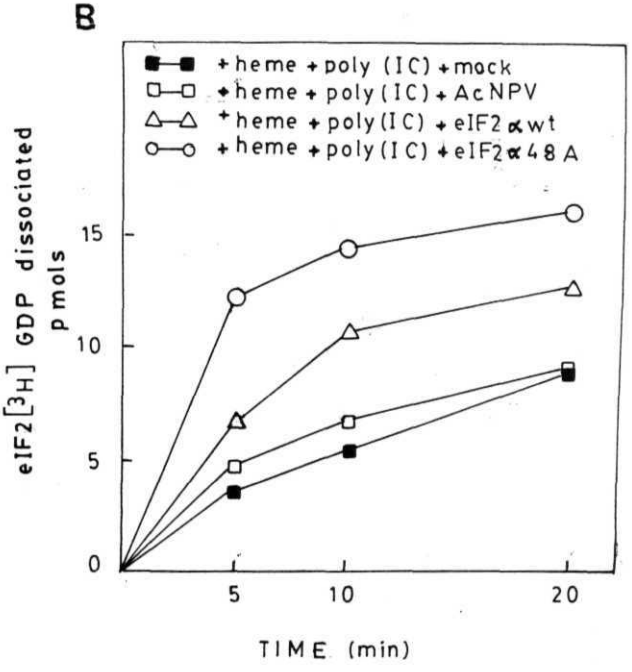

B
+heme+poly (IC)+mock
+heme+poly (IC)+AcNPV
+heme+poly (IC)+eIF2αwt
+heme+poly (IC)+eIF2α48A
eIF2[3H] GDP dissociated pmols
0
5
10
15
5
10
20
TIME (min)

TABLE 9

EFFECT OF DIFFERENT CONCENTRATIONS OF INSECT CELL EXTRACTS OVERPRODUCING eIF2α 48A MUTANT ON eIF2.GDP DISSOCIATION

Experimental conditions	pmol of eIF2.[H]GDP dissociated 5 min
+heme	**11.71**
+heme +poly (IC)	7.57
+heme +poly (IC) +mock, 100 μg	1.50
+heme +poly (IC) +eIF2α wt, 100μg	**2.31**
+heme +poly (IC) +eIF2α 48A, 50 μg	7.64
+heme +poly (IC) +eIF2α 48A, 100 μg	9.53

As described in the legends to Figs. 27A and 27B, reticulocyte binary complex was prepared. Protein synthesis was carried out at 30°C for 10 min in reticulocyte lysates (20 μl) under the various conditions shown in the table 9. 20 μM of hemin and 300 ng/ml poly (IC) were used wherever mentioned. To the lysates, 20 μl of preformed binary complex (18.26 pmol) was added and incubated for 5 min at 30°C. Reactions were terminated by addition of cold wash buffer and the pmol of eIF2.[H]GDP dissociated was determined as described (Naresh Babu and Ramaiah, 1996).

Fig. 28. Dissociation of reticulocyte eIF2.[H]GDP binary complex in insect cell extracts.

Reticulocyte eIF2.[^{3}H]GDP binary complex was prepared as described in Materials and Methods. The following insect cell extracts (21 µl, 87.5 µg) 1) mock-infected (•-•) 2) AcNPV-infected (D-D) 3) recombinant virus-infected, eIF2a wild type (A-A) and 4) recombinant virus-infected, eIF2a 48A mutant (o-o) were incubated for 10 min at 30°C. 70 µl (102.16 pmol) of preformed binary complex was added to each extract and incubated at 30°C. 26 ul aliquots were taken at various time points to determine the pmol of eIF2.[^{3}H]GDP dissociated.

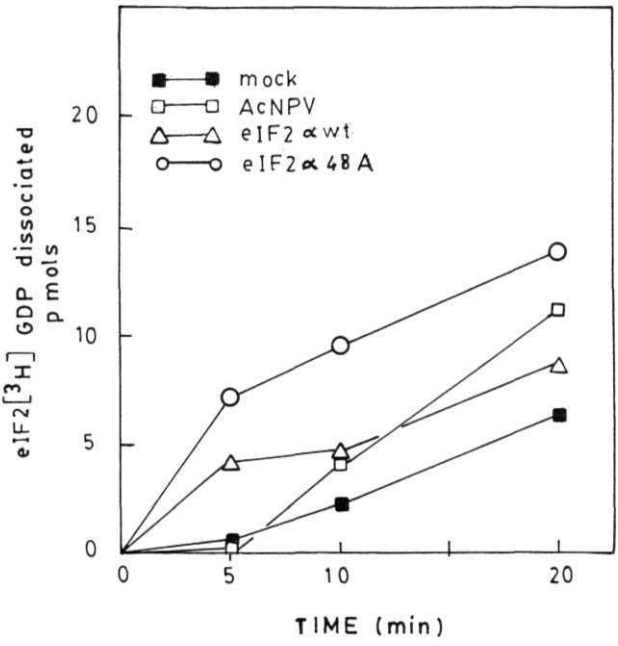

mock
AcNPV
eIF2 α wt
eIF2 α 48 A
eIF2[3H] GDP dissociated p mols
0
5
10
15
20
0
5
10
20
TIME (min)

Fig. 29 Detection of [eIF2(aP).eIF2BJ complex in reticulocyte lysates by immunoblot analysis of eIF2 in the 10-30 % gradient fractions.

Protein synthesis reactions of reticulocyte lysates (100 μl) was carried out for 15 min at 30°C in the presence of following agents.

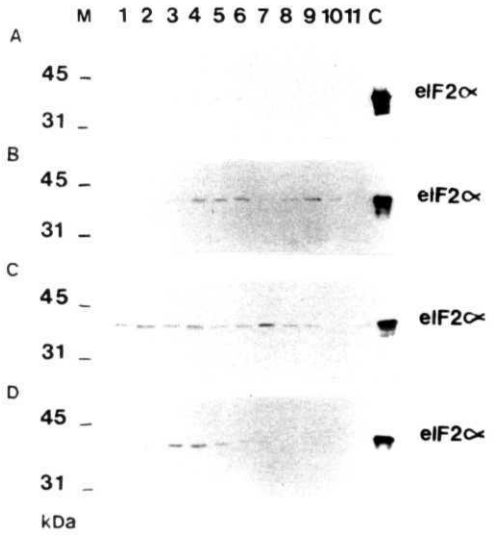
Panel A: +20 μM heme. (h) +uninfected Sf9 cell extract (25 μg).

Panel B: +h + poly (IC), 300 ng/ml +non-recombinant virus infected cell extract (25 ug).

Panel C: +h +poly (IC), 300 ng/ml +recombinant virus infected **cell** extract overexpressing eIF2a wt and

Panel D: +h +poly (IC), 300 ng/ml +recombinant virus infected cell extract overexpressing eIF2a 48A mutant.

The reaction mixtures were diluted with equal volume of TKM buffer (20 mM Tris-HCl pH 7.8, 80 mM KC1 and 2.5 mM $Mg(OAc)_2$) and layered on a 10-30 % sucrose gradient. The gradients were prepared in TKM buffer and spun with the samples for 6 h at 40,000 rpm in a SW 50.1 rotor. The gradient fractions were (400 μl) were collected and concentrated by pH 5.0 precipitation, separated on 10 % SDS-PAGE and transferred to a nitrocellulose membrane. Reticulocyte eIF2a in the transferred proteins of different fractions were detected with the help of anti-mouse human eIF2α monoclonal antibody as described (Krishna et al., 1997). Purified eIF2 (250 ng) was loaded at the end of each gel to serve as a control.


M 1 2 3 4 5 6 7 8 9 10 11 C
A
45
31
eIF2α
B
45
31
eIF2α
C
45
31
eIF2α
D
45
31
eIF2α
kDa

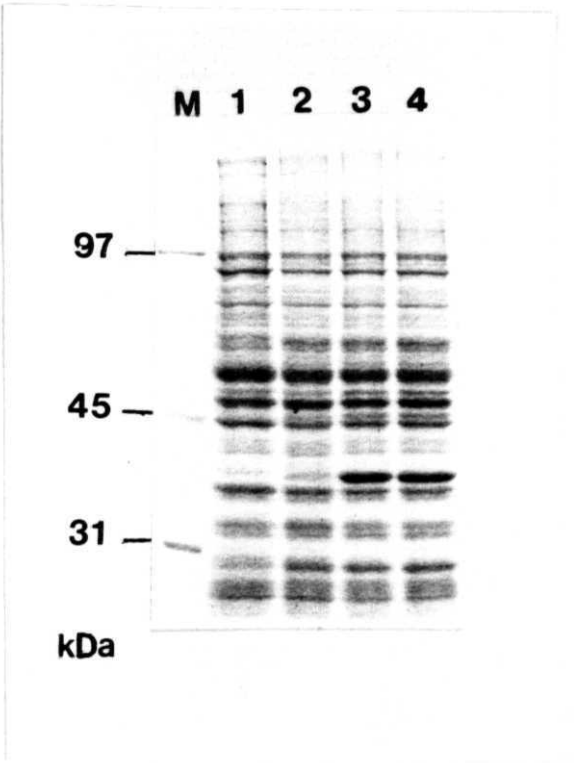
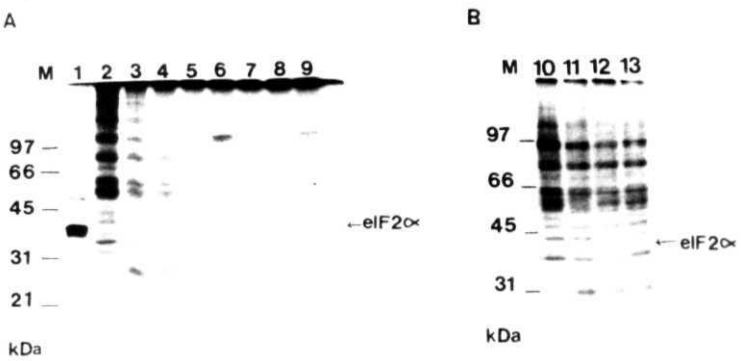


Fig. 30. Insect cell extracts used in cIF2B activity measurement.

Based on protein estimation, equal amount (25 μg) of the insect cell extract was prepared from, 1) mock-infected 2) AcNPV-infected 3) recombinant virus-infected, expressing eIF2α wt and 4) recombinant virus-infected, expressing eIF2a 48A mutant and extracts were resolved on a 10 % SDS-PAGE gel and coomassie stained. The extracts were prepared 48 p.i. as described in Materials and Methods.

Fig. 31. **Phosphorylation** of Sf9 cell extracts in the presence and absence of poly **(IC).**

Three sets of insect cell extracts (25 μg) from uninfected and infected cells (infected with wild type or recombinant virus) prepared 48 p.i. were supplemented with 30 μM ATP and incubated at 30°C for 5 min. In the second set of extracts, 0.4 μg of reticulocyte eIF2 was added while in the third set 500 ng/ml of poly (IC) was added before incubation at 30°C. After 5 min of incubation, the extracts were pulsed with [γ-^{32}P]ATP (20 μCi) for 6 min at 30°C before terminating the reactions by addition of 2X SDS-sample buffer. The samples were resolved on a 10 % SDS-PAGE gel and analyzed later by autoradiography. *In vitro* phosphorylation of reticulocyte eIF2 (0.4 μg) by HRI was also carried out as described in the legend to Fig. 13 and used a control lane to indicate eIF2a phosphorylation. The figure is an autoradiogram. The various lanes represent the following: lane 1, reticulocyte eIF2 phosphorylated *in vitro*; lane 2, uninfected cell extract, mock; lane 3, wild type AcNPV-infected cell extract; lane 4, recombinant virus-infected cell extract expressing eIF2α wild type and lane 5, recombinant virus-infected cell extract expressing eIF2a 48A mutant. Lanes 6, 7, 8 and 9 are similar to lanes 2, 3, 4 and 5 respectively except that the extracts were supplemented with reticulocyte eIF2. Lanes 10, 11, 12 and 13 are also similar to lanes 2, 3, 4 and 5 respectively except that the extracts have poly (IC) in them.


