Cloning and Characterization of a novel eukaryotic Initiation factor-2 alpha (eIF2α) kinase, BeK, from the silkworm, *Bombyx mori*

THESIS SUBMITTED FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

by

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To my

Teachers

First being my mother Girija
Second being my father Muhadevappa
Then at school, college and friends

CERTIFICATE

This is to certify that Mr. M Dharma Prasad has carried out the research work in the present thesis under my supervision and guidance for a full period prescribed under the Ph. D. ordinance of this university. I recommend his thesis entitled **"Cloning and Characterization of a novel eukaryotic Initiation factor-2 alpha (eIF2α) kinase, BeK, from the silkworm, *Bombyx mori*"** for submission for the degree of **Doctor of Philosophy** of this university.

20.8.03

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STATEMENT

I hereby state that the work presented in this thesis entitled "**Cloning and Characterization of a novel eukaryotic Initiation factor-2 alpha (eIF2α) kinase, BeK, from the silkworm, *Bombyx mori***" has been carried out by me under the supervision of Dr. J. Nagaraju at Centre for DNA Fingerprinting and Diagnostics, Hyderabad and that this work is original and has not been submitted in part or full for any degree or diploma of any other university earlier.

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Dharma Prasad

ABBREVIATIONS

°C	: degrees centigrade
μg	: micro grams
μM	: micro molar
a.a.	: amino acid
Ac	: *Autographa californica*
AcMNPV	: *Autographa californica* multiple nuclear polyhedrosis virus
ADV	: Adenovirus
Arg	: arginine
ATF	: activating transcription factor
BeK	: *Bombyx mori* eIF2α Kinase
BeKm	: *Bombyx* eIF2α kinase mutant type
BeKw	: *Bombyx* eIF2α kinase wild type
Bm	: *Bombyx mori*
BmNPV	: *Bombyx mori* nuclear polyhedrosis virus
bp	: base pair
BSA	: bovine serum albumin
Cys	: cystine
Da	: dalton
DES	: *Drosophila* expression system
dPEK	: *Drosophila* PEK
ds	: double strand
dsRBD	: double strand RNA binding domain
dsRNA	: double stranded ribose nucleic acid
eIF2	: eukaryotic Initiation Factor 2
eIF2α	: eukaryotic Initiation Factor 2, alpha subunit
ER	: endoplasmic reticulum
FBS	: fetal bovine serum
HBD	: heme-binding domain
HBM	: heme binding motif
HCV	: hepatitis C virus
HisRS	: histidyl t-RNA synthetase domain
HIV	: human immunodeficiency virus
hr.	: hour
hrs.	: hours
HRI	: heme regulated inhibitor
HRM	: heme regulatory motif
HSV	: herpes simplex virus
IFN	: interferon
ins	: insert domain
IRES	: internal ribosomal entry site
LPS	: lipopolysaccharide

ltr.	: liter
Lys	: lysine
MBP	: maltose binding protein
min.	: minute
ml	: milliliters
mM	: milli molar
NEB	: new england biolabs
nm	: nano meter
NPV	: nuclear polyhedrosis virus
nt	: nucleotide
ORF	: open reading frame
PCR	: polymerase chain reaction
PEK	: Pancreatic eIF2α Kinase
PERK	: PKR like Endoplasmic Reticulum Kinase
PK2	: protein kinase 2 from AcMNPV or BmNPV
PKR	: protein kinase regulated by double stranded RNA
RBD	: double stranded RNA binding domain
reg	: regulatory domain
RNA	: ribose nucleic acid
RNAi	: RNA interference
SP	: signal peptide
SV 40	: simian virus 40
TBST	: tris buffered saline with tween-20
Thr	: threonine
TM	: transmembrane domain
TNF	: tumor necrosis factor
uORF	: upper/upstream open reading frame
UPR	: unfolded protein response
UTR	: untranslated region
ver.	: version
VV	: vaccinia virus

CONTENTS

1 Introduction

Ah, here is our smart new engineer from the chemical plant to see us! Don't look so worried, young fellow: what's the problem?

1.1 Introduction to the subject

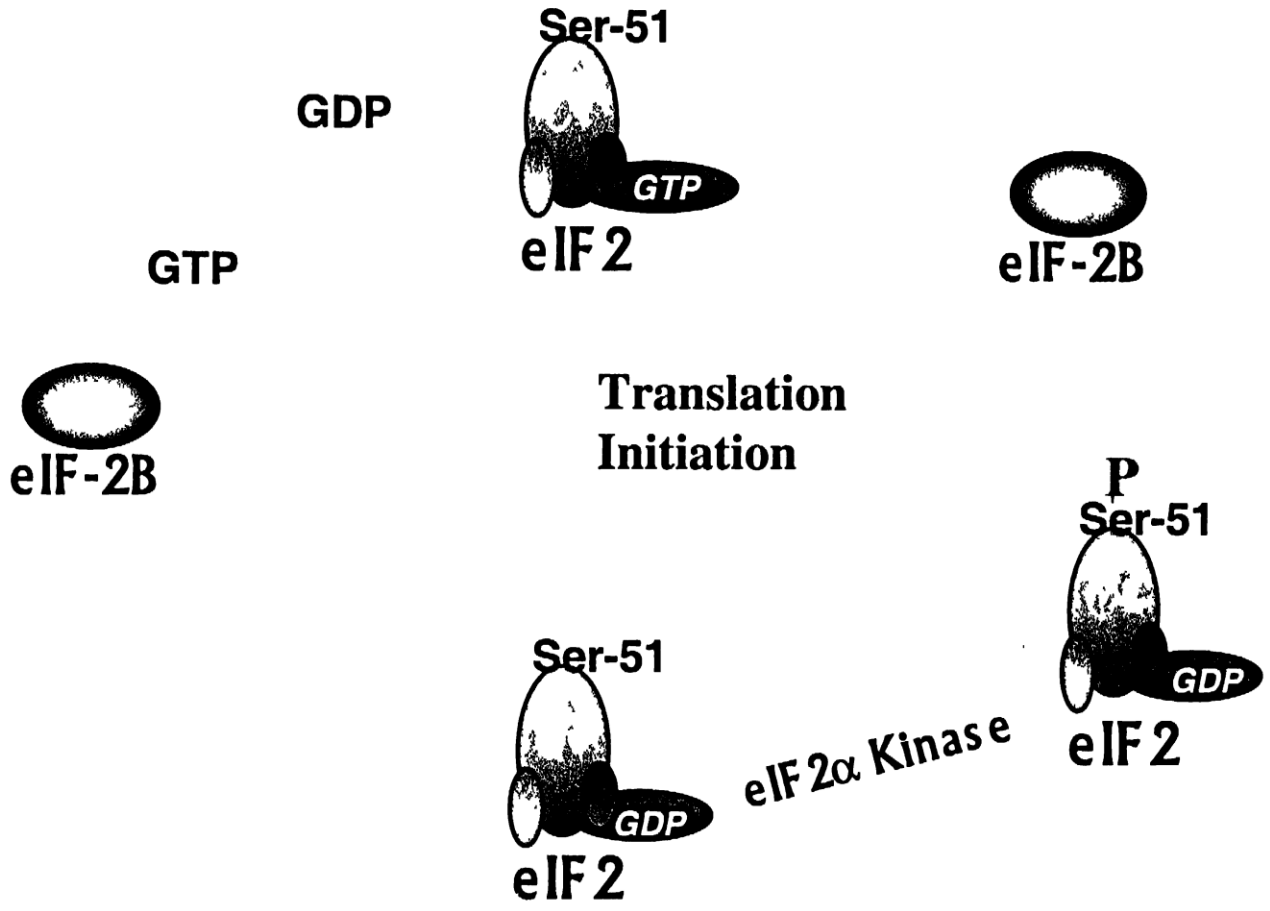
The primary aim of cell under stress is to defend itself and survive the stress. To orient itself towards defense, one of the first and rapid responses is to shut down global protein synthesis machinery or selectively translate a few useful transcription factors directed towards defense. The process of translation where polypeptides are synthesized using mRNA is divided into three phases: initiation, elongation and termination. Initiation is the phase where the assemblage of 80 S ribosome and mRNA complex occurs with the help of soluble proteins called initiation factors. There are at least six (mono and multimeric) initiation factors known so far, of which eukaryotic Initiation Factor 2 (eIF2) is one, which is composed of three subunits, α, β and γ (Merrick and Hershey, 1996). One of the best characterized translation regulation events is phosphorylation of eIF2α subunit, which is influenced by a variety of environmental stresses such as nutrient deprivation, heat shock, heme deficiency, unfolded protein response (UPR) and viral infection (Duncan and Hershey, 1985; Scorsone *et al.*, 1987; Duncan and Hershey, 1984; Chen 1993; Shi *et al.*, 1998; Harding *et al.*, 1999; Mathews *et al.*, 1996). Under these conditions of stress, eIF2α kinase phosphorylates the eIF2α subunit at Ser-51. After initiation of polypeptide synthesis, GTP bound to eIF2α subunit gets hydrolyzed to GDP. The GDP-bound eIF2α subunit is recycled to the active GTP bound form by an exchange factor, called eukaryotic initiation factor 2B (eIF2B). The Ser-50 phosphorylated eIF2α subunit acts as an inhibitor of eIF2B by

