MATERIALS AND METHODS:

Materials and chemicals

Methods

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Materials and Chemicals: Trypan blue, antibiotic-antimycotic solution, ATP, cycloheximide, EGTA, etoposide, cell culture grade DMSO, NBT, BCIP, PMSF, bromophenol blue, agarose, tween 20, triton-x100, coomassie R250, sodium bicarbonate, sucrose, mannitol, glutathione, reduced glutathione, Taq Pol enzyme, BSA, tissue culture flasks, 12 well plates and polystyrene tubes were obtained from Sigma. Ac-DEVD-AFC, the substrate analog of caspase(s), recombinant human caspase-3, and caspase-8 inhibitor Z-IETD-fmk were purchased from BD Pharmingen. Z-VAD-fmk, a cell permeable caspase-3 inhibitor, Ac-DEVD-CHO, bovine PARP, tunicamycin, thapsigargin, calcium ionophore, leupeptin, pepstatin and aprotenin were obtained from Calbiochem. Grace’s insect cell medium, fetal calf serum and lipofectamine were obtained from Gibco BRL. Acrylamide, bis-acrylamide were from Roche Biochemicals. 0.45 uM nitrocellulose membrane, Glutathione Sepharose 4B, BL21 cells were purchased from Amersham-Pharmacia. 0.22 and 0.45 uM filter discs were purchased from Millipore. Whatman 1 and 3 filter papers were obtained from Whatman, UK. Restriction enzymes were bought from New England Biolabs. A phospho specific anti-eIF2a antibody was obtained from Research Genetics, USA. and polyclonal anti-eIF2a were purchased from Santacruz Biotechnology Inc., USA. Anti-PARP P85 fragment antibody, anti-mouse IgG, anti-rabbit IgG were obtained from Promega, Inc, USA. Gel extraction kit and midi plasmid isolation kit were from Qiagen. Tris-HCl, glycine, POP and POPOP were obtained from Spectrochem, India. All other chemicals were purchased from Qualigens, India.

Kind gifts: Mutant p35 virus, vAc5p35, was obtained originally from Dr. Paul D. Friesen’s laboratory, Institute of Virology, University of Wisconsin, Madison, USA. PERK-GST was obtained from Prof. David Ron, Skirball Institute of Molecular Medicine, New York, USA. Anti-PERK was a gift from Randal Kaufamn, Dept. of Biological Chemistry, Howard Hughes Medical Institute, University of Michigan Medical Centre, Ann Arbor, Michigan.
Methods:

1.1 Cell culture:
*Spodoptera frugiperda (Sf9)* cells were grown in TNM-FH medium supplemented with 10% fetal calf serum and 1% antibiotics. Confluent cells with >95% viability were used in all experiments. Trypan blue exclusion test was carried out to assess the viability of the cells. Trypan blue (0.4%) is added to cell suspension in 50 μl at a final concentration of 0.04% and counted in a hemocytometer.

1.2 Preparation of TNM-FH medium:
TNM-FH medium (Hink, 1970) is Grace’s basal insect cell culture medium (Grace, 1962) supplemented with lactalbumin hydrolysate and yeastolate. The medium is enriched with 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 NaHCO₃ 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 added later to make complete medium.

1.3 Freezing and thawing of cells:
*Sf9* cell stocks were made from healthy *Sf9* log-phase cultures. The cells were harvested and the cell pellet was suspended in complete medium containing 10% cell culture grade DMSO. The final cell density was kept at 4×10⁶ cells/ml. The cell suspension was aliquoted (1 ml) and frozen slowly. The cells were initially placed at 4°C for one hour, at -20°C for 2-3 hrs and then at -70°C overnight. The next day cells were transferred to liquid nitrogen. The above mentioned stocks were removed from liquid nitrogen when required and thawed by incubating at 37°C. Once the stock was thawed, the vial was wiped with 70% ethanol before taking in to the hood. The cells were transferred to a T-25 culture flask that contains 3 ml of complete medium. After seeding of cells, the medium can be changed to fresh complete medium, if necessary. Seeding capacity of the flask depends on the surface area - 96 well plate is 10,000; 24 well plate is 0.5 million, 12 well
plate is one million; 6 well plate is two million; 35 mm petri dish is two million; T-25 culture flask is four million; T-75 culture flask is twelve million cells.

1.4 Reconstitution of various chemicals used:
Etoposide, tunicamycin, thapsigargin, calcium ionophore, genestein and staurosporine were reconstituted in DMSO to a final concentration of 50 mM, 12 mM, 15.3 mM, 100 mM, 10 mM and 10 mM respectively. The final concentrations of these chemicals used in various experiments are: etoposide: 20 and 125 μM; tunicamycin: 4 and 20 μM; thapsigargin: 35 μM; calcium ionophore, A23187: 20 and 100 μM; genestein: 25 and 50 mM; staurosporine: 100 nM.