A
M 1 2 3 4 5 6 7 8 9
97
66
45
31
21
kDa
←eIF2α
B
M 10 11 12 13
97
66
45
31
kDa
←eIF2α

eIF2B was measured 5 min after the addition of the substrate as mentioned in the legend. The results of this experiment also indicate that 48A mutant decreases significantly the inhibition of reticulocyte eIF2B activity caused by poly (IC) treatment and appears to be dependent on the concentration of the mutant subunit present in the extracts.

4.0 MECHANISM OF RESCUE OF eIF2B ACTIVITY

4.1 *Analysis of eIF2(αP).eIF2B complex formation in reticulocyte lysates:*

Previously, it was hypothesized that 48A mutation in eIF2a decreases the interaction of eIF2(aP) with eIF2B (Ramaiah *et al,* 1994). To assess such a possibility, the eIF2(αP).eIF2B complex that forms in inhibited poly (IC)-treated reticulocyte lysates due to the ativation of double stranded RNA-dependent eIF2a kinase (PKR) was analyzed as described earlier (Krishna *et al,* 1997) in the presence of insect cell extracts overexpressing eIF2a wt or 48A mutant. Since free eIF2 is lighter than eIF2(aP).eIF2B complex, the top fractions of 10-30 % sucrose gradients contain free eIF2, whereas the bottom fractions contain the complex. The free eIF2 or eIF2 complexed with eIF2B of the gradient fractions can be detected with the help of an eIF2α monoclonal antibody as has been shown previously (Krishna *et al,* 1997). An analysis of the results (Fig. 29) here indicate that the eIF2a signal is seen only in the top fractions of the gradients which contain hemin-supplemented lysates treated with mock insect cell extracts. This is because in the presence of hemin and without poly (IC) or dsRNA being included in the reaction, reticulocyte lysates contain very little or no eIF2a kinase activity which can phosphorylate eIF2a and facilitate the formation of a complex between eIF2(αP) and eIF2B. Hence, very little reticulocyte eIF2 is bound to eIF2B which can be detected in the bottom fractions of the gradient (Fig. 29A). In contrast, eIF2α signal is visible both in the top and bottom fractions of the gradients carrying reticulocyte lysates which are treated with hemin, poly (IC) and non-recombinant virus infected cell extracts (B). This is consistent with the activation of PKR in poly (IC)-treated reticulocyte lysates that facilitates the phosphorylation of eIF2a and formation of eIF2(aP).eIF2B complex in which eIF2B is inactive (Matts and London, 1984; Krishna *et al,* 1997; Thomas *et al,*

1985). A similar result was obtained indicating the presence of eIF2α signal in the top and bottom frations of the gradients of hemin and poly (IC)-treated lysates which are supplemented with insect cell extracts expressing wt eIF2α (C). The intensity of eIF2a signal is however higher in these fractions since the reactions contain overexpressed wt eIF2α subunit. In contrast, eIFα signal is weak and cannot be detected readily in the bottom fractions of the gradients containing hemin and poly (IC)-supplemented reticulocyte lysates which are treated with insect cell extracts expressing 48A mutant (D). These findings suggest that 48A mutation in mammalian eIF2a reduces the formation of a complex between eIF2(aP) and eIF2B in hemin and poly (IC)-treated reticulocyte lysates. These findings are consistent with the eIF2B activity measurements (Fig. 27 and Table 9).

4.2 *Dissociation of reticulocyte eIF2.GDP binary complex occurs significantly in insect cell extracts expressing 48A mutant eIF2α:*

To determine the elF2B like activity of insect cell extracts in the absence of added reticulocyte lysates, the dissociation of reticulocyte elF2.[H]GDP was monitored in Sf9 cell extracts prepared from uninfected or infected with non-recombinant AcNPV or recombinant virus expressing wt or 48A mutant eIF2a (Fig. 30). An analysis of the results indicate that the dissociation of eIF2.GDP is relatively higher in the insect cell extracts infected with non-recombinant AcNPV at 10 and 20 min than in control cell extracts (D-D vs •-•). Although, the total eIF2B like activity at 20 min is not altered significantly and remains approximately similar in the cells infected with AcNPV alone or virus expressing 48A mutant eIF2α (D-D vs o-o), the kinetics of eIF2.[H]GDP dissociation indicate that eIF2B like activity of insect cells is more readily available in the extracts expressing 48A mutant eIF2α than in the AcNPV infected cells. These findings suggest that the dissociation of eIF2.GDP dissociation occurs more readily in insect cell extracts expressing 48A mutant eIF2a (o-o) than wt eIF2a (A-A) and also as compared to the non-recombinant AcNPV infected cell extracts (D-D).

4.3 ***eIF2α kinase activity of insect cell extracts and phosphorylation of insect cell proteins:***

To determine whether eIF2α kinase like activity is present in insect cell extracts that **is** responsible for the observed changes in eIF2B like activity, phosphorylation of insect cell proteins have been carried out in the presence and absence of added purified reticulocyte eIF2 or overexpressed eIF2α subunit as shown in Fig. 31. As can be seen, the insect cell extracts are unable to phosphorylate expressed human eIF2a wt or 48A mutant (compare lane 3 vs lanes 4 and 5) or added reticulocyte eIF2 (lanes 6-9). These findings suggest that insect cells do not contain an active eIF2a kinase under those conditions which can phosphorylate expressed or added eIF2 and raise the possibility that the increased dissociation of eIF2.GDP binary complex in insect cell extracts overproducing 48A mutant eIF2α (Fig. 30) may be occurring independent of eIF2B like activity. However consistently it has been observed that the phosphorylation of several proteins is found to be higher in the control Sf9 cell extracts than in the virus-infected cell extracts (lanes 2 vs 3-5 or lane 6 vs 7-9). Although it is difficult to determine whether eIF2α phosphorylation is decreased specifically due to virus infection, the possibility however cannot be eliminated. Also, we have added poly (IC) to determine if insect cells contain a double stranded RNA-dependent eIF2a kinase (PKR) which can phosphorylate the expressed human eIF2α subunit, wt or 48A mutant (Fig. 31B, lanes 10-13). Since expressed eIF2α subunits were not phosphorylated, the findings suggest that insect cells do not have probably a PKR like activity.

DISCUSSION

In this study, for the first time, the wt human eIF2α and the 48A mutant eIF2a which can be phosphorylated on their 51 serine residues have been overproduced in Sf9 cells using the baculovirus system (Figs 24, 26 and 28). The system is found advantageous in many respects over the mammalian and yeast system for producing biologically active recombinant proteins at very impressive levels. Consistent with this notion, we find that the recombinant eIF2α is expressed 25-30% of the total protein (Figs. 24B and 28). In addition Sf9 cells can be infected with multiple recombinant viruses in order to produce all the subunits of oligomeric proteins like eIF2 or their mutants in order to understand their activities *in vivo* or *in vitro*. Recently, the baculovirus system has been used to overproduce wild type and mutants of heme-regulated eIF2α kinase (Chefalo et al.,1994) and also all the subunits of mammalian eIF2B (Fabian *et al.*, 1997) to determine the structure-function relationship in HRI or the subunit assembly that is required in the pentameric eIF2B protein for its catalytic and regulatory activities.

Previous biochemical studies using cell-free systems derived from rabbit reticulocytes and through genetic experiments conducted in yeast, it has been suggested that phosphorylation of eIF2a results in the inhibition of guanine nucleotide exchange activity of eIF2B and thereby protein synthesis. As the affinity of eIF2B for eIF2(aP).GDP is higher than that for eIF2.GDP, eIF2B is trapped in a complex, [eIF2(aP).eIF2B], in which eIF2B becomes non-functional (Rowlands *et al,* 1988b). This type of sequestration can occur because mammalian eIF2(αP) is not a substrate for eIF2B and that eIF2B has a higher affinity for the inhibitor eIF2(aP) than for the substrate eIF2 (Thomas *et al,* 1985). Recently, using purified polyhistidine-tagged yeast eIF2, Pavitt et al. (1997), devised a 'pull-down' assay to demonstrate that binding of eIF2B subunits to eIF2 increases when eIF2 is phosphorylated.Further, these studies point out that regulatory mutations in GCN3, GCD7 and GCD2 subunits of yeast eIF2B (equivalent to a, β and 5 subunits of mammalian elF2B) can overcome the inhibition of protein synthesis caused by eIF2a phosphorylation because these mutant eIF2B complexes can exchange efficiently GDP from both eIF2.GDP and eIF2(aP).GDP.

Similarly, the importance of eIF2a phosphorylation in translational control is highlighted by the expression of wt human eIF2a and serine to alanine mutants at residues 48 and 51 (48A and 51A mutants) in mammalian systems (Kaufman *et al,* 1989; Choi *et al,* 1992; Murtha-Riel *et al,* 1993; Donze *et al.,* 1995; Srivastava *et al,* 1995). Although 51A mutant cannot be phosphorylated, the 48A mutant can be phosphorylated on its 51 serine residue. Interestingly, the expression of either mutant protects protein synthesis in mammalian cells caused by PKR and heat shock (Kaufman *et al,* 1989; Murtha-Riel *et al,* 1993; Srivastava *et al,* 1995). These findings suggest that in addition to 51 serine residue, other residues in eIF2a also appear to regulate the activity of phosphorylated eIF2α in mammalian systems. In addition, genetic studies in yeast indicated that mutations within 40 amino acids of the phosphorylation site can overcome the inhibitory effects caused by eIF2a phosphorylation (Vazquez de Aldana *et al,* 1993). These findings highlight the importance of various amino acid residues in the subunits of the substrate, eIF2 and enzyme eIF2B which are important for their interaction.

Our studies suggest that baculovirus-expressed 48A mutant human eIF2α can decrease the inhibition of eIF2B activity caused by poly (IC) treatment in reticulocyte lysates that occurs via the activation of PKR (Fig. 27 and Table 9). These results are consistent with our previous observations (Ramaiah *et al,* 1994) where it had been shown that the inhibition of eIF2B activity that occurs in CHO cells by heat shock or purified heme-regulated eIF2a kinase, HRI, can be decreased in the presence of overproducing 48A or 51A eIF2α mutants. In this above study (Ramaiah *et al,* 1994), it has been hypothesized that serine-48 residue in human eIF2α is required to maintain a high affinity between phosphorylated eIF2a and eIF2B. Hence, we have analyzed here the formation of [eIF(αP).eIF2B] complex in hemin and poly (IC)-treated reticulocyte lysates. Consistent with the earlier prediction (Ramaiah *et al,* 1994), the insect cell extracts expressing 48A mutant eIF2a are able to decrease readily the formation of eIF(αP).eIF2B complex (Fig. 29), whereas a similar decrease in the complex formation does not occur in the presence of extracts expressing wt eIF2a.

In addition, we observed that the eIF2B activity of insect cell extracts overexpressing the 48A mutant eIF2α is more readily available to dissociate the preformed reticulocyte eIF2.[^{3}H]GDP binary complex than the extracts expressing wt eIF2a or infected with non-recombinant AcNPV (Fig. 30). Since the eIF2B activity of control Sf9 cell extract is lower and the phosphorylation of various proteins is found to be relatively higher than in other AcNPV infected extracts (Fig. 31), it is likely that a low level of insect cell eIF2α phosphorylation may also be occurring in control Sf9 cell extracts which can inhibit eIF2B activity.