binding to eIF2B and sequestering it. Because eIF2B is quantitatively limiting, phosphorylated eIF2α is sufficient to sequester all the available eIF2B and stop the initiation of translation (Proud, 1992) (Panel 1).

1.2 eIF2α kinase family

Among eIF2α kinase family, four different classes of kinases have been identified, they are: yeast GCN2 like protein kinase (GCN2), double stranded **R**NA dependent **P**rotein **K**inase (PKR), **H**eme **R**egulated **I**nhibitor (HRI), and **P**KR like **E**ndoplasmic **R**eticulum **K**inase or **P**ancreatic **e**IF2α **K**inase (PERK or PEK) (Panel 2).

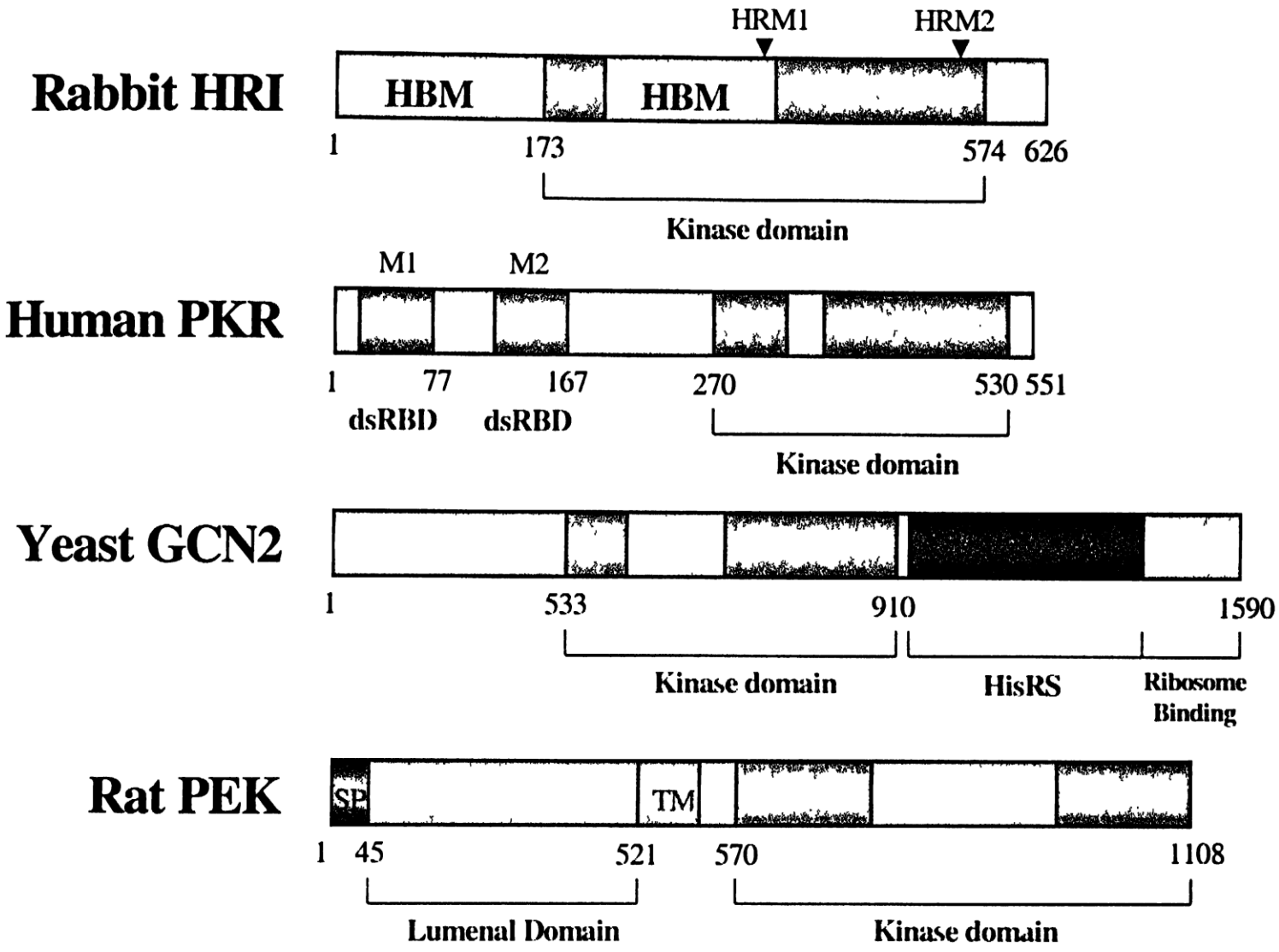
Catalytic domain of kinases are classified into 12 subdomains based on the significant sequence homology they share (Hanks and Hunter, 1995). eIF2α kinases are different from other kinases by having an insert between IV and V subdomains. V subdomain, which is less conserved in other kinases (Hanks and Hunter, 1995) is well conserved in eIF2α kinases. Point mutations in the conserved LFIQMEFCD region of the V subdomain showed varying levels of auto and substrate phosphorylation, whereas deletion of this region completely abolished both the activities. Glutamine (Q) in this region was found to be very important for substrate binding (Cai and Williams, 1998). The kinase insert domain and the conserved V subdomain are the signature features of this kinase family.