The protease inhibitor pepstatin (1.4 mg/ml) was prepared in methanol; aprotenin (5 mg/ml) and leupeptin (5 mg/ml) were prepared in water; PMSF (50 mM) was prepared in isopropanol.

1.5 Induction of Apoptosis:
Sf9 cells were irradiated with UV-B light (312 nm) for 30-60 seconds and incubated at 27°C for 15 hrs. Alternatively cells were treated with different agents like etoposide, cycloheximide, EGTA, A23187 and tunicamycin and incubated at 27°C for 15 hrs. Apoptosis was also studied in the presence of cell permeable caspase-3 and 8 inhibitors z-VAD-fmk, and z-IETD-fmk. For these experiments the apoptotic stimulus and caspase inhibitor were given to cells simultaneously. Effect of kinase inhibitors on UV-B induced apoptosis was studied by treating the cells with either genestein or staurosporine for 30 min, cells were exposed to UV-B light and incubated at 27°C for 15 hrs.

1.6 Assays for Apoptosis:
4x10^6 cells were taken for each experiment and scored for apoptosis by monitoring plasma membrane blebbing. A small aliquot, 45 μl of cell suspension, was stained with 0.04% trypan blue and viewed under an inverted microscope (Labovert) equipped with a digital camera and the software MV500 DEMO to score (20x magnification) the apoptosed cells.
1.7 Preparation of cell lysate:

4 million cells were pelleted at low speed, washed with ice cold PBS twice at 4°C and lysed in 100 µl of lysis buffer (20 mM Tris-HCl pH 7.8, 1 mM Mg²⁺, 1 mM DTT, 3 µg/ml of pepstatin, 10 µg/ml of aprotinin, 10 µg/ml of leupeptin, 250 µM PMSF). Cells were incubated on ice for 10 min in lysis buffer. Lysed cells are centrifuged at 12,000 rpm at 4°C for 12 min and the supernatant is collected into a fresh eppendorf and stored at -70°C.

1.8 Ac-DEVD-AFC hydrolysis:

Caspase activity of the cells undergoing apoptosis was determined using Ac-DEVD-AFC hydrolysis in the cell extracts. Approximately, 400 µg extract protein was taken in 50 µl of lysis buffer and was diluted to 750 µl with 20 mM Tris-HCl buffer (pH 7.2) containing 1 mM Mg²⁺, 80 mM KCl and 1 mM DTT for each reaction. Ac-DEVD-AFC hydrolysis was monitored by fluorescence emission of the released AFC (excitation, 400 nm; emission, 500 nm) using Fluoromax-3, Jobin Yvon, Horiba Spectrofluorimeter (Bhuyan et al., 2000). The final concentration of Ac-DEVD-AFC used in the reactions was 10.9 µM.

1.9 PARP Cleavage:

In addition to monitoring the cell morphology and caspase activity of the cells undergoing apoptosis, the relative induction of apoptosis caused by various treatments was also studied by the cleavage of pure bovine Poly ADP Ribose Polymerase Protein (PARP). Pure bovine PARP protein (150 ng) was incubated with the SJF9 extracts (containing 60 µg of protein) at 30°C for 90 mins in a 40 µl reaction mixture consisting of 10 mM Tris-HCl, pH 7.5; 2.5 mM KH₂PO₄; 2 mM NaCl; 68 mM sucrose and 220 mM mannitol. The PARP reactions were processed by the addition of equal volume of 2x reducing buffer containing 62.5 mM Tris, pH 6.8, 6 M urea, 10% glycerol, 2% SDS, 5% β-mercaptoethanol and 0.005% bromophenol blue. The PARP reaction samples were boiled at 65°C for 15 minutes, and then separated on 10% SDS-PAGE, transferred to nitrocellulose membrane and probed with an antibody (anti-rabbit antibody) that recognizes the 89 kDa cleaved fragment of PARP.
1.10 Virus infection:
80-90% confluent flasks were selected for virus infection. The required MOI for infection was calculated before infection. The complete medium in which the cells were maintained was replaced by 500 µl of complete medium containing a virus for a 35 mm petri dish. The infection was given for one hour with intermittent rocking of the petri dishes or culture flasks once in 15 mins. After one hour, 1ml of complete medium for 35 mm petri dish or 3ml to T-25 culture flask was added and incubated in dark humid environment (preferably in a box with a moist tissue) for 48 hrs at 27°C in the incubator. In order to evaluate the importance of eIF2α phosphorylation in apoptosis, cells were treated with UV-B radiation before and after the expression of wt and mutants of human eIF2α. Expression of recombinant eIF2α wt and mutants was carried out by infecting the cells with recombinant virus expressing wt and mutants of eIF2α.