One possibility for the increased eIF2B like activty observed here in insect cell extracts expressing 48A mutant eIF2a (Fig. 30) may be that the mutant is able to overcome the inhibition in insect cell eIF2B activity (if any) caused by small amounts of endogenous eIF2a phosphorylation. However, when analyzed, none of the insect cell extracts (uninfected, recombinant or non-recombinant virus infected) contain any significant eIF2α kinase activity that can phosphorylate expressed human eIF2a or purified reticulocyte eIF2 (Fig. 31). Hence, the findings raise a possibility that the purified rabbit eIF2.GDP binary complex may be able to dissociate the bound GDP independent of an eIF2B like protein when the 48A mutant eIF2a replaces the eIF2α of the trimeric rabbit eIF2 in the binary complex.

SUMMARY

This thesis reports that both vanadate (+V oxidation state) and different vanadyl species (+IV oxidation state) such as vanadyl D-glucose, vanadyl diascorbate and vanadyl sulfate impair the formation of polysomes and inhibit the initiation of protein synthesis in hemin-supplemented rabbit reticulocyte lysates. Vanadate inhibits protein synthesis more severely than vanadyl species and is consistent with the idea that vanadate is reduced to vanadyl state intracellularly. The inhibition of protein synthesis caused by low concentrations (10-20 μM) of vanadate and vanadyl species is effectively mitigated by reducing agents such as dithiothreitol (DTT), reduced glutathione (GSH) or pyridine dinucleotide (βNADPH). A significant decrease in the protein synthesis inhibition in vanadate-treated lysates by GSH suggests that the mechanism of protein synthesis inhibition by vanadate is different from the action of other oxidants such as heavy metal ions and oxidized glutathione. This suggestion is also consistent with the findings that vanadium compounds do not stimulate phosphorylation of the alpha (a) -subunit of initiation factor 2 (eIF2) or decrease the guanine nucleotide exchange activity of eIF2B, which is required to exchange GDP for GTP in eIF2 . GDP binary complex. The reduction of vanadate to vanadyl state and the subsequent complex formation of vanadyl species with the endogenous reducing compounds or with the -SH groups of certain proteins may be the cause for protein synthesis inhibition in lysates.

Further, inorder to determine the defects caused by eIF2a phosphorylation on eIF2B activity and to assess the interaction of eIF2B with phosphorylated and unphosphorylated eIF2α, we have overexpressed here for the first time human eIF2α wild type (wt) and a mutant human eIF2a in which serine 48 was replaced by an alanine

(48A mutant) in the ovarian cells *of Spodoptera frugiperda* with the help of baculovirus system. Both the wt as well as the 48A mutant of eIF2α were recognized by a monoclonal eIF2α antibody and were phosphorylated by the heme-regulated eIF2α kinase. It was observed that the inhibition in eIF2B activity that occurs due to eIF2a phosphorylation in hemin and poly (IC)-treated reticulocyte lysates was readily decreased in the presence of insect cell extracts overproducing the human 48A eIF2a mutant, but not the wild type. In addition, it has been observed here that the insect cells expressing 48A mutant decreases the formation of [cIF2(aP).eIF2B] complex that occurs between reticulocyte eIF2(αP) and eIF2B in inhibited heme and poly (IC)-treated reticulocyte lysates. These findings support the hypothesis that the 48A mutant of eIF2a reduces the affinity for eIF2B when eIF2α is phosphorylated.

Our studies on insect cell extracts alone indicate that a poly (IC)-induced eIF2α kinase (homologous to human dsI) is absent in insect cell extracts. eIF2B like activity increases in insect cell extracts overexpressing eIF2a 48A mutant. Under non-induced conditions, the over-expressed human eIF2a wild type and 48A mutant do not get phosphorylated in insect cell extracts. These observations in insect cell extracts suggest that a) the eIF2B activity of control insect cell extracts may be inhibited partially due to a low or basal level of eIF2α phosphorylation which can be relieved more efficiently by the expression of 48A mutant or/and b) the dissociation of eIF2.GDP binary complex probably occurs independent of an eIF2B like protein in the presence of eIF2a 48A mutant.

REFERENCES

Akkaraju, G. R., Hansen, L. J., and Jagus, R. (1991) *J. Biol. Chem.* **266,** 24451-24459.

Amesz, H., Goumans, H., Haubrich-Morree, T., Voorma, H. O., and Benne, R. (1979) *Eur. J. Biochem.* **98,** 513-520.

Andrew, N. C, Levin, D. H., and Baltimore, D. (1985) *J. Biol. Chem.* **260,** 7628- 7635.

Anjali Jain (1996) 'Studies on the cis-trans activation of transcription from the baculovirus very late promoters' Ph. D. thesis.

Anthony, D. D., Kinzy, T. G., and Merrick, W. C. (1990) *Arch. Biochem. Biophys.* **281,** 157-162.

Aroor, A. R., Denslow, N. D., Singh, L. P., O'Brien, T. W., and Wahba, A. J. (1994) *Biochemistry* 33, 3350-3357.

Aroor, A. R., Singh, L. P., and Wahba, A. J. (1995) *Exp. Hematol.* 23, 1204-1211.

Ayres, M. D., Howard, S. C, Kuzio, J., Lopez-Ferber, M, and Possee, R. D. (1994) *Virology* **202,** 586-605.

Balfour, W. E., Grantham, J. J., and Glynn, I. M. (1978) *Nature* **275,** 768.

Barrieux, A., and Rosenfeld, M. G. (1977) J. *Biol Chem.* **252,** 3843-3847.

Benabe, J. E., Echegoyen, L. A., Belinda, P., and Marnitez-Maldonado, M. (1987)./. *Biol. Chem.* **262,** 9555-9560.

Blenis, J., and Erickson, R. L. (1986) *Proc. Natl. Acad. Sci. USA* 83, 1733-1737.

Bosch, F., Arino, J., Gomez-Foix, A. M., and Guniovar, J. J. (1987) *J. Biol. Chem.* **262,** 218-222.

Cantley, L. C. Jr., Josephson, L., Warner, R., Yanagisawa, M., Lechene, C, and Guidotti, G. (1977) *J. Biol. Chem.* **252,** 7421-7423.

Charlton, C. A., and Volkman, L. E. (1993) *Virology* **197,** 245-254.

Chasteen, N. D. (1983) *in* Structure and Bonding. Copper, Molybdenum and Vanadium in Biological Systems (Clarke, M. J. et al., Eds.) pp 103-136, Springer-Verlag, Heidelelberg, Federal Republic of Germany.

Chaudhuri, A., Stringer, E. A., Valenzuela, D., and Maitra, U. (1981) *J. Biol. Chem.* **256,** 3988-3994.

Chefalo, P. J., Yang, J. M., Ramaiah, K. V. A., Gehrke, L., and Chen, J.-J. (1994) *J. Biol. Chem.* **269,** 25788-25794.

Chen, J.-J., Yang. J., Petryshyn, R., Kosower, N., and London, I. M. (1989) *J. Biol. Chem.* **264,** 9559-9564.

Chen, J.-J. (1993) in Translational Control of Gene Expression (Ilan, J., ed.) vol. 2. pp. 349-372, Plenum Press, NY

Chen, J.-J., and London, I. M. (1995) *Trends Biochem. Sci.* **20,** 105-108.

Choi, S.-Y., Scherer, B. J., Schnier, J., Davies, M. V., Kaufman, R. J., and Hershey, J. W. B. (1992) J. *Biol. Chem.* **267,** 286-293.

Cigan, A. M., Pabich, E. K., Feng, L., and Donahue, T. F. (1989) *Proc. Natl. Acad. Sci. USA* 86, 2784-2788.

Clark, S. J., Ashford, A. J., Price, N. T., and Proud, C. G. (1989) *Biochim. Biophys. Acta* 1010,377-380.

Clarke, A. S., Fagan, J. M., and Mitch, W. E. (1985) *Biochem. J.* **232,** 273-276.

Clemens, M. J., Pain, V. N., Wong, S., and Henshaw, E. C. (1982) *Nature* **296,** 93-95.

Clemens, M. J., Galpine, A., Austin, S. A., Panniers, R., Henshaw, E. C, Duncan, R., Hershey, J. W. B., and Pollard, J. W. (1987) J. *Biol. Chem.* **262,** 767-771.

Clemens, M. J. (1990) *Trends Biocehm. Sci.* 15, 172-175.

Clemens, M. J. (1992) *Nature* **360,** 210-211.

Clemens, M. J., Liang, k. G., Jeffery, I. W., Schofield, A., Sharp, T. V., Elia, A., Matys, V., James, M. C., and Tilleray, V. J. (1994) *Biochimie* 76, 770-778.

Clemens, M. J. (1996) in Translational Control (Hershey, J. W. B., Mathews, M. B., and Sonenberg, N., eds) pp. 139-172, Cold Spring Harbor Laboratory Press, Plainview, NY

Das, A., Ralston, R. O., Grace, M., Roy, R., Ghosh-dastidar, P., Das, H. K., Yaghmai, B., Palmeiri, S., and Gupta, N. K. (1979) *Proc. Natl. Acad. Sci. USA* 76, 5076-5079.

Dasso, M. C, Milburn, S. C, Hershey, J. W. B., and Jackson, R. J. (1990) *Eur. J. Biochem.* 187,361-371.

Datta, B., Chakrabarti, D., Roy, A. L., and Gupta, N. K. (1988a) *Proc. Natl. Acad. Sci. USA* 85, 3324-3328.

Datta, B. Ray, N. K., Chakrabarti, D., and Gupta, N. K. (1988b) *Indian J. Biochem. Biophys.* 25, 478-482.

Datta, B., Ray, M. K., Chakribarti, D., Wylie, D. E., and Gupta, N. K. (1989) *J. Biol. Chem.* **264,** 20620-20624.

Davies, M. V., Furtado, M., Hershey, J. W. B., Thimmappaya, B., and Kaufman, R. J. (1989) *Proc. Natl. Acad. Sci. USA* 86, 9163-9167.

Davies, M. V., Elroy-Stein, O., Jagus, R., Moss, B., and Kaufman, R (1992) *J. Virol.* **66,** 1943-1950.

Dever, T. E., Chen, J.-J., Barber, G. M., Cigan, A. M., Feng, L., Donahue, T. F., London, I. M., Katze, M. G., and Hinnebusch, A. G. (1993) *Proc. Natl. Acad. Sci. USA* 90, 4616-4620.

Dholakia, J. N., Mueser, T. C, Woodley, C. L., Parkhurst, L. J., and Wahba, A. J. (1986) *Proc. Natl. Acad. Sci. USA* 83, 6746-6750.

Dholakia, J. N., and Wahba, A. J. (1988) *Proc. Natl. Acad. Sci. USA* 85, 51-54.

Di Segni, G., Rosen, H., and Kaempfer, R. (1979) *Biochemistry* 18, 2847-2854.

Donahue, T. F., Cigan, A. M., Pabich, E. K., and Castilho-Valavicius, B. (1988) *Cell (Cambridge, Mass.)* 54, 621-632.

Donze, O., Jagus, R., Koromilas, A. E., Hershey, J. W. B., and Sonenberg, N. (1995) *EMBOJ.* 14,3828-3834.

Duncan, R. F., and Hershey, J. W. B. (1985) *J. Biol. Chem.* **260,** 5493-5497.

Elberg, G., Li, J., and Shecter, Y. (1994) J. *Biol Chem.* **269,** 9521-9527.

Ernst, V., Levin, D. H., and London, I. M. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 4110-4114.