Panel 1. Schematic depiction of eIF2α recycling after protein initiation.

eIF2α kinases share a highly conserved Lysine (Lys-296) in II domain, mutation of which to alanine, arginine or proline results in inactive kinase (Chong *et al.*, 1992). Two Threonine (Thr) residues at 446th and 451st positions in human PKR, located between VII and VIII subdomains were proved to be the auto-phosphorylation sites (Romano *et al.*, 1998). Corresponding Thr-882 and Thr-887 of yeast GCN2, Thr-980 and Thr-985 of human PEK were also shown to be the auto-phosphorylation sites (Romano *et al.*, 1998; Ma *et al.*, 2002).

All the eIF2α kinases use eIF2α subunit as a common substrate suggesting that eIF2α phosphorylation by eIF2α kinase is a central signaling event for the inhibition of general translation in response to various stress conditions (Samuel, 1993). The known sources of different eIF2α kinases and their activating signals are as shown in Panel 3.



Panel 2. Schematic representation of structural differences in different members of eIF2α kinase protein family (for expansions please refer abbreviations section).

eIF2α kinase	Known source	Activating signals
	Mammals	Viral infection ds RNA Cytokines PACT/RAX
	Mammals *P.falciparum* *S.pombe*	Low heme NO
	Mammals *D.melanogaster* *C.elegans* *S.cerevisiae* *S.pombe*	Low amino acids Uncharged tRNA
	Mammals *D.melanogaster* *C.elegans*	ER stress Unfolded proteins Hypoxia UV

Panel 3. Different class of eIF2α kinases, organisms from which these kinases were identified and the conditions under which they are activated. PK is protein kinase domain, dsRBD is double stranded RNA binding domain, HisRS is Histidyl tRNA synthetase domain, SP is signal peptide, and TM is transmembrane domain.

1.3 GCN2 like kinase

The yeast GCN2 kinase is activated by amino acid starvation and generates a signal that leads to enhanced expression of genes involved in amino acid biosynthesis (Dever *et al.*, 1992; Hinnebush, 1994). GCN2, which selectively translates GCN4 mRNA in yeast cells, has been identified in mouse (Sood *et al.*, 2000a; Berlanga *et al.*, 1999), *Drosophila melanogaster* and *Caenorhabditis elegans* (Santoyo *et al.*, 1997; Olsen *et al.*, 1998) and shown to functionally substitute yeast GCN2 in *Saccharomyces cerevisiae.*

Binding of uncharged tRNA to histidyl-tRNA synthetase regulatory domain (Zhu *et al.*, 1996) activates GCN2. After activation, it phosphorylates eIF2α and facilitates selective translation of GCN4 mRNA which were otherwise repressed because of the four short upstream ORFs (uORF) before the actual GCN4 start codon. A model was proposed for GCN2 action to overcome the uORFs: when eIF2α gets phosphorylated under starvation, the concentration of eIF2/GTP/Met-tRNA$_i^{Met}$ ternary complex decreases. Ribosomes scanning from uORF1 bypasses uORFs 2-4 because of non-availability of ternary complex and reach GCN4p to reinitiate translation after rebinding to ternary complex. Under non-starved normal condition, eIF2α is not phosphorylated and ternary complex is abundant. All ribosomes scanning from 5′ end translate uORF1 and reinitiates at uORF4, which is an inhibitory ORF and later they dissociate from mRNA before binding to GCN4p (Gaba *et al.*, 2001).

Three GCN2 isoforms varying at N terminal end isolated from mouse were differentially expressed in different tissues (Sood *et al.*, 2000a). GCN2 expression in *Drosophila* is developmentally regulated. Although GCN2 mRNA is expressed in several tissues during early embryogenesis, at the later stages, its expression gets selectively restricted to a few cells of the ventral chord, which may indicate that it is involved in determining neural cell identity for these cells (Santoyo *et al.*, 1997). This kind of distribution was proposed to be involved in helping to differentiate three different germ layers of mesoderm, endoderm and ectoderm. This is supported by the early expression in the mesoderm suggesting that dGCN2 could be involved in determining mesoderm germ layer identity during early development (Santoyo *et al.*, 1997).

Deprivation of leucine, tryptophan or glycine in GCN2 knockout mice induced significant prenatal and neonatal mortality (Zhang *et al.*, 2002a). In mammals GCN2 phosphorylation of eIF2α was found to selectively increase translation of Activating Transcription Factor (ATF) 4 and *cat-1*. ATF4 has 4 uORFs and its regulation is similar to that of GCN4. Contrastingly, *cat-1*, an arginine/lysine transporter was regulated by indirect method through Internal Ribosomal Entry Site (IRES) (Harding *et al.*, 2000; Fernandez *et al.*, 2002).

1.4 Double stranded RNA activated protein kinase

PKR is induced by interferons (IFN) and is activated by low concentrations of double stranded RNA (dsRNA) produced during viral infections (Meurs

et al., 1990; Thomis *et al.*, 1992). Human interferon γ mRNA activates PKR through a phylogenetically conserved mechanism known as pseudoknot. Pseudoknot present in the 5′ Untranslated Region (UTR) activates PKR and inhibits its translation thus serving to avoid over-expression of IFN γ protein in normal conditions, which might lead to diseases. Disturbance in the pseudoknot due to mutation in the first 14 nucleotides (nt), render it more effective for translation overcoming PKR based inhibition (Ben-Asouli *et al.*, 2002).

PKR, having two dsRNA Binding Domains (RBD) is identified only in mammals (Feng *et al.*, 1992; Green and Mathews, 1992). PKR regulated by dsRNA, phosphorylates its primary substrate eIF2α and controls protein synthesis. Both dsRBDs are required for activation of PKR by dsRNA. Deletion of dsRBD1 severely reduced eIF2α kinase activity, whereas deletion of 25 amino acids of auto-inhibiting/negative acting region at the C terminal junction of dsRBD2 increased kinase activity by 30 times independent of dsRNA (Vattem *et al.*, 2001a; Lee *et al.*, 1994). Other than dsRNA, binding of PACT/RAX proteins to dsRBD under stress treatments like arsenite, Thapsigargin and H_2O_2 also activates PKR (Saelens *et al.*, 2001, Ito *et al.*, 1999). dsRBDs mediate ribosome binding and they are not necessary for PKR to access eIF2 substrate *in vivo* (Vattem *et al.*, 2001b). Native proteins like p58 and p74 are found to be regulating PKR in cells (reviewed in Clemens and Elia, 1997 and Williams, 1999; Polyak *et al.*, 1996; Coolidge and Patton, 2000).