1.11 Virus amplification:
For virus amplification, cells were infected in T-75 culture flasks. Virus infected cells were left in the incubator at 27°C for 10-15 days for the virus to amplify. Lysis of cells occurred when ~80% of the cells were infected. Cell lysate was harvested and viral supernatant was collected by centrifuging the cells at 3000 rpm for 10 min at room temperature. The virus was filtered through 0.22 µ filters. The virus was stored in liquid nitrogen for long storage. It is stable at -70°C for 1-2 years and few weeks at 4°C.

1.12 Virus titre calculation by End Point Dilution assay:
The infection efficiency of the virus changes on long storage. It’s necessary that its titre be calculated before use after long storage. Virus titre was calculated by end point dilution assay (Reilly et al., 1992). The aim of this procedure is to dilute the virus and see if such diluted inoculum is able to infect cell cultures. The titre of the virus is obtained as pfu/ml (plaque forming units or number of virus particles per ml). The pfu/ml value is converted to MOI (number of virus particles per cell) during infection.

The experiment was performed in 96 well plates. Cells were diluted to a concentration of 1x10^5 ce/ml. Ten fold serial dilutions of the virus stock in tissue culture medium were
made. Dilutions till $10^{-5}$ to $10^{-8}$ are preferred for each virus stock. Cells were seeded at a concentration of $1 \times 10^4$ cells per 100 ml in each well of 96 well plates. Each row was used for one dilution of the virus. In each row first well was kept as control. 10 μl of the diluted virus was added to each well except for the controls. The plate was sealed with parafilm and incubated in humid environment at 27°C in the incubator for four days. After four days of infection, virus infected cells were identified under microscope by the appearance of polyhedra particles. For each dilution number of infected wells and uninfected wells were noted and tabulated. This method is based on the assumption that (Reed and Muench, 1938) cultures infected at a particular dilution would have been infected at all lower dilutions: cultures uninfected at a particular dilution would have been uninfected at all higher dilutions.

For eg:

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Infected wells</th>
<th>Uninfected wells</th>
<th>Dilution</th>
<th>Infected</th>
<th>Uninfected</th>
<th>% Infected</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-5}$</td>
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<td>$10^{-5}$</td>
<td>21</td>
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<td>$10^{-6}$</td>
<td>9</td>
<td>4</td>
<td>69.2</td>
</tr>
<tr>
<td>$10^{-7}$</td>
<td>1</td>
<td>11</td>
<td>$10^{-7}$</td>
<td>1</td>
<td>15</td>
<td>6.3</td>
</tr>
<tr>
<td>$10^{-8}$</td>
<td>0</td>
<td>12</td>
<td>$10^{-8}$</td>
<td>0</td>
<td>27</td>
<td>0</td>
</tr>
</tbody>
</table>

pfu/ml is calculated by multiplying the dose or dilution that gives 50% response (TCID$_{50}$) and concentration of virus particles at that dilution. TCID$_{50}$ is calculated by the formula: TCID$_{50}$ (dose that gives 50% response) = log of dilution giving a response greater than 50% - PD (proportionate distance) of that response.

$10^{-7}$ respectively. The dilution that gives more that 50% of infection, $10^{-6}$ was taken as A in calculating the PD value and dilution that gives less than 50% of infection, $10^{-8}$ is taken as B. Proportionate distance (PD) of a 50% response is calculated using the formula $PD=(A-50)/(A-B)$. $PD = (69.2-50)/(69.2-6.3) = 0.305$. TCID$_{50}$ (dose that gives 50% response) = log of dilution giving a response greater than 50%- the PD of that response.

Log TCID$_{50}$ = -6-0.305

4!
The titre of the virus is the reciprocal of this = 2.02 \times 10^6/10\mu l or 2.02 \times 10^6/ml. This can be converted to pfu/ml (plaque forming units per ml) using the relationship
\[ \text{pfu} = \text{TCID}_{50} \times 0.69 \]
Thus titre of the virus is \(1.4 \times 10^8\) pfu/ml. Infection to cells is given in terms of MOI (multiplicity of infection) i.e., no. of infectious virus particles or plaque forming units per cell. So if the MOI is set at 10 for \(2 \times 10^6\) cells (35 mm petri dish), no. of virus particles that has to be infected is \(2 \times 10^7\). This no. of virus particles is present in 143 \(\mu\)l of virus which has titre value of \(1.4 \times 10^8\) pfu/ml.