Ernst, V., Levin, D. H., Leroux, A. L., and London, I. M. (1980) *Proc. Natl. Acad. Sci. USA* 77, 1286-1290.

Ernst, H., Duncan, R., and Hershey, J. W. B. (1987) J. *Biol. chem.* **262,** 1206-1212.

Fabian, J. R., Kimball, S. R., Heinzinger, N. K., and Jefferson, L. S. (1997) *J. Biol. Chem.* 272,12359-12365.

Farrell, P. J., Balkow, K., Hunt, T., Kackson, R. J., and Trachsel, H. (1977) *Cell*

(Cambridge, Mass.) **11,** 187-200.

Fraser, M. J. (1986) *J. Ultrastruct. Mol. Struct. Res.* **95,** 189-195.

Gaspar, N. J., Kinzy, J. G., Scherer, B. J., Humbelin, M., Hershey, J. W. B., and Merrick, W. C. (1994) J. *Biol. Chem.* **269,** 3415-3422.

Gil, J., Miralpeix, M., Carreras, J., and Bartrons, R. (1988) J. *Biol. Chem.* **263,** 1868-1871.

Gonsky, R., Lebendiker, M. A., Harary, R., Banai, Y., and Kaempfer, R. (1990) *J. Biol. Chem.* **265,** 9083-9089.

Gonsky, R., Itamar, D., Harary, R., and Kaempfer, R. (1992) *Biochimie* **74,** 427-430.

Grace, ML, Ralston, R. O., Banerjee, A. C, and Gupta, N. K. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 6517-6521.

Grace, T. D. C. (1962) *Nature* **195,** 788-789.

Gross, M., and Rabinowitz, M. (1972) *Biochim. Biophys. Acta* 287, 340-352.

Gross, M, Redman, R., and Kaplansky, D. A. (1985) *J. Biol. Chem.* **260,** 9491-9500.

Gross. M., Rubino, M. S., and Starn, T. K. (1988) J. *Biol. Chem.* **263,** 12486-12492.

Gross, M., Olin, A., Hessefort, S., and Bender, S. (1994) *J. Biol. Chem.* **269,** 22738-22748.

Gupta, N. K. (1993) *in* Translational Regulation in Gene Expression (llan, J., ed.) vol 2, pp 405-431, Plenum Press, New York.

Hannig, E. M., Cigan, A. M, Freeman, B. A., and Kinzy, T. G. (1993) *Mol. Cell. Biol.* **13,** 506-520.

Harary, R., and Kaempfer, R. (1990) *Biochim. Biophys. Ada* **1050,** 129-133.

Hasnain, S. E., Nakhai, B., Ehtesham, N. Z., Sridhar P., Ranjan, A., Talwar, G. P., and Jha, P. K. (1994) *DNA Cell Biol.* **13,** 275-282.

Hershey, J. W. B. (1989) *J. Biol. Chem.* **264,** 20823-20826.

Hershey, J. W. B. (1991) *Ann. Rev. Biochem.* **60,** 717-755.

Heyliger, C. E., Tahiliani, A. G., and McNeill, J. H. (1985) *Science* 227, 1474-1476.

Hink, W. F. (1970) *Nature* **226,** 466-467.

Hinnebusch, A. G. (1988) *Microbiol. Rev.* **52,** 248-273.

Hinnebusch, A. G. (1993) *Mol. Microbiol.* **10,** 215-223.

Hinnebusch, A. G. (1994) *Cell Biol.* 5, 417-426.

Horton, H. M., and Burand, J. P. (1993) *J. Virol* 67, 1860-1868.

Hunt, T., Vanderhoff, G. A., and London, I. M. (1972) *J. Mol. Biol.* **66,** 471-481.

Hunt, T. (1979) *Miami Winter Symp.* **16,** 321-346

Hunt, T. (1980) *Mol. Aspects Cell Regul.* 1, 175-202.

Hurst, R., Schatz, J. R. and Marts, R. L. (1987) J. *Biol. Chem.* **262,** 15939-15945.

Jackson, R. J. (1991) *in* Translation in Eukaryotes (Trachsel, H., Ed.), pp. 193-229, CRC Press, Boca Raton, FL.

Janaki, N., Krishna, V. M., and Ramaiah, K. V. A. (1995) *Arch. Biochem. Biophys.* **324,** 1-8.

Janssen, G. M. C, and Moller, W. (1988) J. *Biol. Chem.* **263,** 1773-1778.

Jeffrey, I. W., Kadereit, S., Meurs, E. F., Metzger, T., Bachman, M, Schwemmle, M., Hovanessian, A. G., and Clemens, M. J. (1995) ***Exp.*** *Cell Res.* **218,** 17-27.

Jimenez-Garcia, L. F., Green, S. R., Mathews, M. B., and Spector, D.L. (1993) *J. Cell. Sci.* **106,** 11-22.

Kaempfer, R. (1974) *Biochem. Biophys. Res. Commun.* 61, 591-597.

Kaempfer, R., Hollender, H., Soreq, H., and Nudel, U. (1979) *Eur. J. Biochem.* 94, 591-600.

Kaempfer, R., van Emmelo, J., and Fiers, W. (1981) *Proc. Natl. Acad. Sci. USA* 78, 1542-

1546.

Kaempfer, R., and Konijin, A. M. (1983) *Eur. J. Biochem:* **131,** 545-550.

Kaempfer, R. (1984) *Compr. Virol.* 19, 99-175.

Kan, B., London, I. M., and Levin, D. H. (1988) J. *Biol. Chem.* **263,** 15652-15656.

Kan Liao, and Daniel Lane, M. (1995) *J. Biol. Chem.* 270, 12123-12132.

Kaufman, R. J., Davies M. V., Pathak, V. K., and Hershey, J. W. B. (1989) *Mol. Cell. Biol.* 9, 946-958.

Kimball, S. R., and Jefferson, L. S. (1990) J. *Biol. Chem.* **265,** 16794-16798.

Kimball, S. R., Karinch, A. M., Feldhoff, R. C, Mellor, H., and Jefferson, L. S. (1994) *Biochim. Biophys. Acta* **1201,** 473-481.

Kimball, S. R., and Jefferson, L. S. (1995) *Biochem. Biophys. Res. Commun.* **217,** 1074-1081.

Kitts, P. A., Ayres, M. D., and Possee, R. D. (1990) *Nucleic. Acids Res.* **18,** 5667-5672.

Kitts, P. A., and Possee, R. D. (1993) *Biotechniques* **14,** 810-817.

Koniecizny, A., and Safer,B. (1983) *J. Biol. Chem.* **258,** 3402-3408.

Koromilas, A. E., Lazaras-Karatzas, A., and Sonenberg, N. (1992) *EMBOJ.* **11,** 4153-4158.

Kosower, N. S., Vanderhoff, G. A., and Kosower, E. M. (1972) *Biochim. Biophys. Acta* **272,** 623-627.

Kozak, M. (1992) *Critical Reviews in Biochemistry and Molecular Biology* 27, 385-402.

Kramer, H. 1, Krampitz, G., Backer, A., Krampitz, G. Jr., and Meyer-Lehnert, H. (1995) *Biochem. Biophys. Res. Commun.* **213,** 289-294.

Krishna, V. M., Janaki, N., and Ramaiah, K. V. A. (1997) *Arch. Biochem. Biophys.* **346,** 28-36

Kumar, A. J., Haque, J., Lacoste, J., Hiscott, J., and Williams, B. R. G. (1994) *Proc. Natl. Acad. Sci. USA* 91, 6288-6292.

Laemmli, U. K. (1970) *Nature* **277,** 680-685.

Langland, J. O., Langland, L. A., Browning, K. S., and Roth, D. A. (1996) *J. Biol. Chem.* 271,4539-4544.

Lengyel, P. (1993) *Proc. Natl. Acad. Sci. USA* 90, 5893-5895.

Leroux, A., and London, I. M. (1982) *Proc. Natl. Acad. Sci. USA* 79, 2147-2151.

Levin, D. H., Petryshyn, R., and London, I. M. (1981) J. *Biol. Chem.* **256,** 7638-7641.

Li, X., and Chang, Y.-H. (1996) *Biochem. Biophys. Res. Commun.* 227, 152-159.

London, I. M., Levin, D. H., Matts, R. L., Thomas, N. S. B., Petryshyn, R., and Chen, J.-J. (1987) *in* The Enzymes (Boyer, P. D. and Krebs, E. G., Eds.) Vol. 17 pp. 359-380, Academic Press, New York.

Macara, I. G., Kustin, K., and Cantley, L. C. Jr. (1980) *Biochim. Biophys. Acta* **629,** 95-106.

Maeda, S. (1989)*Annu. Rev. Entomol.* **34,** 351-372.

Manche, L., Green, S. R., Schmedt, C, and Mathews, M. B. (1992) *Mol. Cell. Biol.* **12,**

5238-5248.

Marsh, S., Banai, Y., Na'amad, M., and Kaempfer, R. (1990) *in* Post-Transcriptional Control of Gene Expression (McCarthy, J. E. G., Tuite, M. F., eds), NATO ASI Books, Springer-Verlag, Heidelberg pp.249.

Matthews, R. E. F. (1982) *in* Classification and Nomenclature of Viruses. Fourth Report of the International Committee on Taxonomy of Viruses. Karger, Basel,

Matts, R. L., Levin, D. H., and London, I.M. (1983) *Proc. Natl. Acad. Sci. U. S. A.* 80, 2559-2563.

Matts, R. L., and London, I. M. (1984)./ *Biol. Chem.* **259,** 6708-6711.

Matts, R. L., Levin, D. H., and London, I. M. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 1217-1221.

Matts, R. L., Schatz, J. R., Hurst, R., and Kagen, R. (1991) *J. Biol. Chem.* **266,** 12695-12702.

Matts, R. L., Xu, Z., Pal, J. K., and Chen, J.-J. (1992) *J. Biol. Chem.* 267, 18160-18167.

Mendez, R., and C. de Haro (1994) *J. Biol. Chem.* **269,** 6170-6176.

Merrick, W. E. (1992) *Microbiol. Rev.* **56,** 291-315.

Meurs, E., Chang, K., Galabru, J., Thomas, N. S. B., Kerr, I. M., Williams, B. R. G., and Hovanessian, A. G. (1990) *Cell* **62,** 379-390.

Meurs, E. F., Galabru, J., Barber, G. N., Katze, M. G., and Hovanessian, A. G. (1993) *Proc. Natl. Acad. Sci.* 90, 232-236.

Meyerovitch, J., Farfel, Z., Sack, J., and Shechter, Y. (1987) *J. Biol. Chem.* **262,** 6658-6662.

Murtha-Riel, P., Davies, M. V., Scherer, B., Choi, S.-Y., Hershey, J. W. B., and Kaufman, R. J (1993) *J. Biol. Chem.* **268,** 12946-12951.

Naranda, T., Sirangelo, I., Fabbri, B., and Hershey, J. W. B. (1995) *FEBS Letts.***372,** 249-252.

Naresh Babu, S. V., and Ramaiah, K. V. A. (1996) *Arch. Biochem. Biophys.* **327,** 201-208.

Naresh Babu, S. V. (1996) 'Regulation of protein synthesis in rabbit reticulocyte lysates

mediated by initiation factor 2 alpha (eIF2a) phosphorylation' Ph. D. thesis.

Nechay, B. R., Nanninga, L. B., Nechay, P. S. E., Post, R. L., Grantham, J. J., Macara, I. G., Kubena, L. F., Phillips, T. D., and Nielson, F. H. (1986) *Fed. Proc. Am. Soc. Exp. Biol.* 45, 123-132.