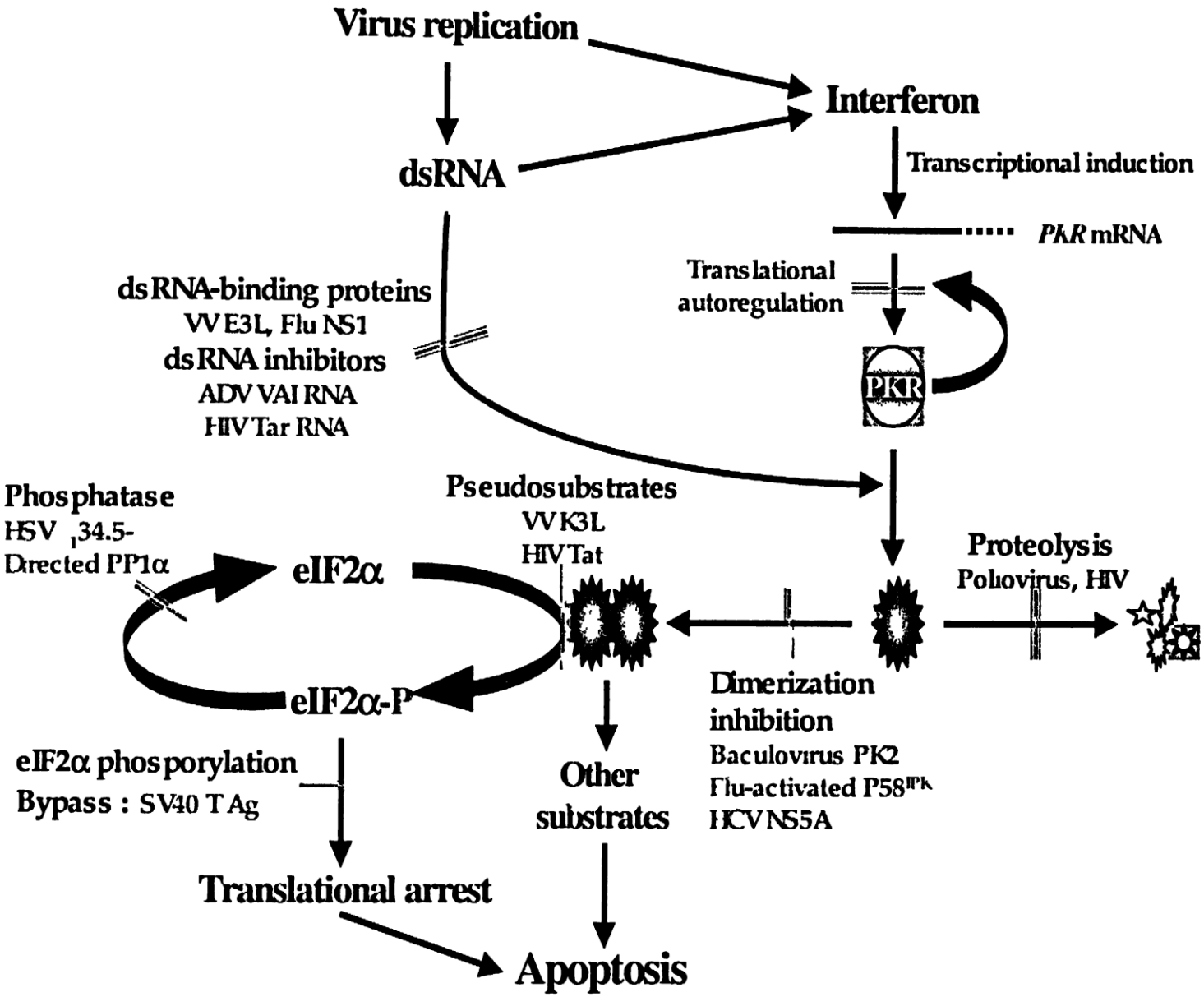
PKR activates many other proteins like NF-kB and pathways like tumor suppression and apoptosis (Kumar *et al.*, 1997; Williams, 1999). PKR kinase domain and its activity is essential to activate NF-kB through IKK activation and dsRBDs are not necessary for this function (Zamanian-Daryoush *et al.*, 2000; Gil *et al.*, 2001). Mutant cells did not undergo apoptosis in response to dsRNA, Tumor necrosis factor (TNF) α or Lipopolysaccharide (LPS) indicating that PKR regulates apoptosis under these conditions (Der *et al.*, 1997). During apoptosis, PKR is cleaved at Asp-251 by Caspase- 3, 7 and 8 and not by Caspase- 1 and 11. This cleavage between the regulatory and kinase domains liberates an active eIF2α kinase domain (Saelens *et al.*, 2001).

Model proposed for PKR function is as follows: dsRNA produced during viral infections associates with the dsRBD1 and 2, leading to overcoming of N-terminal inhibitory sequences and an activated conformational change in PKR. Interaction between PKR molecules is enhanced by oligomerization and ribosome association, which leads to elevated localized concentrations of the eIF2α kinase. The dsRBDs and the N-terminal portion of the catalytic domain contribute to PKR dimerization. Increased localized concentrations of the eIF2α kinase may enhance interaction between PKR molecules through association with a common RNA molecule. The combination of an activated protein conformation and interaction between PKR molecules leads to trans-autophosphorylation at multiple serine and threonine residues. Autophosphorylation leads to release of RNA before binding to eIF2α. Upon autophosphorylation, PKR

would retain high levels of eIF2α kinase activity and low affinity for dsRNA until the protein was dephosphorylated by phosphoprotein phosphatases or subjected to proteolysis (Vattem *et al.*, 2001a; Jammi and Beal, 2001).

During cell cycle, PKR activity showed two peaks, one in early G1 and other in G1/S boundary. Cells expressing mutant PKR showed longer G1 phase and fewer cells in S phase, thus having a slower growth compared to normal cells. PKR activation was stated to regulate cell cycle by regulating the orderly expression and selective down regulation of the regulatory genes and proteins (Zamanian-Daryoush *et al.*, 1999).

Because of the important role played by PKR in antiviral defense, many viruses are found to possess PKR inhibitors like C8L protein (Swinepox virus), E3L protein (Vaccinia virus), NS1 protein (Influenza virus), NS5A protein (Hepatitis C virus) TAR RNA (HIV-1), VA_1 RNA (Adenovirus) etc. (Kawagishi-Kobayashi *et al.*, 2000; Kaufman, 1999; Tan and Katze, 1999; Gale *et al.*, 1999) (Panel 4).



Panel 4. Flow chart showing different viral inhibitors at multiple stages of PKR pathway in translation regulation (Please refer abbreviation section for expansions).

1.5 Heme regulated inhibitor

HRI, activated under heme deficient condition in reticulocytes is identified in mammals (Chen *et al.*, 1991; Mellor *et al.*, 1994; Corsby *et al.*, 1994) and malarial parasite *Plasmodium falciparum* (Mohrle *et al.*, 1997). Two HRI related kinases Hri1p and Hri2p were recently identified in *Schizosaccharomyces pombe* which were inhibited by hemin *in vitro*, although they need higher concentration of hemin than mammalian HRIs (Zhan *et al.*, 2002).