1.13 Purification of PKR-GST and PERK-GST proteins:
A recombinant PKR-GST construct (kind gift of Prof. Bryan Williams, Cleveland Clinic Foundation, USA) and was overexpressed in BL21 cells. PERK-GST transformed cells were obtained from Prof. David Ron, Skirball institute for molecular medicine, New York. The transformed cells (100 \(\mu\)l) were inoculated in a 10 ml of LB with ampicillin (100 \(\mu\)g/ml) and grown overnight. A secondary culture of 100 ml was set up with 2 ml (2\%) of primary culture for 37\(^\circ\) C for 10-12 hrs in LB with ampicillin. From this 50 ml was inoculated in 500 ml of LB with ampicillin and incubated in a orbital shaker at 200 rpm at 37\(^\circ\) C for 2-3 hrs. O.D. of the cell growth was allowed to reach OD\(_{600}\) 0.6-0.7. Cells were then induced with IPTG at 0.1 mM. Induced cells were grown at 100 rpm at 27\(^\circ\) C for 10-12 hrs. Cells were pelleted at 3000 rpm for 10 min at 4\(^\circ\) C. Cell pellet was lysed in 10 ml of PBS lysis buffer containing 1 mM DTT, 0.1\% triton x100, 10 \(\mu\)g/ml of leupeptin, 10 \(\mu\)g/ml of aprotenin, 3 \(\mu\)g/ml of pepstatin, 250 \(\mu\)M of PMSF. The lysate was then subjected to sonication at 4\(^\circ\) C; four strokes of 10 amp of frequency were given to 3 ml of lysate each time for 15 sec with an interval of 1.5 min. The sonicated lysate was loaded on to the Glutathione-sepharose-4B affinity matrix. The column was washed with 50 mM Tris (pH 8) and low throw was collected. The GST tagged protein was eluted with 50 mM Tris (pH 8) containing 10 mM GSH. Fractions of high O.D. were checked. The protein was eluted in the first 3-4 fractions. The eluted protein was dialyzed against 50 mM Tris buffer (pH 8) to remove reduced glutathione. The samples were stored at
Figure 2: Purification of recombinant human PKR-GST

Panel A represents coomasie gel of the purified PKR-GST protein. PKR-GST fusion construct was transformed in BL21 E.coli cells. The cells were treated with IPTG in their log phase to induce the expression. Cells were harvested, sonicated and lysed. The crude lysate was passed through glutathione sepharose 4B column. The bound protein was eluted with 10 mM GSH in 20 mM Tris-HCl, pH 8.

Panel B: Phosphorylation of human recombinant eIF2α by PKR-GST. Recombinant human wt eIF2a was expressed in Sf9 cells and partially purified (Sudhakar et al., 2000). This purified protein (~50 ng) was incubated with Sf9 cell extracts (~30 μg) and phosphorylated with different concentrations (~40, 80, 200, 400 ng) of PKR-GST in the presence of a reaction mixture consisting of (80 mM Tris-HCl, 2 mM Mg²⁺, 1 mM DTT, 30 uM ATP) at 30° C for 12 min. The reaction was terminated with 4x SDS loading buffer, proteins were separated by 10% SDS-PAGE, transferred to a nitro cellulose membrane and analysed with a phosphospecific anti eIF2a antibody.

Panel C: Phosphorylation of the recombinant human wt eIF2a protein and S/9 (~30 μg) cell extract was carried out by of PKR-GST (~40 ng) at different time periods at 30° C as mentioned in the previous legend.
Figure 3: Purification of PERK-GST protein

PERK-GST protein was purified from transformed E. coli, BL21 strain as described in the legend to figure 3.

Panel A: Coomasie gel for PERK-GST purification. Lanes in the figure are: lanes 1 and 2, crude BL21 cell extract; lane 3, IPTG induced cell extract; lanes 4 and 5, PERK-GST protein.

Panel B: The figure is the western blot showing the immunoreactivity of the purified protein with mouse anti-PERK.

Panel C: Phosphorylation of Sf9 eIF2α phosphorylation by purified PERK-GST. ~30 μg of Sf9 extract prepared from control, uninfected cells was incubated with increasing concentrations of PERK (~20, 50, 100 ng) of enzyme in a phosphorylation buffer (20 mM Tris-HCl pH 7.8, 1 mM DTT, 2 mM Mg²⁺, 80 mM KCl and 30 μM ATP) at 30° C for 10 min. The samples were terminated with 4xSDS sample buffer; proteins were separated by SDS-PAGE, transferred to a membrane probed with a phosphospecific anti-eIF2a antibody. The figure is a western blot.
70° C. 10 μl of the high O.D. fraction was loaded on to the 10% SDS-PAGE and analysed for the pure protein, PKR-GST or PERK-GST (Figures 2 and 3).