Ochoa, S. (1983) *Arch. Biochem. Biophys.* **223,** 325-349.

Oldfield, S., and Proud, C. G. (1992) *Eur. J. Biochem.* **208,** 73-81.

O'Reilly, D., Miller, L. K., and Luckow, V. A. (1992) *in* "Baculovirus Expression Vectors, A Laboratory Manual" W. H. Freeman and Company, New York, NY.

Pain, V. M. (1994) *Biochimie* 76, 718-728.

Palhan, V. B., Sumathy, S., and Gopinathan, K. P. (1995) *Biotechniques* 19, 97-104.

Palomo, C, Vincente, O., Sierra, J. M., and Ochoa, S. (1985) *Arch. Biochem. Biophys.* 239,497-507.

Panniers, R., and Henshaw, E. C. (1983) J. *Biol. Chem.* **258,** 7928-7934.

Panniers, R. (1994) *Biochimie* 76, 737-747.

Parra-Diaz, D., Wei, Q., Lee, E. Y. C, Echegoyen, L., and Puett, D. (1995) *FEBS Letters* 376, 58-60.

Patel, R. C, and Sen, G. C. (1992) *J. Interferon Res.* 12, 389-393.

Patel, R. C, Stanton, P., and Sen, G. C. (1994) J. *Biol. Chem.* **269,** 18593-18598.

Pathak, R. C, Nielsen, P., Trachsel, H., and Hershey, J. W. B. (1988a) *Cell* 54, 621-632.

Pathak, V. K., Schindler, D. and Hershey, J. W. B. (1988b) *Mol. Cell. Biol.* 9, 946-958.

Pavitt, G. D., Ramaiah, K. V. A., Kimball, S. R., and Hinnebusch, A. G. Communicated.

Price, N. T., Welsh, G. I., and Proud, C. G. (1991a) *Biochem. Biophys. Res. Commun.* 176,993-999.

Prostko, C. R., Brostrom, M. A., Malara, E. M., and Brostrom, C. O. (1992) *J. Biol. Chem.* **267,** 16751-16754.

Proud, G. G. (1992) *Curr. Top. Cell. Regul.* 32, 242-369.

Ramaiah, K. V. A., and Davies, E. (1985) *Plant and Cell Physiol.* 26, 1223-1231.

Ramaiah, K. V. A., Dhindsa, R. S., Chen, J.-J., London, I. M., and Levin, D. H. (1992) *Proc. Natl. Acad. Sci. USA* 89, 12063-12067.

Ramaiah, K. V. A., Davies, M. V., Chen, J.-J., and Kaufman, R. J. (1994) *Mol. Cell Biol.* 14,4546-4553.

Ramaiah, K. V. A., Chen, J.-J., Gallop, P. M., and London, I. M. (1997) *Bl. Cells. Mol. and Dis.* **23,** 177-187.

Ramasarma, T., and Crane, F. L. (1981) *Curr. Top. Cell. Regul.* **20,** 247-301.

Ramirez, M., Wek, R. C, and Hinnebusch, A. G. (1991) *Mol. Cell. Biol.* **11,** 3027-3036.

Ramirez, M., Wek, R. C, Vazquez de Aldana, C. R., Jackson, B. M., Freeman, B., and Hinnebusch, A. G. (1992) *Mol. Cell Biol.* 12, 5801-5815.

Ranu, R. S. (1981) *Proc. Natl. Acad. Sci. USA* 80, 3148-3152.

Redpath, N. T., and Proud, C. G. (1989) *Biochem. J.* 262, 69075.

Rehder, D. (1991) *Angew. Chem.* **103,** 152-172.

Rhoads, B. E. (1993) *J. Biol. Chem.* **268,** 3017-3020.

Reichel, P., Merrick, W. C, Sierkierka, J., and Mathews, M. B. (1985) *Nature* **313,** 196-200.

Rose, D. W., Welch, W. J., Kramer, G., and Hardesty, B. (1989) *J. Biol. Chem.* 264, 6239-6244.

Rosen, H., Knoller, S., and Kaempfer, R. (1981) *Biochemistry* **20,** 3011-3020.

Rosen, H., Di Segni, G., and Kaempfer, R. (1982) *J. Biol. Chem.* **257,** 946-952.

Rowlands, A. G., Panniers, R., and Henshaw, E. C. (1988a) *J. Biol. Chem.* **263,** 5526-5533.

Rowlands, A. G., Montine, K. S., Henshaw, E. C, and Panniers, R. (1988b) *Eur. J. Biochem.* **175,** 93-99.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *in* "Molecular Cloning: A Laboratory Manual" Cold Spring Harbor Laboratory Press, New York.

Samuel, C. E. (1993) J. *Biol. Chem.* **268,** 7603-7606.

Santoyo, J., Alcade, J., Mendez, R., Pulido, D., and de Haro, C. (1997) *J. Biol Chem.* 272, 12544-12550.

Scheper, G. C., Thomas, A. A. M., and Voorma, H. O. (1991) *Biochem. Biophys. Acta* **1089,** 220

Siekierka, J., Meuser, L, and Ochoa, S. (1981) *Proc. Natl Acad. Sci. U. S. A.* 78, 220-

223.

Simons, T. J. B. (1979) *Nature* **281,** 337-338.

Singh, L. P., Aroor, A. R., and Wahba, A. J. (1994) *Biochemistry* 33, 9152-9157.

Singh, L. P., and Wahba, A. J. (1995) *Biochem. Biophys. Res. Commun.* **217,** 616-623.

Singh, L. P., Denslow, N. D., and Wahba, A. J. (1996) *Biochem.* 35, 3206-3212.

Spirin, A. S. (1986) *in* 'Ribosome Structure and Protein Biosynthesis'. The Benjamin/Cummings Publishing Company, Inc.

Sreedhara, A., Srinivasa, R. M. S., and Rao, C. P. (1994) *Carb. Res.* **264,** 227-236.

Sreedhara, A., Susa, N., Anjali, P., and Rao, C. P. (1996) *Biochem. Biophys. Res. Commun.* 224, 115-120.

Srivastava, S. P., Davies, M. V., and Kaufman, R. J. (1995) J. *Biol. Chem.* **28,** 16619-16624.

Summers, M. D., and Smith, G. E. (1987) *in* A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures, Texas Agric. Station. Bull. No. 1555, College Station, TX.

Surolia, N., and Padmanaban, G. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 4786-4790.

Thanuja, K., Sreedhara, A., Rao, C. P., and Ramaiah, K. V. A. (1997) *Arch. Biochem. Biophys.* **348,** in press.

Thomas, N. S. B., Matts, R. L., Petryshyn, R., and London, I. M. (1984) *Proc. Natl. Acad Sci. USA* **81,** 6998-7002.

Thomas, N. S. B., Matts, R. L., Levin, D. H., and London. I. M. (1985) *J. Biol. Chem.* **260,** 9860-9866.

Vaughn, J. L., Goodwin, R. H., Tomkins, G. J., and McCawley, P. (1977) *In Vitro* 13, 213-217.

Vazquez de Aldana, C. R., Dever, R. E., and Hinnebusch, A. G. (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90,7215-7219.

Venema, R. C, Peters, H. 1., and Traugh, J. A. (1991a) *J. Biol. Chem.* **266,** 11993-11998.

Venema, R. C, Peters, H. I., and Traugh, J. A. (1991b) J. *Biol. Chem.* **266,** 12574-12580.

Vialard, J., LaLumiere, M., Vernet, T., Briedis, D., Alkhatib, G., Henning, D., Levin, D., and Richardson, C. (1990) J. *Virol.* 64, 37-50.

Volkman, L. E., and Goldsmith, P. A. (1985) *Virology* **143,** 185-195.

Watson, J. D., Hopkins, N. C, Roberts, J. W., Steitz, J. A., and Weiner, A. M. (1987) *in* Molecular Biology of the Gene, IV edition, vol 1, The Benjamin/Cummings Publishing Company Inc., California

Welsh, G. I., and Proud, C. G. (1992) *Biochem. J.* **284,** 19-23.

Welsh, G. I., Price, N. T., Bladergroen, B. A., Bloomberg, G., Proud, C. G. (1994) *Biochem. Biophys. Res. Commun.* **201,** 1279-1288.

Welsh, G. I., Miyamoto, S., Price, N. T., Safer, B., Proud, C. G. (1996) *J. Biol. Chem.* **271,** 11410-11413.

Wek, R. C. (1994) *Trends Biochem. Sci.* 19, 491-496.

Wettenhall, R. E. H., Kudlicki, W., Kramer, G., and Hardesty, B. (1986) *J. Biol. Chem.* **261,** 12444-12447.

Weyer, U., Knight, S., and Possee, R. D. (1990)./ *Gen. Virol.* 71, 1525-1534.

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Reducing Agents Mitigate Protein Synthesis Inhibition Mediated by Vanadate and Vanadyl Compounds in Reticulocyte Lysates

Thanuja Krishnamoorthy,* A. Sreedhara,† Chebrolu P. Rao,† and Kolluru V. A. Ramaiah*,[1]

Department of Biochemistry, School of Life Sciences, University of Hyderabad, Hyderabad-500046, Andhra Pradesh, India; and †Bioinorganic Laboratory, Department of Chemistry, Indian Institute of Technology, Powai, Mumbai-400 076, Maharashtra, India

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Recently, we synthesized and characterized vanadyl saccharides to evaluate the effects of various vanadate and vanadyl complexes, which differ in their oxidation states on various biomacromolecules and cellular activities (1, 2). Here, we report that both vanadate (+V oxidation state) and different vanadyl species (+ IV oxidation state) such as vanadyl D-glucose, vanadyl diascorbate, and vanadyl sulfate, impair the formation of polysomes and inhibit the initiation of protein synthesis in hemin-supplemented rabbit reticulocyte lysates. Vanadate inhibits protein synthesis more severely than vanadyl species and is consistent with the idea that vanadate is reduced to vanadyl state intracellularly. The inhibition of protein synthesis caused by low concentrations (10-20 μM) of vanadate and vanadyl species is effectively mitigated by reducing agents such as dithiothreitol, reduced glutathione (GSH), orpyridine dinucleotide. A significant decrease in the protein synthesis inhibition in vanadate-treated lysates by GSH suggests that the mechanism of protein synthesis inhibition by vanadate is different than the action of other oxidants such as heavy metal ions and oxidized glutathione. This suggestion is also consistent with the findings that vanadium compounds do not stimulate phosphorylation of the alpha (a) subunit of initiation factor 2 (eIF2) or decrease the guanine nucleotide exchange activity of eIF2B, which is required to exchange GDP for GTP in eIF2 · GDP binary complex. The reduction of vanadate to vanadyl state and the subsequent complex formation of vanadyl species with the endogenous reducing compounds or with the -SH groups of certain proteins may be the cause for protein synthesis inhibition in lysates.

Vanadium is known as an essential nutrient required by living organisms. High levels of vanadium are toxic as it causes bronchitis and pneumonia. The major sources of vanadium are from burning fossil fuels and from rnining (3). Intracellular concentrations of vanadium, e.g., in erythrocytes, may occur in the micromolar range (4). Vanadate $(+V)^2$ is a diuretic, a natriuretic, and a vasoconstrictor (5-9). Vanadium ions are known to be potent inhibitors of Na^+, K^+ ATPase, Ca^{2+} ATPase, Mg^{2+} ATPase, and adenylate kinase (9, 10).

