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.


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: $[^{14}\text{C}]$leucine (330 mCi/mm, 100 μCi/ml) and $[^{3}H]$GDP (2 μM, 9 Ci/mm).

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 BCIP.

Qiagen, USA: Qiagen kit for DNA purification.
Qualigens, India: KCl, NaCl, glucose, HCl, EDTA, magnesium chloride, acetone, toluene, glycerol, ammonium sulphate, TCA, isomyl alcohol, isopropanol, potassium dihydrogen phosphate, disodium hydrogen phosphate, sodium fluoride 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 MgCl₂, 5 mM KCl, 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 [¹⁴C]leucine, 6 μM leucine, 0.004 M creatine phosphate and 0.1 mg/ml creatine phosphokinase (Ernst et al., 1980). 20 μM 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$_2$O$_2$ (30 % H$_2$O$_2$:H$_2$O, 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

\[ [^{14}C] \text{Leucine incorporated} \times 10^3 \]

\[ \text{CPM} \times 10^3 \]

\[ \text{TIME (min)} \]

---

\[ [^{14}C] \text{Leucine incorporated} \times 10^3 \]

\[ \text{CPM} \times 10^3 \]

\[ \text{TIME (min)} \]

---

\[ [^{14}C] \text{Leucine incorporated} \times 10^3 \]

\[ \text{CPM} \times 10^3 \]

\[ \text{TIME (min)} \]

---

\[ [^{14}C] \text{Leucine incorporated} \times 10^3 \]

\[ \text{CPM} \times 10^3 \]

\[ \text{TIME (min)} \]
Fig. 4. Protein synthesis in heme insensitive and in dead lysates.

Protein synthesis was carried out as described in the legend to Fig. 3. in different batches of reticulocyte lysates. Protein synthesis was measured at 30 and 60 min.
eIF2 was purified from ribosomal salt wash as well as from postribosomal supernatant by ion-exchange chromatography as described in 'Materials and Methods'.

Fig. 5. Schema for rabbit reticulocyte eIF2 purification.
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/200 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 eIF2a (38 kDa) monoclonal antibody to determine the eIF2α 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 KCl 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 γ- 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[3H]GDP

3.1 Formation of eIF2[3H]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 KCl fraction; 3- Phosphocellulose 0.7 M KCl fraction; 4- CM-Sephadex 0.4 M KCl 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

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>pmols of eIF2.[$^3$H]GDP dissociated 5 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>-HRI -GDP</td>
<td>3.82</td>
</tr>
<tr>
<td>-HRI +GDP</td>
<td>5.26</td>
</tr>
<tr>
<td>+HRI -GDP</td>
<td>2.29</td>
</tr>
<tr>
<td>+HRI +GDP</td>
<td>2.42</td>
</tr>
</tbody>
</table>

* 15.55 pmols (0.5μg) 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°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.
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 \(\mu\)M) in a 20 \(\mu\)l reaction mixture containing 20 mM Tris-Hcl pH 7.8, 80 mM KCl, 100 \(\mu\)g/ml CPK and 1 mM DTT at 30\(^{\circ}\)C for 10 min followed by another 10 min on ice. The binary complex was stabilized by the addition of 1 mM Mg\(^{2+}\) and incubated on ice for 10 more min. The amount of 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 KCl and 1 mM Mg\(^{2+}\)). The reactions were then filtered through 0.45 \(\mu\)m 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 \(\mu\)M) at 30\(^{\circ}\)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 [\(^3\)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 (HR1 incubated with 100 μM 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 μl), prepared as described above. Reactions were stopped at specified time points by the addition of cold buffer...
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.\( ^{3} \text{H} \)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:

\textit{In vitro} phosphorylation assays were carried out by incubating eIF2 with HRI and \( [\gamma^{32}\text{P}]\text{ATP} (3000 \text{ Ci/mmol}) \) in a 20 µl cocktail containing 20 mM Tris-HCl pH 7.8, 2 mM \( \text{Mg}^{2+} \), 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, \( \beta\)-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 \( [\gamma^{32}\text{P}]\text{ATP} \) 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 ul 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 eIF2(aP).eIF2B15S 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 KCl 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 et 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 $4 \times 10^6$ cells/ml. The cell suspension was aliquoted (1 ml) and IVcezed slowly. The cells were initially placed at -$20^\circ$C for one h, then at -$70^\circ$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 1E 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\textsubscript{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°C. 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 H2O 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 Denhard'ts solution (10 g each of Ficoll, PVP and BSA were dissolved in 500 ml of H2O, to prepare 50X Denhar'dt'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, MgCl₂ and |3-mercaptopethanol), [α³²P]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 µl volume. To the recombinant plasmid DNA (500 ng, 100 ng/ml), 5 µl 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 µm minisart 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 solidifies, 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 Na₂HPO₄ and 0.02 g of KH₂PO₄ in 100 ml of H₂O, 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 µl 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:

\[ \text{pfu/ml} = \text{Average No. of plaques} \times \text{I/ml of inoculum per plate} \times \text{I/dilution factor} \]

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 \( \times 10^6 \) 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 \( \times 10^6 \) 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 cm² 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 10⁶ 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 KCl (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-PAGE):

Proteins were separated on a modified Laemmli method (1970). The 10 % separation gel mix, 30 ml, contained 7.5 ml of 1.5 M Tris-HCl pH 8.8, 10 ml of 30:0.8
acrylamide:BIS-acrylamide mixture, 0.3 ml of 10% SDS, 0.1 ml of 10% ammonium per sulphate, 7.5 μl TEMED and 12.1 ml water. The 4.5% stacking gel mix in a total volume of 6 ml contained 0.9 ml of 30:0.8 acrylamide:BIS-acrylamide mixture, 15 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 μl) and BCIP (33 μl) as substrates in 10 ml of AP buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM MgCl₂). 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.