1. 14 Sf9 eIF2α phosphorylation in cell extracts:
Equal protein (~25 μg) from cell extracts prepared from cells treated with various agents was separated on a 10% SDS-PAGE. The separated proteins were transferred to a nylon membrane and probed with a phosphospecific anti-eIF2α antibody and the membrane was developed with AP-conjugate substrate developer, NBT-BCIP. The blots were scanned at a resolution of 200 dpi by using a Hewlett Packard Scanjet 3400C. Band intensities were quantified using the Quantity 1™ Image Analysis software using Biorad Model GS-800 Calibrated Imaging Densitometer.

1.15 In vitro phosphorylation:
In vitro phosphorylation assays were carried out by incubating the Sf9 cell extract with purified PERK-GST/PKR-GST in a 15 μl cocktail containing 20 mM Tris-HCl pH 7.8, 2 mM Mg2+, 80 mM KCl and 50 μM ATP at 30° C for a period of 12 min. The protein kinase assay was terminated by the addition of 4x SDS sample buffer (0.25 M 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 analysed on 10% polyacrylamide gel.

1.16 Cleavage of PKR-GST and PERK-GST in vitro:
Purified recombinant PKR-GST (~800 ng) and PERK-GST (~3 μg) was incubated in vitro at 37° C (~ 40 μg) with Sf9 cell extracts prepared from cells undergoing apoptosis and non-apoptotic cells containing equal amount of protein for different time periods as described in figure to legends and terminated in 4x SDS-loading dye. The cleavage inhibition was demonstrated with caspase-3 inhibitor, Ac-DEVD-CHO (50 μM) which was incubated simultaneously with extracts and PERK protein. The extracts were then separated on 10% SDS-PAGE for PKR samples and 7.5% SDS-PAGE for PERK samples. The separated proteins were transferred to a nylon membrane and probed with a
polyclonal anti-PKR or monoclonal anti-PERK antibody to determine the cleavage of recombinant PKR or PERK in the extracts prepared from apoptotic cells.

1.17 Cleavage of PERK-GST by pure caspases:
~1.5 μg of PERK-GST protein in 2 μl is incubated with pure recombinant caspase-3 and caspase-6 proteins at 300 and 600 ng in the cleavage buffer (10 mM Tris-HCl, pH 7.5, 2.5 mM KH$_2$PO$_4$; 2 mM NaCl, 68 mM sucrose and 220 mM mannitol) for about 3 hrs at 30° C. The reaction was terminated with 4x SDS loading dye and proteins were separated on a 7.5% SDS-PAGE transferred to a nitro cellulose membrane and probed with monoclonal anti-PERK antibody.

1.18 Sf9 eIF2a phosphorylation by caspase cleaved PERK:
This reaction was performed in two steps. In the first step PERK-GST was allowed to be cleaved with caspase-3 protein in the cleavage buffer (10 mM Tris-HCl, pH 7.5, 2.5 mM KH$_2$PO$_4$; 2 mM NaCl and 220 mM mannitol) for about 3 hrs at 30° C. Then the cleaved PERK was allowed to phosphorylate the Sf9 eIF2a in the extract in the phosphorylation buffer (20 mM Tris-HCl pH 7.8, 2 mM Mg$^{2+}$, 30 μM ATP, 1 mM DTT and 80 mM KCl) at 30° C for 12 min. The reaction was terminated with 4x SDS loading dye.

1.19 In vivo protein synthesis:
2x10$^6$ cells were seeded in 35 mm petridish and treated with various agents as mentioned above for 15 hrs. The cells were pelleted at low speed, washed with complete media-methionine and incubated with complete medium-methionine for one hour at 27° C to deplete the endogenous methionine. Cells were then pelleted, washed, transferred to a fresh medium containing 20 uxi of labeled [$^{35}$S] methionine in 20 μl, and incubated again for one hour at 27° C. Cells were then washed and lysed in 60 μl lysis buffer. 15 ui (~ 20 μg protein) of the reaction mixture was spotted on a whatman filter paper, dried, and counted in a Wallac 1409 liquid scintillation counter to obtain the amount of labeled methionine taken up by the cells. A duplicate filter was processed through trichloroacetic acid (TCA) through a series of steps, first incubated on ice for 10 min in 10% TCA, boiled in 5% TCA for 3 min, and incubated at room temperature in 5% TCA for 5 min.
The filters were washed first in alcohol and then in acetone. The filters were dried, taken in scintillation fluid (50 mg POPOP, 4 g POP per litre of toluene) and measured in a liquid scintillation counter to determine the amount of radioactivity incorporated into the TCA precipitable portion of the protein. Percent incorporation of labeled methionine into the protein was calculated based on the above uptake and incorporation values. Protein synthesis with caspase inhibitors was performed in 24 well plates to minimize the usage of the inhibitor.

