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

The availability of site-specific mutants of human eIF2α like the 48A or 51A in which the serine residues in the respective positions of eIF2α have been replaced by alanine has advanced our understanding in identifying that a) serine 51 residue is the only phosphorylation site in mammalian eIF2α (Pathak et al., 1988), b)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 pphosphorylation 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. eIF2α wt represents wild type human eIF2α cDNA (1.6 kb). This subunit can be phosphorylated on its serine-51 residue. This is the only site of phosphorylation in human eIF2α. Phosphorylation of eIF2α inhibits the guanine nucleotide exchange activity of eIF2B and thereby inhibits protein synthesis. In eIF2α 48A mutant, the serine-48 residue has been replaced by alanine by site specific mutagenesis (Kaufman et al., 1989).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 eIF2α 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

\[ \rightarrow \]

Ligation of eIF2a cDNA insert into baculovirus transfer vector, pBacPAK8

Transformation of DH5a cells by recombinant vector pBacPAK8 and purification

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Preparation of a radioactive probe of the insert to identify colonies containing recombinant vector using Colony hybridization technique

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

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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

\[ \rightarrow \]

Plaque assay and amplification of recombinant virus

\[ \rightarrow \]

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 eIF2α 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 origin 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 (Bsu36 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 eIF2α cDNA to amplify the parent vector. Vector DNA was isolated using the Qiagen column and digested with EcoRI to separate the 1.6 Kb eIF2α insert. The insert was electroeluted from 1% agarose gel and purified by Qiaquick spin column. Figs. 17A and 17B show the linearized pBacPAK8 (EcoRI digested) vector (5.5 Kb) and both the eIF2α cDNAs. The eIF2α inserts were ligated to the transfer vector, pBacPAK8 (EcoRI 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 eIF2α wild type as well. DH5α cells were transformed with the recombinant pBacPAK8 vector and the positive colonies containing the vector with eIF2α 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 EcoRI; lane 3, 1.6 Kb fragment of eIF2α cDNA wild type.

Panel B: Lanes 1 and 2 are similar as in Panel A. Lane 3, 1.6 Kb fragment of eIF2α cDNA 48A mutant.
Fig. 18. In gel hybridization.

A $[^{32}P]dCTP$ 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 eIF2α 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 transfer vector with eIF2α 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.
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*I 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*I 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.
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 BamHI and SphiI. Lane 1 DNA ladder; lane 2, vector, pBacPAK8 cut with BamHI and SphiI; 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: pBacTA1 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 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 represents cells infected with non-recombinant AcNPV virus. Well 8 contains a positive control, i.e., eIF2α insert.
digestion using \textit{Mlu} I and \textit{Sea} I or \textit{Sph} I and \textit{BamH} I enzymes for pBacPAK8 vector carrying wt or 48A mutant eIF2a cDNA. Six positive colonies, identified by colony hybridization, carrying the eIF2\(\alpha\) wt were picked up randomly and their plasmid DNA was digested with \textit{Mlu} I and \textit{Sca} I (Figs. 20A and B). \textit{Mlu} I cuts the vector pBacPAK8 (5.5 Kb) at 546 bp. \textit{Sea} I cuts the vector at 3990 bp while it cuts the eIF2\(\alpha\) wt insert at 561 bp. If the eIF2\(\alpha\) 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 eIF2\(\alpha\) using \textit{Sph} I and \textit{BamH} I enzymes. The insert has a single \textit{Sph} I site at 493 bp while the vector has none. \textit{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 recombiant constructs with the insert in the right orientation are shown in Figs. 22A and 22B.

1.2 \textit{Recombinant baculoviruses:}

Sf9 cells were co-transfected with the recombiant pBacPAK8 viral transfer vector with \textit{Bsu36} I digested BacPAK6 viral DNA in order to obtain an infectious complete virus genome with the eIF2\(\alpha\) 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 recombiant viruses from a single clone and the positive plaques were identified by dot blot hybridization. Amplification of the recombiant viruses were carried out to increase the titre of the recombiant 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 eIF2\(\alpha\), 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-recombiant 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 expression of 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, pBacTAl, 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 up to 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 eIF2α by 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 eIF2α 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 up to 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 eIF2α monoclonal antibody.

3.0 FUNCTIONAL CHARACTERIZATION OF THE EXPRESSED PROTEINS

3.1 Phosphorylation of the expressed protein by eIF2α kinase, HRI:

Since the wild type eIF2a and 48A mutant eIF2α expressed in mammalian systems are shown to be substrates for eIF2α kinases, the ability of baculovirus-expressed eIF2α 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.
Fig. 25. Detection of recombinant eIF2α wild type and 48A mutant subunits by a human eIF2α 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 eIF2α 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 μM ATP at 30°C for 5 min). The extracts were incubated for another 5 min before they were pulsed with [$γ$-³²P]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 eIF2α 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 eIF2α and not by wt eIF2α in Chinese hamster ovary cells and in their extracts (Ramaiah et al., 1994). To check the functional characteristics of baculovirus-expressed human wt and 48A mutant eIF2α, 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 eIF2α, 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 eIF2α (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 eIF2α) (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 eIF2α. 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 μl) 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 (○-○).

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.
### TABLE 9
EFFECT OF DIFFERENT CONCENTRATIONS OF INSECT CELL EXTRACTS OVERPRODUCING eIF2α 48A MUTANT ON eIF2.GDP DISSOCIATION

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>pmol of eIF2.[H]GDP dissociated 5 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>+heme</td>
<td>11.71</td>
</tr>
<tr>
<td>+heme +poly (IC)</td>
<td>7.57</td>
</tr>
<tr>
<td>+heme +poly (IC) +mock, 100 μg</td>
<td>1.50</td>
</tr>
<tr>
<td>+heme +poly (IC) +eIF2α wt, 100 μg</td>
<td>2.31</td>
</tr>
<tr>
<td>+heme +poly (IC) +eIF2α 48A, 50 μg</td>
<td>7.64</td>
</tr>
<tr>
<td>+heme +poly (IC) +eIF2α 48A, 100 μg</td>
<td>9.53</td>
</tr>
</tbody>
</table>

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.[3H]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.[3H]GDP dissociated.
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 μg).

**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 KCl 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.
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 2) 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-32P]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 eIF2α 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 eIF2α 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.
eIF2B was measured 5 minutes 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(αP) 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 activation 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(αP).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 eIF2α 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 eIF2α kinase activity which can phosphorylate eIF2α 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 eIF2α and formation of eIF2(αP).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 fractions of the gradients of hemin and poly (IC)-treated lysates which are supplemented with insect cell extracts expressing wt eIF2α (C). The intensity of eIF2α 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 eIF2α 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 eIF2B like activity of insect cell extracts in the absence of added reticulocyte lysates, the dissociation of reticulocyte eIF2.[ 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 eIF2α (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 eIF2α (o-o) than wt eIF2α ( 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 *eIF2α* 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 *eIF2α* 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 *eIF2α* 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(αP) 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 α, β and 5 subunits of mammalian eIF2B) 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(αP).GDP.
Similarly, the importance of eIF2α phosphorylation in translational control is highlighted by the expression of wt human eIF2α 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 eIF2α 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 eIF2α 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 eIF2α 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 eIF2α 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 eIF2α 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 eIF2α.
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.\[^3\text{H}\]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 activity 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.