HRI transcript is not restricted to reticulocytes, but also found in non-erythroid cells of mouse like liver, brain and NIH 3T3 cells, with a relatively high transcript in liver, kidney and testis suggesting its role other than maintaining equilibrium between heme and globin (Mellor *et al.*, 1994; Berlanga *et al.*, 1998). PfPK4 from *P. falciparum* is expressed during all the stages of parasite development and is proposed to help parasite in sensing its environment during the invasion process. In the final stage of parasite development, PfPK4 was found to associate with the host erythrocyte membrane suggesting its involvement in erythrocyte rupture (Mohrle *et al.*, 1997).

Mammalian HRIs were proposed to have two heme regulatory motifs (HRM), whereas only one heme-regulatory motif was identified in PfPK4 from *P. falciparum*. When the 10 amino acid (a.a.) HRM consensus peptide was studied, the cystine moiety was found to be mandatory, whereas the proline residue reduced the efficiency of heme binding (Zhang and

Guarente, 1995; Mohrle *et al.*, 1997). Contrary to this, the second HRM found in the C terminus (cys-548/Pro-549) was showed not to bind heme by deletion studies. It was stated that HRMs may not be sufficient to bind heme or they are just regulatory domains and may not bind heme (Rafie-Kolpin *et al.*, 2000). Two heme binding domains (HBD), one in N terminus region and the other in a.a. position 301 to 420 (rabbit HRI) of kinase insertion domain were identified in mammalian HRI (Chefalo *et al.*, 1998; Rafie-Kolpin *et al.*, 2000). Stable binding of heme to the N terminal HBD is important for proper folding and stability. At this stage HRI is autophosphorylated and still possesses eIF2α kinase activity. Binding of heme to the second HBD rapidly represses HRI activity. Heme binding in second HBD is reversible and helps in rapid regulation of kinase activity (Chefalo *et al.*, 1998; Rafie-Kolpin *et al.*, 2000; Zhan *et al.*, 2002). Histidine 83 in the N terminal HBD played a critical role in the stable binding of heme (Uma *et al.*, 2000).

HRI is regulated by autophosphorylation in two stages. Autophosphorylation of the newly synthesized HRI stabilizes low phosphorylated first stage against aggregation. At this stage HRI is an active autokinase although it does not have kinase activity. Formation of stable dimeric kinase actively regulated by heme requires additional multiple phosphorylation in the second stage (Bauer *et al.*, 2001).

Decrease in hemin concentration activates HRI leading to inhibition of protein synthesis thereby coupling α- and β-globin synthesis with the

availability of hemin in reticulocytes (Proud, 1992; Chen and London, 1995). Expression of three inactive mutants in mouse erythroleukemic cells resulted in increased hemoglobin production and proliferation of these cells upon DMSO induction of erythroid differentiation (Crosby *et al.*, 2000). In HRI knockout mouse, globins devoid of heme aggregates within the erythrocytes and its precursors resulting in a hyperchromic normocytic anemia with decreased RBC counts, compensatory erythroid hyperplasia and accelerated apoptosis in bone marrow and spleen (Han *et al.*, 2001).

Although regulation of globin synthesis in erythrocytes is the primary function of this protein, it is observed to get activated under oxidative stress induced by arsenite, heat shock and osmotic stress in erythrocyte and fetal liver nucleated erythroid progenitor (Lu *et al.*, 2001). Hsc 70, which is a critical component of Hsp 90 chaperone is very important for proper folding of HRI. Hsc 70 is found to interact even after HRI is transformed to right conformation. Disruption of this interaction hyperactivates HRI in heme deficient rabbit reticulocyte lysate, which is proposed to be the case under heat shock stress (Uma *et al.*, 1999). NO and CO also influence HRI by directly binding to the N-terminal heme binding domain. NO is a very potent activator whereas CO is a suppressor of NO induced activation (Uma *et al.*, 2001).

1.6 PKR like Endoplasmic Reticulum Kinase

The new addition to this family of eIF2α kinases is PERK or PEK, a glycoprotein, which is activated by unfolded protein response in Endoplasmic Reticulum (ER). In addition to mammals, homologues of this protein were identified in *D. melanogaster* and *C. elegans* and *Fugu rubripes* (Shi *et al.*, 1998; Harding *et al.*, 1999; Sood *et al.*, 2000b). PEK functionally substituted endogenous GCN2 in yeast. In mammals this protein is primarily expressed in pancreas and in elevated levels in different secretory tissues (Shi *et al.*, 1998; Sood *et al.*, 2000b), although activity was detected only in pancreas and pancreatic islets cells (Shi *et al.*, 1999).

PEK has a transmembrane domain in the regulatory region and localizes in ER of islet delta-cells (Shi *et al*, 1999). PEK is found to associate with BiP, a chaperonin protein in unstressed cells thus preventing oligomerization. Upon ER stress, BiP dissociates to bind misfolded proteins relieving negative regulation. It is observed that BiP levels increase under hypoxic and ER stresses but it is not sufficient to bind to PEK because of competitive binding to misfolded proteins (Koumenis *et al.*, 2002; Ma *et al.*, 2002). PEK contains an IRE1 like sequence between position 102 to 209, which was found to be the oligomerization region, whereas region between position 411- 481 was required for binding BiP (Ma *et al.*, 2002).

PEK is activated under hypoxic stress and UV exposure other than ER stress (Wu *et al.*, 2002; Koumenis *et al.*, 2002). Cells without PEK tend to have lower survival rate under prolonged exposure to hypoxic condition. The translation regulation by PEK under hypoxic condition may allow the cells to adopt to the fluctuating oxygen concentration which are known to occur in tumors (Koumenis *et al.*, 2002). PEK was found to be necessary for the development of skeletal system in postnatal growth and viability of pancreas in mouse (Zhang *et al.*, 2002b).

1.7 Studies in insects

In insects studies on the eIF2α kinase family are scanty as compared to mammalian and yeast systems. Only recently, GCN2 and PEK homologues were identified in *Drosophila* (Santoyo *et al.*, 1997; Olsen *et al.*, 1998; Sood *et al.*, 2000b) and eIF2α phosphorylation in *Drosophila* Schneider S2 cells under starvation and ER stress were studied (Williams *et al.*, 2001). eIF2B subunits were cloned from *Drosophila* and shown to bind to native and phosphorylated forms of eIF2α (Williams *et al.*, 2001).