1.20 Autoradiography:
The labeled proteins were separated by 10% SDS-PAGE and the gel was dried using a Bio-rad gel drier. The dried gel was exposed to Kodak X-ray film and kept -70°C. The film, after exposure for the required time was developed with a set of photographic solutions as per the manufacturer's instructions. Alternatively the dried gel was exposed to the cassette of the phosphor imager and the developed image was scanned by phosphor imager.

1.21 Protein Estimation:
Cell lysate total proteins were estimated by a Bio-rad protein estimation kit as per the instructions given by the manufacturer.

1.22 Cloning:
Wild type and mutants of human eIF2a cDNAs were cloned in to a PNN1 vector under an Hsp promoter for transient expression of the eIF2a protein in Sf9 cells.

Steps in cloning:
Following steps were used: a) PCR amplification of eIF2α cDNA; b) gel elution of PCR amplified product; c) restriction digestion of gel eluted PCR product; d) ligation of PCR amplified insert and vector; e) transformation of the ligation mixture; inoculation of randomly selected transformed colonies; f) DNA isolation from the inoculum; confirmation of positive colonies; g) midi preparation of positive clone and transfection of Sf9 cells with pure plasmid DNA (Flow chart # 1).
Flow chart for **cloning**:

1. **PNN1 vector-p35**
   - EcoRI and BamH1 digested
   - BamH1 1490

2. **Ligated with T4 DNA ligase at 16°C for 18 hrs**
   - T7 promoter 1
   - Apa 14
   - AatII 20
   - SphI 26
   - XbaI 31
   - EcoRI 511
   - KpnI 521

3. **PCR amplification**
   - Human elf2α cDNA (1.6 kb)
   - EcoRI → BamH1 (948 bp)
**PCR amplification:**

Human eIF2α cDNA was amplified using primers (32 mer).

Forward primer: cgGAATTCatgccgggtctaagttgtagattt with site for Eco RI enzyme at its 5’ end (melting temp of primer with restriction enzyme: 66.73°C; primer alone: 54.3°C).

Reverse Primer: cgGGATCCttaatcttcagctttggcttccat with site for Bam HI enzyme at its 5’ end (melting temp with restriction enzyme: 69.02°C; primer alone: 55.4°C) (Figure 4A). Primers were reconstituted in autoclaved water at a concentration of 4 μg/μl at stored at -20°C. The method involved was step up PCR to promote proper binding of primer on the template. After denaturation of the plasmid DNA at 94°C for 120 sec, primer was annealed to its template at lower temperature, i.e., at 52°C for 30 sec. Extension of 5’ and 3’ overhangs was done at 72°C for 90 sec. This cycle was run for five times. Once again the plasmid was denatured at 92°C for 30 sec, primers were annealed at 60°C for 30 sec and overhangs were extended at 72°C for 90 sec. These cycles were continued for 25 times for amplification of the insert. Extension temperature for the last cycle was continued for 7 min. PCR reaction was set up with 50 ng of plasmid DNA in 1x PCR buffer, 1.5 mM of MgCl₂, 200 mM of dNTPs, 50 ng of forward and reverse primers, 1 enzymatic unit of Taq polymerase in a reaction volume of 50 μl and reaction is represented as:

**Step up PCR for eIF2α cDNA:**

<table>
<thead>
<tr>
<th>5 cycles</th>
<th>25 cycles</th>
</tr>
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<tbody>
<tr>
<td>94°C</td>
<td>94°C</td>
</tr>
<tr>
<td>2'</td>
<td>60'</td>
</tr>
<tr>
<td>0.5°C</td>
<td>0.5°C</td>
</tr>
<tr>
<td>52°C</td>
<td>60°C</td>
</tr>
<tr>
<td>1.5°C</td>
<td>1.5°C</td>
</tr>
<tr>
<td>72°C</td>
<td>72°C</td>
</tr>
</tbody>
</table>

**Gel elution of PCR products:**

PCR amplified products were checked on 0.8% agarose gel in TAE buffer by comparing with DNA marker (Figure 4B). Gel containing the DNA was carefully cut with a clean blade and DNA was eluted with gel extraction kit. Gel was solubilised with 3 volumes of gel extraction buffer with silica slurry added (1 μg of DNA=15 μl of silica slurry) and incubated at 52°C for 15 min. Agarose was pelleted at high speed. The agarose pellet was washed with wash buffer. The supernatant containing the DNA+silica complex was
**Figure 4: Cloning of eIF2α cDNA into a PNN1 plasmid**

PCR amplification of human wt and mutants of eIF2α cDNA was carried out using gene specific primers as described in ‘Materials and Methods’.