Vanadate inhibits a number of enzymes of the glycolytic pathway (4, 9). Vanadate compounds are being used as an oral insulin mimetic, although vanadium does not mimic all the actions of insulin (11, 12). Vanadium therapy is given to diabetic patients to normalize blood glucose levels and also to alleviate many of the abnormalities associated with hyperglycemia (13—16). Vanadate is a potent protein tyrosine phosphatase inhibitor (17). It is also known that vanadyl ions (+IV) inhibit receptor tyrosine kinases like insulin receptor, while the vanadate form stimulates cytoplasmic protein tyrosine kinases in rat adipocytes (13).

Vanadate that enters the erythrocytes via the phosphate channel is reduced to its vanadyl state by cytoplasmic glutathione, in a nonenzymatic process (4, 18, 19). Earlier studies indicate that vanadyl binds to ATP as well as to a variety of metalloproteins (4, 18, 20). Recently, we have demonstrated that vanadyl (+IV) saccharide complexes introduce nicks in pUC18 DNA and cause lipid peroxidation in isolated rat hepatocytes (2). Protein synthesis in reticulocyte lysates is known to be regulated by phosphorylation-dephosphorylation

[1] To whom correspondence should be addressed. Fax: (91) (040) 3010120 / 3010145 E-mail: kvarsl@uohyd.ernet.in

[2] Abbreviations used: +V, vanadate; +IV, vanadyl ions; DTT, dithiothreitol; eIF2α, alpha Bubunit of initiation factor 2; vadex, vanadyl D-glucose; vasc, vanadyl diascorbate.

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of protein factors and also by redox levels (21–23). A previous study (24) suggests that vanadate impairs protein synthesis initiation but does not reveal its mechanism. In this study, the effect of vanadium compounds in +IV and +V oxidation states on protein synthesis has been investigated. In addition, the ability of reducing agents to counter the vanadium-mediated inhibition of protein synthesis has been investigated. This is the first report to our knowledge which suggests that protein synthesis inhibition mediated by vanadium is decreased by reducing agents such as GSH, NADPH, and DTT and that vanadyl ions are less inhibitory than vanadate.

Both the species of vanadium do not increase the phosphorylation of the alpha subunit of initiation factor 2 (eIF2) or decrease the guanine nucleotide exchange activity of eIF2B protein, which is required in the recycling of eIF2 • GDP binary complex. These negative findings suggest that the mechanism of protein synthesis inhibition in the presence of vanadium species is different than that of other oxidizing agents such as heavy metals or oxidized glutathione or pyrroloquinoline quinone (25—31). The findings are consistent with the result that GSH can prevent significantly the inhibition of protein synthesis in vanadate-treated hemin-supplemented lysates.

MATERIALS AND METHODS

Materials

[^{14}C]Leucine (337.5 μM, 330 mCi/mmol) and [^{3}H]GDP (9 Ci/mmol) were purchased from Dupont NEN. [γ-^{32}P]ATP (3000 Ci/mmol) was obtained from BRIT., Mumbai and Jonaki Center in CCMB (Hyderabad, India). DTT was purchased from Boehringer Mannheim. Poly (IC) was purchased from Calbiochem. Heparin was obtained from Biological E. Limited (Hyderabad, India). Vadex and vasc were prepared by one of us (A.S.). All other chemicals were obtained from Sigma (St. Louis, MO). New Zealand white male rabbits were procured from Indian Immunologicals and from Biological E. Limited.

Experimental Procedures

Protein synthesis in reticulocyte lysates. Heme-deficient rabbit reticulocyte lysates were prepared as described (32). Standard lysate protein synthesis assays [15–25 μl] were carried out at 30°C for 60 min as previously mentioned (31, 33) in the presence or absence of 20 μM hemin. Addition of various other agents are mentioned in the legends to the figures and tables. The extent of protein synthesis was measured by the incorporation of labeled leucine into TCA-precipitable protein at specified time points in 5-μl aliquots.

Purification of reticulocyte eIF2. Rabbit reticulocyte eIF2 was purified from postribosomal supernatant by column chromatography CDEAE, phosphocellulose, and CM-Sephadex) as detailed earlier (34, 35). The preparation was checked by 10% sodium dodecyl sulfate - polyacrylamide gel electrophoresis and also by an eIF2α monoclonal antibody.

Phosphorylation of lysate proteins. Protein synthesizing lysates were pulsed with [γ-^{32}P]ATP (3000 Ci/mmol) for a brief period (5 min) and samples were pH 5 precipitated (31, 36), separated on 10% polyacrylamide and 0.1% sodium dodecyl sulfate gels, and analyzed by autoradiography.

Binary complex formation, eIF2 [^{3}H]GDP and its dissociation. Binary complex (eIF2 • [^{3}H]GDP) was prepared as described (33, 37). Purified reticulocyte eIF2 (2 μg) was incubated in a 20-μl reaction mixture containing 20 mM Tris-HCl, pH 7.6. 80 mM KC1, 100 μg/ml CPK (as carrier), and 2 μM PHIGDP (1100 cpm/pmol). Reducing agent, DTT (1 mM), was generally added to the above reaction mixtures unless otherwise stated in the figure legends. The reactions were incubated at 30°C for 10 min, followed by 10 min on ice, for the binary complex to be formed as described (35, 38); 1 mM $Mg(OAc)_2$ was added to stabilize the binary complex and incubated further for 10 min on ice. Reactions were terminated at this stage by the addition of 3 ml of cold wash buffer (20 mM Tris-HCl, pH 7.8, 1 mM $Mg(OAc)_2$, and 80 mM KC1) to determine the formation of binary complex.

Dissociation of preformed binary complex was also studied in protein synthesizing lysates under different conditions at 30°C as described in the figure legends. Protein synthesis (20-25 μl) was carried out at 30°C without the addition of any labeled amino acid for 10-15 min prior to the addition of preformed labeled binary complex (20 μl). Reactions were terminated at the specified time points by the addition of cold wash buffer, and the amount (pmol) of eIF2 • [^{3}H]GDP bound to the membrane was ascertained as described (33, 35, 37).

Analysis of polysomes. Protein synthesis mixes (100 μl) were chilled on ice and diluted with an equal volume of ice-cold buffer (20 mM Tris-HCl, pH 7.8, 1 mM $Mg(OAc)_2$, 80 mM KC1, and 0.5 mM EDTA) and layered on 10-50% sucrose gradients prepared as described (36). Samples were centrifuged at 45,000 rpm for 45 min in a SW 50.1 rotor. Ribosomal profiles were monitored at 254 nm by upward displacement of the gradient with an Isco (Model 185) density gradient fractionator.

RESULTS

Vanadate and Vanadyl Ions Inhibit Protein Synthesis Initiation

An earlier study described vanadate inhibition of protein synthesis inhibition in reticulocyte lysates (24). Most of the earlier studies involving vanadium effects on various biological processes or enzyme reactions suffer from a lack of knowledge about the oxidation states of vanadium. In contrast to the earlier studies on protein synthesis in the presence of vanadate, we have evaluated here the effects of the following four compounds: sodium orthovanadate (+V), vanadyl D-glucose (+IV, vadex), vanadyl diascorbate (+IV, vasc), and vanadyl sulfate (+ IV). Vadex is a novel anionic saccharide complex which is very stable (1).

Protein synthesis of typical heme-deficient and hemin-supplemented lysates are shown in Fig. 1. In the absence of heme, protein synthesis was impaired (legend to Fig. 1) as expected and described (reviewed in 22, 23). Protein synthesis was linear over a period of 30 min in the presence of heme. All vanadium compounds mentioned above inhibited protein synthesis of both heme-deficient (data not shown) and hemin-supplemented lysates (Fig. 1). The inhibition was concentration dependent. Among the vanadium compounds tested, sodium orthovanadate (+V) was the most potent and produced maximum inhibition of 70% at 10 μM (Fig. 1). Vanadyl iiascorbate and vanadyl sulfate (+IV) were less inhibitory while vadex (+IV) was relatively more inhibitory (Fig. 1).

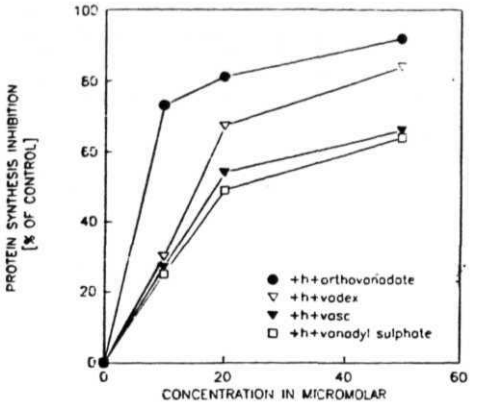

FIG. 1. Percent inhibition in protein synthesis by vanadium compounds at different concentrations in hemin-supplemented reticulocyte lysate at 60 min. Hemin-supplemented (20 μM) protein-synthesizing lysates (15 μl) were incubated at 30°C with increasing concentrations of different vanadium compounds (added at 0 min) in four separate assays. Protein synthesis was measured by studying [^{14}C]-leucine incorporation in 5-μl aliquots at 60 min as described (31). The percent inhibition was calculated taking hemin-supplemented lysates as the control (mentioned below as cpm x 10^{-3} at 60 min) which were as follows for the different vanadium compounds: 37.1 for orthovanadate, 48.6 for vadex, 43.6 for vasc, and 38.7 for vanadyl sulfate. The values for the heme-deficient lysates were as follows: 14.2, 19.0, 15.8, and 17.4 for the four separate assays as described above.

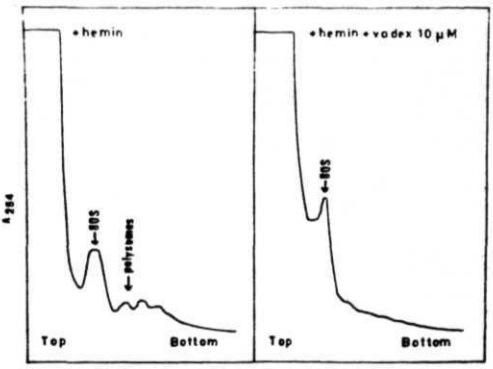

FIG. 2. Effect of vadex on lysate polysomes. Standard reticulocyte lysate protein synthesis mixtures were incubated for 10 min at 30°C with 20 μM hemin (left) or 20 μM hemin with 10 μM vadex (right). The mixtures were then diluted, loaded on the sucrose gradient, centrifuged, and analyzed as described under Materials and Methods. At 254 nm, the OD was taken with a chart speed of 300 cm/h, absorption of 1.0, and a flow rate of 3 ml/min.

Vanadium Impairs Polysome Formation

Addition of one of the compounds, vanadyl D-glucose, to hemin-supplemented lysates decreased the polysomes and increased monosomes (Fig. 2). This finding suggests that protein synthesis inhibition occurs at the initiation step and is in accordance with the previous results (24) that vanadate inhibits the protein chain initiation.

Reducing Agents like DTT, βNADPH, and GSH Mitigate Vanadium-Mediated Inhibition

Vanadate is hypothesized to interact with protein-SH groups of the enzymes like glyceraldehyde 3-phosphate dehydrogenase and consequently inhibits its activity (4). Also, there are reports that vanadate is converted to its vanadyl state in erythrocytes by intracellular glutathione (GSH) (4, 18, 19). Hence, we have studied here the effects of DTT, βNADPH, and GSH, as well as low and high concentrations of sugar phosphates, on protein synthesis in vanadium-treated lysates. DTT and βNADPH mitigated significantly the inhibition caused by all vanadium compounds at a low concentration (10 μM, Tables IA and IB) but were unable to act effectively to counter the inhibition caused by high concentrations of vanadate on protein synthesis (Table IC). We have also studied the effect of GSH and Glu-6-P in vanadate-treated lysates. It was observed that while GSH could decrease the inhibitory effect of vanadate on protein synthesis, a similar effect could not be obtained with the addition of low and high

TABLE IA

Effect of βNADPH and DTT on Protein Synthesis in 10 μM Vanadium-Treated Lysates

Reducing agent present: / Addition	None	Incorporation (% of control) NADPH	DTT
None	100	80	105
Vanadate	36	69	77
Vadex	50	88	87
Vasc	73	87	82
$VOSO_4$	103	85	95

Note Protein synthesis was carried out as described in the legend to Fig. 1 under the following conditions: (i) plus hemin (+h, 20 μM), (ii) +H +10 μM vanadium compound, (iii) +h +1 mM DTT, (iv) +h +1 mM βNADPH, (v) +h +10 μM vanadium compound +1 mM DTT, and (vi) h + 10 μM vanadium compound +1 mM βNADPH. Protein synthesis was measured in 5-μl aliquots at 60 min as described under Materials and Methods.