1.8 Baculovirus and translation shut down

ORF 123 or Protein kinase2 (PK2), a truncated kinase homologue was identified from the insect baculovirus *Autographa californica* Multiple Nuclear Polyhedrosis Virus (AcMNPV). PK2 is a truncated protein with only VI to XI kinase subdomains. It also lacks the phosphorylation loop and potential autophosphorylation sites present between VII and VIII subdomains. It was found to express both in early and late stages of

infection. PK2 was shown to inhibit eIF2α phosphorylation during viral replication, although it was not necessary for infection and virulence as indicated by deletion mutant virus (Li and Miller, 1995). PK2 was also found to inhibit both human PKR and yeast GCN2 in *S. cerevisiae*. It specifically interacts with human PKR by forming heterodimer and prevents its auto-phosphorylation activity *in vivo* (Li and Miller, 1995; Dever *et al.*, 1998). This gives a clue that a native eIF2α kinase must be interacting with PK2 protein produced by baculovirus and thus helping the insect cells to defend against viral replication.

AcMNPV infection of Ld652Y gypsy moth cell line globally shut down protein synthesis at late times of infection. p35 was shown to be involved in inducing translation arrest in the absence of late viral gene expression (Du and Thiem, 1997).

1.9 RNA interference and gene knockdown

RNA interference (RNAi) first discovered by Fire *et al.*, in 1998 is a technology based on silencing the gene of interest targeted by using specific double stranded RNA (dsRNA). Fire *et al.*, demonstrated that presence of small amount of dsRNA is sufficient to almost abolish the expression of corresponding homologous gene. RNAi occurs in two different steps; in the first step sequence specific silencing complex is generated, which processes long dsRNAs to 21-23-nucleotide (nt) RNAs. In the second step, RNAi silencing complex uses the anti-sense strand of the processed 21-23-nt RNA and cleaves mRNA (Panel 5). This process

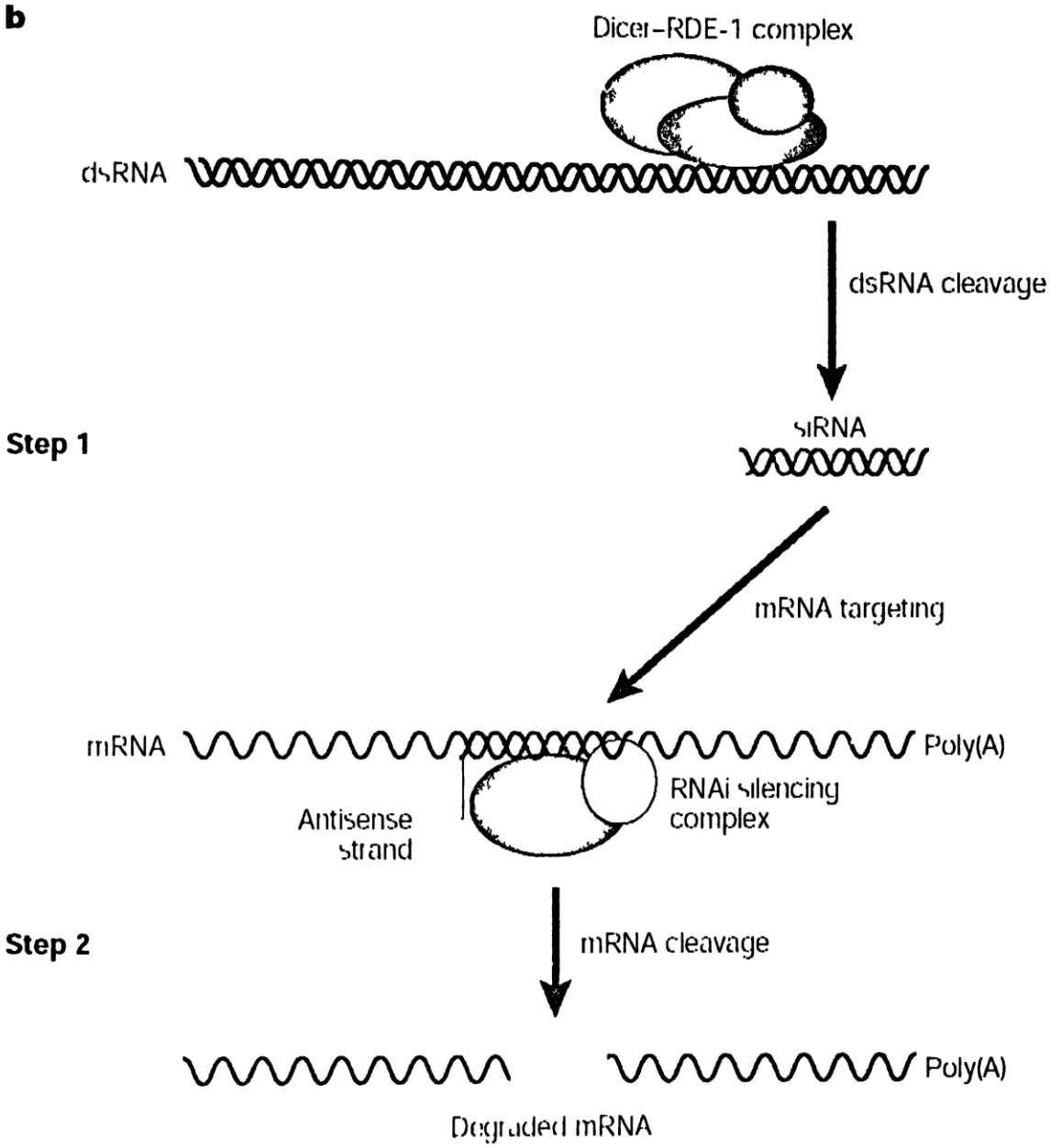
was first elucidated in *C. elegans* and *Drosophila* S2 cells, and is now known to be widely conserved in plants, fungi, insects, metazoans and mammals. RNAi technology has been applied to decipher the function of a number of genes in mammalian system. Recently this technology is being applied to develop antiviral strategy against Respiratory Syncytial Virus and HIV. (reviewed by McManus and Sharp, 2002). In mammals, long dsRNA cannot be used as it activates PKR which shuts down translation, thus, short 21bp dsRNA were used. In insects and lower organisms, PKR like activity is not identified and thus is safer to use longer fragments of dsRNA for gene knockdown studies. Using RNAi technology recombinant baculoviral infection was prevented *in vitro* and *in vivo* showing that this technology works in lepidopteran insects as well (Valdes *et al.*, 2003).

1.10 Project

Bombyx mori is one of the well-studied insects after *Drosophila*. This insect is domesticated for over 4000 years for silk production. Baculovirus, one of the major pathogens of silkworm, causes huge mortality. Given the fact that viral resistance is less studied in this economically important insect, we took up a project to identify and characterize anti-stress and anti-viral proteins. An offshoot of our project is the identification of a novel eIF2α kinase family member, the activity of which is reduced by baculoviral recombinant PK2 *in vitro*.