Panel A represents the products of PCR amplification on a 0.8% agarose gel. Lanes are as follows: Lane 1, eIF2α wt; lane 2, eIF2α S51A; lanes 3, 4 and 5 eIF2α S51D.

Panel B represents vector DNA and gel eluted PCR products of eIF2α cDNA on an agarose gel. PNN1 vector DNA was isolated and digested with EcoRI and Bam HI. Vector DNA and gel eluted PCR products of eIF2α cDNA were separated on a 0.8% agarose gel. The various lanes represent different amounts of the following. Lanes 2-4 represent different amounts of vector DNA; lane 1, gel eluted eIF2α wt cDNA; lane 5 gel eluted eIF2α S51A cDNA; lane 6 gel eluted eIF2α S51D cDNA lane 7, X marker DNA; lane 8, 150 ng of 1 kb DNA ladder.

Panel C represents the restriction analysis of plasmid DNA isolated from transformed colonies to determine the positive colonies containing the eIF2α cDNA insert. The vector DNA was ligated with eIF2α cDNA and the ligation mix was transformed as mentioned in ‘Materials and Methods’. Colonies were grown in agar medium containing ampicillin (100 µg/µl) and tetracycline (15 µg/µl). Plasmid DNA was isolated from randomly selected colonies and was double digested with Eco RI and Bam H1. Digested DNA products were separated on 0.8% agarose gel to identify the 945 bp insert containing positive colonies. Lanes 8, 17 and 27 represent marker lane where as all other lanes contain plasmid DNA with or without eIF2α cDNA insert as indicated in the figure.
collected. The supernatant was transferred to a fresh centrifuge tube, spun at high speed for 10 min to obtain the silica+DNA pellet. This pellet was washed thrice and the pellet was air dried. DNA was eluted in to 20 μl of autoclaved water from silica by incubating at 37°C for 30 min. DNA was collected in to a fresh tube and stored.

**Generation of PNN1 vector and insert with compatible restriction sites:**

PNN1 plasmid was double digested with Eco RI and Bam H1 in bulk and checked on 0.8% agarose gel and the vector released was gel eluted. The gel purified PCR products (insert) were also double digested with Eco RI and Bam H1 and were ethanol precipitated. They were checked on an agarose gel and quantified comparing with the 1 kb ladder (3 kb marker of X ladder is 80-90 ng per 150 ng of the total marker loaded).

**Ethanol precipitation of DNA:**

DNA after restriction digestion was incubated with two volumes of absolute ethanol and one tenth volume of 3 M sodium acetate pH 5.2 and incubated at -20°C overnight. DNA was pelleted by spinning at 13 k for 20 min. Pellet was washed with 70% ethanol and dried at 37°C for 20 min. The dried DNA was suspended in 10 μl of autoclaved water, allowed to dissolve by incubating at 37°C for 20 min and stored at -20°C.

**Ligation:**

Vector and insert digested with Eco RI and Bam H1 were ligated with T4 DNA ligase in a 1X ligation buffer in a final reaction volume of 10 ul. Insert and vector were ligated at a molar ratio of 3:1. As vector is 3.5 kb and insert 948 bp, equal amount of insert and vector (100 ng) were taken for ligation. Ligation was performed at 16°C for 18 hrs. The entire ligation mixture was transformed in XL1 blue cells (E.coli strain with tetracycline and ampicillin resistant genes).

**Transformation:**

Competent XL1 blue cells were incubated with ligation mix on ice for 30 min (Sambrook et al., 1989). The cells were given heat shock at 42°C for 30 sec, immediately transferred to ice, 500 μl of LB with ampicillin and tetracycline was added, and incubated at 37°C
for 30 min. The transformed cells were plated on LB agar plates containing ampicillin (100 µg/ml) and tetracycline (15 µg/ml). The plates were incubated for 12 to 14 hr to allow the growth of transformed bacterial colonies. Colonies are selected randomly and inoculated in 3 ml of LB medium with ampicillin and tetracycline. Cells were allowed to grow for 10-12 hrs at 37° C and plasmid was isolated by alkaline lysis method (Sambrook et al., 1989).

**Identification of positive clone:**
The plasmid DNA was digested with Eco RI and Bam HI enzymes. These are the enzymes used for PCR amplification of the insert. A 948 bp insert was expected to release upon digestion of the plasmid DNA from positive clone (Figure 4C).