Effect of GSH and Glucose 6-Phosphate on Protein Synthesis in 15 μM Vanadium-Treated Lysates

Vanadate concentration:	0	15 μM
Addition		
None	100	56
DTT, 1 mM	113	83
GSH, 1 mM	118	85
Glu-6P, 50 μM	111	57
Glu-6P, 1 mM	97	55

Note. Separate protein synthesizing lysates (20 μl) were carried out at 30°C in the presence of hemin, 20 μM; 15 μM sodium orthovanadate and 1 mM each DTT and GSH were used wherever indicated in the table. Two different concentrations of Glu-6-P, 50 and 1 μM, were used as shown. All additions were done at 0 min. Protein synthesis was monitored in 5-μl aliquots at 60 min.

concentrations of Glu-6-P (Table IB), suggesting that vanadate does not inhibit protein synthesis simply by generating an oxidizing stress which depletes the Glu-6-P of the lysate. However, the results do not reveal if vanadate can affect the enzyme, Glu-6-P dehydrogenase. The effect of reducing agents to overcome the inhibition of protein synthesis caused by low concentrations of vanadate or vanadyl compounds is so impressive that it compels us to suggest that the mechanism of action is similar for both oxidation states of vanadium. Since vanadate is converted to vanadyl state intracellularly, an experiment was conducted to follow the fate of one of the vanadyl compounds, vadex, in lysates by electron paramagnetic spectra (EPR). The results of this experiment indicated that vadex remained in the +IV oxidation state (data not shown).

eIF2 Alpha Phosphorylation and eIF-2B Activity Is Not Affected in Lysates Treated with Low Concentration of Vanadate

Previous studies have shown that the inhibition of protein synthesis in reticulocyte lysates occurs under a variety of conditions which include addition of oxidants or sulfhydryl reactive agents, heavy metals, and heat stress (reviewed in 22, 23). Under all these conditions, the protein synthesis inhibition occurs apparently due to the activation of a kinase with properties similar to heme-regulated inhibitor which stimulates phosphorylation of the alpha or small subunit of initiation factor 2 (eIF2α) (reviewed in 39). Phosphorylated eIF2α inhibits the guanine nucleotide exchange activity of eIF2B protein, which is required in the exchange of GTP for GDP in eIF2 • GDP binary complex, and thereby inhibits the recycling of eIF2 and initiation of protein synthesis (33, 35, 37). In addition to eIF2α phosphorylation, eIF2B activity is regulated by changes in redox levels (31. 40–42) and the phosphorylation of the largest subunit (82 kDa) in eIF2B (43, 44). Hence, the eIF2α phosphorylation and eIF2B activity of vanadate-treated lysates have been studied here to determine if there are any changes in them which can explain the protein synthesis inhibition and the effect of reducing agents to overcome the inhibition of protein synthesis.

The findings indicated that none of the vanadium compounds affected eIF2α phosphorylation in lysates or *in vitro* (data not 6hown).

We have also studied the eIF2B activity of vanadium-treated lysates by monitoring the release of labeled GDP from a preformed eIF2 · [^{3}H]GDP binary complex as has been described (33, 35, 37). As DTT is able to mitigate the inhibition of protein synthesis caused by vanadium compounds and DTT is also used generally in the preparation of binary complex *in vitro*, a control experiment was conducted in the presence and absence of DTT, to determine the changes in lysate eIF2B activity under standard protein synthesizing conditions, i.e., (a) hemin-supplemented lysates which can carry active protein synthesis and (b) hemin and poly (IC)-treated lysates where protein synthesis is inhibited due to eIF2α phosphorylation and inhibition in eIF2B activity (Table II). The assay was carried out using a binary complex, eIF2 • [^{3}H]GDP, which was prepared with or without DTT. The dissociation of the labeled GDP was monitored for a period of 5 min at 30°C. The dissociation of eIF2 • [^{3}H]GDP binary complex (prepared in the presence or absence of DTT) was typically inhibited in poly (IC)-treated lysates as expected due to enhanced eIF2α phosphorylation. However, the levels of dissociation of preformed binary complex in lysates differ depending on the presence or absence of DTT. The above preformed binary complex was more rapidly dissoci-

TABLE IC

Effect of DTT and βNADPH on Protein Synthesis in 10-50 μM Vanadate-Treated Lysates

Vanadate concentration:	0	Incorporation (% of minus vanadate) 10	20	50
Addition				
None	18,800(0)	12,500(0)	7,200(0)	3160(0)
DTT	23,700 (+26)	19,500 (+56)	11,000 (+57)	4200 (+35)
NADPH	20,000 (+7)	15,700 (+25)	9,500 (+33)	3700 (+19)

Note. Protein synthesis was carried out in 15 μl volume in the presence of hemin or hemin and different concentrations of vanadate at 30°C as described in the legends to Tables 1A and IB. The extent of protection in protein synthesis offered by reducing agents (1 mM DTT or βNADPH) in lysates treated with different concentrations of vanadate (10, 20, and 50 μM) has been determined.

TABLE II

Effect of DTT on eIF2 • [3H]GDP Dissociation in Heme or Heme and Poly (IC)-Treated Ly6ates

Protein synthesis conditions	eIF2 · [³H]GDP bound (cpm x 10^{-3})	eIF2 · [³H]GDP dissociated (pmols, 5 nun)
-DTT +h, 0′	20.7	
-DTT+h	7.9	11.61
-DTT +h +Poly (IC)	15.2	5.0
+DTT +h, 0′	21.8	
+DTT +h	3.5	16.6
+DTT +h +Poly (IC)	12.6	8.4

Note. Protein synthesizing lysates (25 μl) were incubated at 30°C in the presence of hemin or hemin and poly (IC) |20 μM hemin, 300 ng/ml poly (IC)| for 10 min. Labeled binary complex, eIF2 • [³H]GDP was prepared in the presence and absence of 1 mM DTT and added to the above translating lysates at 10 min or protein synthesis. The reactions were incubated for a period of 5 min at 30°C to determine the dissociation of labeled GDP from the binary complex.

a ted in -theme lysates and strongly inhibited in +heme and poly (IC)-treated lysates in the presence of DTT than without DTT (Table II). These findings are consistent with the idea that reducing conditions stimulate eIF2B activity (31, 41–43) and also eIF2α kinase activity (reviewed in 22, 23, 39) of heme-deficient reticulocyte lysates.

Since omission of DTT in the preparation of binary complex does not alter the trend in the inhibition in eIF2B activity of lysates caused by poly (IC) treatment, a similar experiment was carried out to determine the eIF2B activity of lysates treated with different concentrations of vanadate or vanadate and DTT (Table III). Presence of low concentrations (10 and 20μM) of vanadate inhibited marginally the dissociation of binary complex, whereas poly (IC) treatment caused a maximum inhibition in the dissociation of binary complex. This comparison reveals that the minimal inhibition in eIF2B activity by vanadate is unlike the inhibition in eIF2B activity caused by poly (IC). Addition of 1 mM DTT to vanadate-treated lysates (10 or 20 μM) could overcome this marginal inhibition in eIF2B activity as the dissociation of binary complex increased and was on par with +heme lysates. The ability of DTT to reverse the small decrease in eIF2B activity observed in the presence of vanadate (Table III) can be best explained by the ability of DTT to stimulate eIF2B activity (Table II) and protein synthesis (Tables IA–IC) in untreated lysates. This may be a consequence of the natural depletion of the lysate-reducing ability during prolonged incubation of the lysate. Hence the findings suggest that low concentrations of vanadate which inhibit protein synthesis significantly do not appear .o affect eIF2B activity.

In contrast, higher concentrations (50 μM) of vanadate which inhibited protein synthesis more severely (Fig. 1) were also found to enhance the dissociation of eIF2 · GDP binary complex in lysates (Table III). This latter finding suggests that the dissociation of eIF2 • GDP binary complex does not truly represent the functional eIF2B activity. To confirm this artifactual situation, the dissociation of preformed eIF2 • [³H]GDP binary complex and protein synthesis were monitored in poly (IC)-treated lysates which were supplemented with the increasing concentrations of vanadate. The results suggest that the dissociation of the above binary complex increases with increasing concentrations of vanadate in poly (IC)-treated lysates, whereas the protein synthesis is further reduced in poly (IO-treated lysates (Table III). Hence the dissociation of binary complex in the presence of higher concentrations of vanadate does not appear to be mediated by eIF2B protein. eIF2 • [³H]GDP dissociation varies with higher vanadate concentrations in lysates and the variation is also contributed by lysates in which eIF2α is unphosphorylated or phosphorylated (+heme or +heme +poly (IC)). This apparent discrepancy may be due to the fact that vanadate at higher concentrations may be

TABLE III

Effect of Different Concentrations of Orthovanadate on the Dissociation of eIF2 · [³H]GDP Complex and on Protein Synthesis in Heme or Heme and Poly (IC)-Treated Lysates

Protein synthesis incorporation conditions	eIF2 · [³H]GDP dissociated (pmol, 15 min)	[¹⁴C]Leu (cpm x 10^{-3}, 30 min)
+h	16.88	137
+h +10 μM Na_3VO_4	15.76	9.2
+h +20 μM Na_3VO_4	16.24	3.7
+h +50 μM Na_3VO_4	16.66	2.5
+h +Poly (IC)	0.0	10.1
+h +Poly (IC) + 10 μM Na_3VO_4	1.91	5.8
+h +Poly (IC) +20 μM Na_3VO_4	2.37	3.0
+h +Poly (IC) +50 μM Na_3VO_4	3.76	2.6
+h +DTT +10 μM Na_3VO_4	16.98	—
+h +DTT +20 μM Na_3VO_4	17.07	—
+h + DTT + 50 μM Na_3VO_4	17.76	—

Note. Hemin-supplemented (20 μM) protein synthesizing lysates (25 μl) were incubated at 30°C in the presence of various agents as shown in the table. DTT (1 mM) and 300 ng/ml of poly (IC) were used where indicated. At 15 min of protein synthesis, 20 μl of performed binary complex, formed in the absence of DTT (21 pmol, eIF2 • (³H)GDP] was added and its dissociation was assayed for 5 min at 30°C as described under Materials and Methods. In another set of protein synthesizing lysates, using [¹⁴C]leucine cocktail, protein synthesis was carried out in hemin-supplemented lysates in the presence of poly QC) or poly (IC) and orthovanadate. Protein synthesis was carried out in 25 μl under the fallowing conditions: (i) plus hemin (+ h), (ii) + h + paly (IC), 300 ng/ml; (iii) +h + orthovanadate; and (iv) +h + orthovanadate + poly (IC). Protein synthesis was carried out at 30°C and monitored in 5-μl aliquots at 30 min as described under Materials and Methods

competing with a phosphate for the enzyme binding site (9, 24).