In this thesis, I report a novel eIF2α kinase family protein, called BeK (**Bombyx eIF2α Kinase**) from *B. mori*. This gene encodes a complete ORF

of 579 amino acids and contains all 11 catalytic domains of protein-serine/threonine kinases. Notably, it contains an "Leu-Xaa-Ile-Gln-Met-Xaa-Xaa-Cys" motif, which is highly conserved from yeast to mammalian eIF2α kinases. BeK does not show any significant homology in the NH_2 terminal regulatory domain, suggesting a distinct regulatory mechanism of this novel eIF2α kinase. BeK is ubiquitously expressed in the various tissues of the final instar larvae. We also found its expression in early embryonic stages. Importantly, BeK is activated in *Drosophila* Schneider cells following heat shock, osmotic stress and bacterial cell wall stress, and activated-BeK has been shown to phosphorylate an eIF2α subunit at the Ser50 site. However, other forms of stress, such as fungal cell wall stress, endoplasmic reticulum stress and oxidative stress, cannot significantly elicit BeK activity. Interestingly, the baculovirus gene product, PK2 can inhibit BeK enzymatic activity suggesting that BeK may be an endogenous target for a viral gene product. The effect of eIF2α phosphorylation under heat shock and osmotic stress was significantly reduced under BeK knockdown indicating that this kinase is the major eIF2α kinase active under these stresses. Taken together, these data indicate that BeK is a novel eIF2α kinase involved in the stress response in *B. mori*.



Panel 5. Two step RNA interference mechanism. Long double-stranded RNA (dsRNA) is processed by the Dicer–RDE-1 complex to form siRNAs. The anti-sense strand of the siRNA is used by an RNA interference (RNAi) silencing complex to guide mRNA cleavage, so promoting mRNA degradation. (McManus and Sharp, 2002)

2 Materials and methods

Wipe off that I-told-you-so look!

2.1 Organism and library

Silkworm larvae were reared on mulberry leaves. Tissues from day 4 of 5th instar larvae were dissected and flash frozen in liquid nitrogen and later used for total RNA isolation. cDNA library was kindly provided by Dr. Hirano H, Tottori University, Japan.

2.2 Cells and transfection

Drosophila Schneider S2 cells were maintained in *Drosophila* Schneider medium (Life Technologies) with 10% Fetal Bovine Serum (FBS). S2 cells were maintained in T25 flasks and incubated at 25°C for optimum growth.

Transfections for transient expression and for establishing transformed cell lines of Schneider cells were done as per the recommendations of the supplier (DES, Invitrogen). Stably expressing (BeKw, BeKm and dPEK) *Drosophila* Schneider cells were maintained in *Drosophila* Schneider medium (Invitrogen) with 10% FBS.

Bombyx BmN cells were maintained in TC100 medium (Invitrogen) with 10% FBS. These cells were maintained in T25 flasks incubated at 27°C for optimum growth. Schneider cells maintained in T25 flask were incubated at 27°C for optimum growth.

2.3 PCR on cDNA library

To identify different stress related kinases from *B. mori*, an anti-sense degenerate primer, P1, AANAYNACRTTNSWNGGYTT was synthesized based on amino acids (KPSNV/IF) in the conserved kinase subdomain VI. We used primer, P1, and λgt-10 upper or lower primer as sense primer to do Polymerase Chain Reaction (PCR). PCR was performed using 1μM of P1 and 1μM of λgt-10 upper or lower primer in 10mM Tris-HCl, pH8.0, 50mM KCl, 1.5mM $MgCl_2$, 200μM dNTPs and 2.5 units of Taq Polymerase. An aliquot (5 μl) of bacteria-challenged λgt-10 *Bombyx* fat body cDNA library (Morishima *et al.*, 1990) was used as template, after denaturing for 5 min. at 70°C. The amplified products were cloned into pMos blue vector and sequenced using bigdye terminator technology (Perkin Elmer).

2.4 cDNA library screening

To obtain full-length cDNA clone, 1300 bp fragment obtained by PCR was used as probe to screen bacteria-challenged λgt-10 *Bombyx* fatbody cDNA library (Morishima *et al.*, 1990). About 400,000 plaques were screened using the standard method (Sambrook and Russell 2001). Two of the six positive clones identified were sequenced using bigdye terminator technology (Perkin Elmer).

2.5 Sequence analysis

Sequence editing, translation, ORF detection were done using DNA strider program (http://www.cellbiol.com/soft.htm). All the submitted sequences of eIF2α kinase family proteins were downloaded from Genbank, the accession numbers of which are as follows- P15442 (*S. cerevisiae* GCN2), T13826 (*Drosophila* GCN2), NP_038747 (Mouse GCN2), XP_031612 (Human GCN2), NP_496781 (*C. elegans* GCN2), CAC21489 (*S. pombe* GCN2), P33279 (Rabbit HRI), NP_037355 (Rat HRI), NP_038585 (Mouse HRI), NP_055228 (Human HRI), T28139 (*P. falciparum* PfPK4), AAN04053 (*S. pombe* HRI1), AAN04054 (*S. pombe* HRI2), A39650 (Human PKR), S50216 (Rat PKR), NP_035293 (Mouse PKR), T17455 (Rat PEK), T14351 (Mouse PEK), NP_004827 (Human PEK), AAF61200 (*Drosophila* PEK), AAF61201 (*C. elegans* PEK) and 3318705 (Rat Erk2). Multiple alignment of BeK kinase domain with other eIF2α kinases was done using Clustal W (Thompson *et al.*, 1994). Aligned sequences were shaded using Boxshade program (http://www.ch.embnet.org/software/BOX_form.html) for a better view. A phylogenetic tree was generated using the above mentioned aligned kinase domain sequences by parsimony method with 100 bootstrap replications using RatErk2 as outgroup with the help of Phylip (ver. 3.573c) software (Felsenstein, 1993). The alignment gaps were assumed to be unknown sequences while constructing the tree (- were replaced with ?).

2.6 Plasmid construction

BeK cDNA sequence was excised from λgt-10 and cloned in pMal-C2 fusion vector (NEB) giving rise to pMalC2BeK plasmid.

Drosophila eIF2α subunit was amplified from cDNA clone and cloned in pQE-30 expression vector to obtain pQEdeIF2α plasmid.

BeK gene sequence was amplified by PCR in 20µl reaction volume using FBKp primer ctcgagatgcatcaccatcaccatcacgataaacatagccaagac and RBKp primer gggcccggtaccatggaaaagttaat in 10mM Tris-HCl, pH8.0, 50mM KCl, 1.5mM $MgCl_2$, 100µM dNTPs and 2 units of Taq Polymerase at annealing temperature of 60°C. FBKp primer consists of hexa histidine tag. Lambda clone, containing BeK cDNA as insert was used as template and the amplicon obtained was cloned in metal inducible *Drosophila* expression vector pMT/V5 (DES, Invitrogen) and named pMTBeKw. A mutant clone of BeK was constructed by point mutating highly conserved Lysine amino acid (Lys-174) in Kinase domain into Arginine (codon AAA to AGA). BeK mutant was also cloned in metal inducible *Drosophila* expression vector, pMT/V5 and named pMTBeKm. Both BeK wild type (BeKw) and BeK mutant (BeKm) were cloned in constitutively expressing *Drosophila* expression vector pPacPl with a 5′ poly Histidine tag introduced during PCR.