**Midi preparation of the positive clone:**
After confirmation of the positive clone; cells were transformed with 100 ml of the positive clone and were inoculated in 100 ml of LB containing 100 µg/ml ampicillin and 15 µg/ml of tetracycline. The culture was grown for 15 hrs until significant growth of culture was obtained. Plasmid was isolated using Qiagen plasmid midi preparation kit-
Cells were pelted at 4000 rpm for 10 min. Cell pellet was lysed with 5 ml of lysis buffer (PI). The cell lysate was incubated with 5 ml of P2 for 10 min at room temperature. 5 ml of P3 was added, centrifuged at 15,000 rpm at 4° C for 20 min. Meanwhile the DNA columns were equilibrated with 10ml of equilibration buffer. On to this equilibrated columns the supernatant was loaded, washed with 20 ml of wash buffer. DNA was eluted with 15 ml of elution buffer in to centrifuge tubes. DNA was precipitated with 0.8 volumes of isopropanol. DNA was pelleted by centrifuging at 15,000 rpm for 10 min and washed with 70% ethanol. The DNA pellet was suspended in 500 µl of autoclaved water and stored at -20° C.

**Transfection:**
Cells were washed with incomplete medium with out serum and antibiotics twice or thrice. Around one million cells were seeded in 35 mm petri dish. Meanwhile DNA-lipofectamine complexes were made. DNA at the required concentration (5-20 µg) was
incubated in 50 µl of incomplete media for about 15 min in an eppendorf. Lipofectamine in 50 µl of incomplete media was incubated in a polystyrene tube. Volume of lipofectamine is equal to the concentration of the DNA that is transfected. DNA was added to lipofectamine vial and mixed thoroughly and incubated for about 30 min in hood. Thus 100 µl of DNA-lipofectamine mix was added on to the cells drop wise covering the whole surface of the petri dish containing 900 µl of incomplete media. Cells were incubated in the incubator at 27° C for 4-6 hrs. Incomplete medium was then changed to complete medium. After 10-12 hrs, cells were given heat shock for half an hour at 42° C. Cells were incubated in the incubator at 27° C for 48 hrs and analysed for the expression of the protein.

1.24 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE):
Proteins were separated by a modified Laemmli method. The 10% separation gel mix, 8ml, contained the following: 1.875 ml of 2 M Tris-HCl pH 8.8, 2.5 ml of 30:0.8 acrylamide:Bis-acrylamide, 75 µl of 10% SDS, 50 µl of 10% ammonium per sulphate, 8 µl of TEMED and 3.75 ml of water (7.5% gel mix contained 2 ml of acrylamide:bisacrylamide solution, 4.25 ml of water and the rest being the same). The 5% stacking gel mix in a total volume of 2.5 ml contained: 1.875 ml of water, 0.375 ml of Tris-HCl pH 6.8, 0.325 ml ol acrylamide:bisacrylamide solution, 25 ul of 10% SDS, 50 µl of 10% ammonium per sulphate, 8 ul of TEMED. Proteins were prepared in a sample buffer containing 0.25 M Tris-HCl pH 6.8, 10% SDS, 40% glycerol, SDS, β mercaptoethanol and bromophenol blue. Vertical slab gel electrophoresis was carried out at 120 volts with Tris-SDS-glycine buffer (0.3% Tris, 1.5% glycine, 0.1% SDS) until the dye front ran into the lower buffer. The gel was stained either by coomassie or by silver nitrate.

1.25 Western blotting:
The proteins separated on SDS-PAGE were transferred on to a nitro cellulose membrane electrophoretically at 70 volts. The transfer was done for 3 hrs in a transfer buffer (25 mM Tris, 19 5mM glycine, in 20% methanol). After the transfer, membrane was stained with ponceau s red solution and molecular weight marker proteins are marked. The stain
was removed by rinsing the membrane with TBS. The membrane was thereafter soaked in blocking solution (3% blot grade BSA or 5% mild powder in TBS-10 mM Tris-HCl pH 8, 150 mM NaCl). After 1hr, the blocking solution was replaced the primary antibody diluted in TBS and left at cold room for 10-12hrs. The membrane was later washed thrice with TBST (0.05% tween 20). Membrane was incubated with secondary anti-IgG-AP conjugate for 45min. The membrane was once again washed thrice with TBST. The membrane was developed with 10 ml of AP buffer buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM MgCl₂) containing 330 ul of NBT (one tablet of 25 mg from Sigma is dissolved in one ml of water) and 33 ul of BCIP (one tablet is dissolved in one ml of dimethyl formamide). The color 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. Blots were scanned at a resolution of 150 dpi by using a Hewlett Packard Scanjet 3400C. Band intensities were quantified using the Quantity 1™ Image Analysis software using Biorad Model GS-800 Calibrated Imaging Densitometer.