DISCUSSION

Earlier studies indicate that cytoplasmic vanadate is reduced to the +IV oxidation state characteristic of vanadyl ions (4, 8, 19). Effects of such vanadyl complexes on various cellular activities have not been well documented. In an earlier communication (1), we reported the results of reduction of vanadate by multihydroxy molecules such as saccharides and ascorbic acid. The vanadyl complexes thus formed were able to produce nicks in pUC18 DNA and also exhibit lipid peroxidation in isolated rat hepatocytes (2). The saccharide and ascorbate complexes of vanadium were found to be hydrolytically and oxidatively stable in lysates (data not shown). The availability of these vanadyl complexes allowed us for the first time to evaluate the effects of vanadate and vanadyl compounds directly on total protein synthesis in hemin-supplemented rabbit reticulocyte lysates. In addition, reticulocyte lysate is a good translational model system to investigate the effects of various agents such as heavy metals, oxidizing/reducing agents, antibiotics, and lectins, to investigate their mechanism of action on protein synthesis *in vitro*. The reticulocyte extracts contain a number of reducing agents common to cells, like L-ascorbic acid, glutathione, and cysteine, which can convert vanadate (+V) to vanadyl (+IV) state. A very early study (24) is the only report that we are aware of suggesting that vanadate (10-40 μM) inhibits protein chain initiation in reticulocyte lysates and produces defective 80S initiation complexes. This study also points out that higher concentrations of vanadate (above 50 μM) inhibit polypeptide chain elongation to some extent. Consistent with this observation, our results also indicate that vanadate inhibits protein synthesis initiation (Figs. 1 and 2). In addition, we observed that vanadate inhibits protein synthesis more strongly than vanadyl complexes. Also the protein synthesis inhibition caused by low concentrations of vanadium (10 and 20 μM) can be easily mitigated by reducing compounds like βNADPH, DTT, or GSH but not by glucose 6-phosphate (Tables IA and IB).

These findings suggest that both species of vanadium can deplete the reducing power of the lysate. Consistent with the idea that vanadate can be reduced to vanadyl state in the presence of GSH or NADPH (18), we find here that the inhibition of protein synthesis is more severe in the presence of vanadate ions (Fig. 1). This suggests that vanadate can more effectively deplete the lysate GSH or NADPH for its reduction than vanadyl ions. Also, we observed that vanadyl compounds are not reduced any further in the lysate during the reaction period (data not shown).

Previous studies have shown that maintenance of protein synthesis initiation in rabbit reticulocyte lysates requires the presence of hemin, a sugar phosphate (e.g., Glu-6-P), and a reducing system which is capable of reducing disulfide bonds. Sugar phosphates are required as a stimulating "cofactor" affecting protein chain initiation and for NADPH generation by way of metabolism through pentose phosphate shunt (reviewed in 22, 23). The requirement for reducing power is thought to be met by this NADPH generation, together with an active thioredoxin/thioredoxin reductase system. A variety of conditions which include oxidants or sulfhydryl reactive agents, heat stress, and heavy metals (reviewed in 22, 39, 45) have been shown to inhibit protein synthesis in hemin-supplemented reticulocyte lysates due to the activation of heme-regulated eIF2α kinase which subsequently stimulates eIF2α phosphorylation. As a consequence, the guanine nucleotide exchange activity of eIF2B, which is required in the recycling of eIF2 • GDP binary complex, is inhibited. In addition to oxidative stress, the activation of heme-regulated eIF2α kinase is also dependent on its association with heat shock proteins and is influenced by hemin, ATP, and GTP (reviewed in 39).

The inhibition of protein synthesis which is accompanied by enhanced eIF2α phosphorylation in lysates treated with heavy metals (25, 26) or with GSSG (27-30) can be reversed by DTT but not by GSH. These findings suggest that sulfhydryl groups present in some critical protein(s), possibly dithiols present in thioredoxin reductase, thioredoxin, and/or heat shock proteins, are modified in the presence of the above treatments, which lead to the activation of HRI and enhanced eIF2α phosphorylation.

In our studies here, vanadate or vanadyl species have not been able to stimulate eIF2α phosphorylation (data not shown) or to decrease significantly the lysate eIF2B activity (Table III). Moreover, GSH, a monothiol, which cannot prevent or restore protein synthesis caused by oxidizing metals or GSSG (26, 28), has been found here to prevent significantly protein synthesis inhibition caused by vanadate. These findings emphasize that the mechanism of protein synthesis inhibition in vanadate-treated lysate appears to be different from the inhibition caused by other oxidizing metal ions or GSSG. The reducing agents which are found here to prevent significantly the protein synthesis inhibition in vanadium-treated lysates not only are the reductive type of agents but also can complex with metal ions. Hence, it is likely that vanadyl species generated due to reduction of vanadate or added vanadyl compounds are able to deplete the lysate GSH or NADPH by complexing with them and thereby causing inhibition of protein synthesis in reticulocyte lysates. Other possibilities which need to be tested include binding of vanadyl ions

to some critical proteins which are required for protein synthesis and thereby modifying their activities.

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REFERENCES

1. Sreedhara, A., Srinivasa, R. M. S., and Rao, C. P. (1994) *Carbohydr. Res.* **264,** 227–236.
2. Sreedhara, A, Susa, N., Anjali, P., and Rao, C. P. (1996) *Biochem. Biophys. Res. Commun.* 224, 115-120.
8. Ramasarma, T., and Crane, F. L. (1981) *Curr. Top. Cell. Regul.* 20, 247–301.
4. Benabe, J. E., Echegoyen, L. A., Belinda, P., and Marnitez-Maldonado, M. (1987) *J. Biol Chem.* 262, 9555-9560.
5. Nechay, B. R., Nanninga, L. B , Nechay, P. S. E , Post, R. L., Grantham, J. J., Macara, I. G., Kubena, L. F., Phillips, T. D., and Nielson, F. H (1986) *Fed. Proc Am. Soc. Exp. Biol.* **45,** 123–132.
6. Rehder, D. (1991) *Angew. Chem.* **103,** 152–172.
7. Kramer, H. J., Krampitz, G., Backer, A., Krampitz, G., Jr., and Meyer-Lehnert, H (1995) *Biochem. Biophys Res. Commun.* 21S, 289-294.
8. Balfour, W. E., Grantham, J. J., and Glynn, I. M (1978) *Nature* **275,** 768. 708
9. Simons, T.J.B. (1979) *Katun* 281, 337-338.
10. Cantley, L. C, Jr., Josephson, L., Warner, R., Yanagisawa, M., Lechene, C, and Guidotti, G. (1977) *J. Biol. Chem.* **252,** 7421-7423.
11. Bosch, F., Arino, J., Gomez-Foix, A. M., and Guniovar, J. J. (1987) *J. Biol. Chem.* **262,** 218-222.
12. Clarke, A S., Fagan, J. M., and Mitch, W. E. (1985) *Biochem J.* **232,** 273–276.
13. Elberg, G., Li, J., and Shecter, Y. (1994) *J. Biol. Chem.* 269, 9521-9527.
14. Heyliger, C. E., Tahiliani, A. G., and McNeill, J. H. (1985) *Science* **227,** 1474–1476.
15. Meyerovitch, J., Farfel, Z., Sack, J., and Shechter, Y. (1987) *J. Biol Chem.* **262,** 6658–6662.
16. Gil, J., Miralpeix, M., Carreras, J., and Bartrons, R (1988) *J. Biol. Chem.* **263,** 1868–1871.
17. Kan Liao, and Daniel Lane, M. (1995) J. *Biol Chem.* 270,12123-12132.
18. Macara, I. G., Kustin, EL, and Cantley, L. C. Jr. (1980) *Biochim. Biophys. Ada* 629, 95-106.
19. Chasteen, N. D. (1983) *in* Structure and Bonding: Copper, Molybdenum and Vanadium in Biological Systems (Clarke, M. J., *et at.,* Eds), pp 103-136, Springer-Verlag, Heidelelberg, German v.
20. Parra-Diaz, D., Wei, Q., Lee, E. Y. C, Echegoyen, L., and Puett, D. (1995) *FEUS Lett.* 876, 58-60.
21. Hershey, J. W. B. (1989) *J. Biol. Chem.* 264, 20823-20826.
22. Jackson, R. J. (1991) in Translation in Eukaryotes (Trachsel, H., Ed), pp. 193-229, CRC Press, Boca Raton, FL
23 London, I. M , Levin, D. H., Matts, R L., Thomas, N. S. B., Petryshyn, R , and Chen, J .J. (1987) *in* The Enzymes (Boyer, P. D., and Krebs, E. G., Eds.), Vol. 17, pp. 359–380, Academic Press, New York.
24. Ranu, R. S. (1981) Proc. *Natl. Acad. Sci. USA* 80, 3148-3152.
25. Hurst, R , Schatz, J. R., and Matts, R. L. (1987) *J. Biol. Chem.* **262,** 15939–15945.
26. Matte, R. L., Schatz, J. R., Hurst, R, and Kagen, R. (1991) *J. Biol Chem.* 266, 12695-12702.
27. Kosower, N. S., Vanderhoff, G. A., and Kosower, E. M. (1972) *Biochim. Biophys. Acta* 272, 623–627.
28. Palomo, C, Vincente, O., Sierra, J. M., and Ochoa, S. (1985) *Arch. Biochem Biophys.* 239, 497-507.
29. Ernst, V., Levin, D.H., and London, I. M. (1978) Proc. *Natl. Acad. Sci. USA* **75,** 4110-4114.
30. Kan, B , London, I. M., and Levin, D. H. (1988) *J. Biol. Chem.* **263,** 15652–15656.
31. Ramaiah, K. V. A., Chen, J.-J., Gallop, P. M., and London, I. M. (1997) *Bl. Cells. Mol. Dis.* 23, 177-187.
32. Hunt, T., Vanderhoff, G. A , and London, I.M. (1972) *J. Mol Biol.* 66,471-481.
33. Matte, R. L., and London, I. M. (1984) J. *Biol. Chem.* **259,** 6708–6711.
34. Andrew, N. C, Levin, D. H., and Baltimore, D. (1985) *J. Biol. Chem.* 260, 7628-7635.
35. Naresh Babu, S. V., and Ramaiah, K. V. A. (1996) *Arch. Biochem. Biophys.* 827, 201-208.
36. Ramaiah, K. V. A , Dhindsa, R. S., Chen, J.-J., London, I. M., and Levin, D. H. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 12063–12067.
37. Ramaiah, K. V. A., Davies, M. V., Chen, J.-J., and Kaufman, R. J. (1994) *Mol. Cell. Biol.* 14, 4546-4553.
38. Janaki, N., Krishna, V. M., and Ramaiah, K. V. A. (1995) *Arch. Biochem. Biophys.* **324,** 1-8.
39. Chen, J-J., and London, I. M. (1995) *TIBS* 20, 105-108.
40. Dholakia, J. N., Mueser, T. C, Woodley, C. L., Parkhurst, L. J., and Wahba, A. J. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 6746-6750.
41. Akkaraju, G. R, Hansen, L. J., and Jagus, R (1991) *J. Biol Chem.* 266, 24451-24459.
42. Oldfield, S., and Proud, C. G. (1992) *Eur. J. Biochem.* **208,** 73-81.
43. Dholakia, J. N., and Wahba, A. J. (1988) *Proc. Natl. Acad Sci. USA* **85,** 51-54.
44. Welsh, G. I., Miyamoto, S., Price, N.T., Safer, B., and Proud, C.G. (1996) J. *Biol. Chem* 271, 11410-11413.
45. Hunt, T. (1979) *Miami Winter Symp.* 16, 321-346.