Drosophila PEK (dPEK) was amplified by PCR in 20µl reaction volume using LD41715 cDNA clone as template, 1µM each of fDPEK primer cgcggatccgcgtacgatgcaggacgac and rDPEK primer tacgggccctcagtgatggtgatggtgatgctgatgcggctcactgga in 10mM Tris-HCl, pH8.0, 50mM KCl, 1.5mM MgCl2, 100µM dNTPs and 2 units of Taq Polymerase at 60°C annealing temperature. The amplified product of 3.5 kb size obtained was cloned in pMos blue vector (Amersham) and sequenced. The insert with a full reading frame and a 3′ poly Histidine tag was cloned in pMT/V5 vector giving rise to pMTdPEK.

B. mori Nuclear Polyhedrosis Virus (BmNPV) isolate Pa was used as template to amplify BmPK2 and *Autographa californica* Multiple Nuclear Polyhedrosis Viruses (AcMNPV) DNA was used as template to amplify AcPK2; FPK2, as sense primer accatgcatcaccatcaccatcacaaaccegaacaattggtat and RPK2 as anti-sense primer ctcgagcatggtgttctatattcgacaa were used for amplification. Amplification was performed in a 20µl reaction mixture containing 10mM Tris-HCl, pH8.0, 50mM KCl, 1.5mM MgCl2, 100µM dNTPs, 1µM each of primers and 2 units of Taq Polymerase at 60°C annealing temperature. The amplification products were cloned in pMos blue vector (pMosBmPK2 and pMosAcPK2) and sequenced. A poly-histidine tag at 5′ end was introduced in both AcPK2 and BmPK2 during PCR. Both AcPK2 and BmPK2 were then cloned in pQE-30 bacterial expression vector (Qiagen)

to obtain pQEAcPK2 and pQEBmPK2 respectively. All the clones were confirmed for mutations and correct ORF by sequencing.

For *in vitro* synthesis of double stranded RNA, regulatory region, insert region and kinase domain were separately cloned. Regulatory domain region was amplified using iregF aaacatagccaagacaaatgga and iregR ccagacggctgaactataggc primers. Insert domain, region that is identified as insert between IV and V subdomain of kinase, was amplified using iinsF cccatgatagagtctacagttaaaa and iinsR cgagagcaattttcgtactcc primers. Polymerase chain reaction was done in 20µl reaction with pMTBeKw as template using 1µM of the above mentioned primers, 10mM Tris-HCl, pH8.0, 50mM KCl, 1.5mM MgCl2, 100µM dNTPs and 2 units of Taq Polymerase with annealing temperature of 60°C. The 398bp regulatory region and 323bp insert region amplicons obtained by the PCR reactions were cloned in pCR2.1 T vector (Invitrogen). Clones were isolated with regulatory and insert domain insert in both orientations and named as preg and pins respectively. + and – were added to the plasmid name to indicate + orientation and – orientation. All clones were confirmed by sequencing.

AcMNPV *ie1* gene was amplified using 1µM each of FoIE1 ccaaacgactatgacgcaaattaatttt and ReIE1 ttgttaaattggcccaccacacttgt primers in 10mM Tris-HCl, pH8.0, 50mM KCl, 1.5mM MgCl2, 100µM dNTPs and 2 units of Taq Polymerase at annealing temperature of 58°C.

Amplification product of 471bp obtained was cloned in pCR2.1 T vector (Invitrogen) and sequenced for confirmation. From this vector *ie1* gene insert was excised with *Hind*III and *Xba*I restriction digestion and cloned in SK+ vector with compatible ends to create SK*ie1* plasmid.

2.7 Expression, purification and immunodetection of MBP-BeK

The MBP-BeK fusion protein was expressed in *Escherichia coli* (K12TB1) and checked for induction. MBP-BeK fusion protein was induced using 0.3mM IPTG. Induced cells were pelleted after three hrs. and frozen overnight at –20°C. Frozen cells were thawed on ice for few min. To this 30 ml column buffer (200mM Tris-HCl, 200mM NaCl, 1mM EDTA, 1mM Sodium azide and 1mM DTT) containing 1mM PMSF and 1mg/ml lysozyme was added and sonicated for 20 min. with a pulse of 10 sec. every minute on an ice bath. Lysed cells were centrifuged at 10,000rpm for 30 min. Supernatant was decanted and stored on ice. Amylose resin (New England Biolabs) column was prepared using 1ml of resin and washed with 10ml of column buffer. Diluted cell lysate was added onto the column and allowed to flow through at the rate of 1ml/min. Column was washed with 15ml of column buffer. Protein was eluted with elution buffer (column buffer with 10mM maltose) and collected in 10 fractions of 4ml each. Protein samples were then dialyzed and concentration was estimated by Bradford's reagent.

Purified MBP-BeK protein was resolved using SDS-PAGE to check its purity and size. Equal amount of MBP-BeK protein was resolved using

SDS-PAGE and transferred onto Hybond C membrane (Amersham Pharmacia) by semi-dry method. Out of the two strips, which were made with one lane each, one strip was incubated with pre-immune serum and the other was incubated with anti BeK immune serum. Antibodies were diluted 1:2000 times and incubated with the membrane in 1X TBS containing 0.02% Tween-20 and 5% skimmed milk at 4°C, overnight. After washing, the blots were incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase diluted 1:2000 times. The signal was detected using ECL western blot detection kit (Amersham Pharmacia).

2.8 Expression and purification of PK2 and eIF2α subunit proteins

pQEAcPK2, pQEBmPK2 and pQEdeIF2α vectors were transformed in *E. coli* M15 strain. Protein expression was obtained by induction with 1mM IPTG and purified using NiNTA resin. Frozen pellet (from 1 ltr. culture) of induced *E. coli* was thawed on ice and pellet was resuspended completely in 10ml of lysis buffer (50mM NaH_2PO_4, pH 8.0, 300mM NaCl and 10mM Imidazole) containing 1mM PMSF and 1mg/ml lysozyme followed by sonication on an ice bath for about 2-5 min. Crude lysate was centrifuged at 10,000g for 30 min. and the clear lysate thus obtained was stored at 4°C. A Ni-NTA resin column was prepared with 2ml of resin and calibrated with 10ml of lysis buffer. Diluted cell lysate was loaded onto the column and allowed to flow through. The column was washed with 20ml of wash buffer (50mM NaH_2PO_4, pH 8.0, 300mM NaCl and 20mM Imidazole). Protein was eluted by sequentially adding 2ml each of elution

buffer 4 times (50mM NaH_2PO_4, pH 8.0, 300mM NaCl and 250mM Imidazole). The protein thus eluted was dialyzed, concentrated and protein was estimated using Bradford's reagent.

2.9 RNA isolation and transcript detection

Total RNA was extracted from *B. mori* at varying time points of embryonic stages, different tissues of 5th instar larvae, whole pupa and whole 4th instar larvae by using Trizol reagent (Sigma). The frozen embryo and tissue samples were ground to fine powder in liquid nitrogen with the help of mortar and pestle. Trizol was added to this ground tissue and allowed to thaw completely. Once thawed, samples were transferred to microcentrifuge tubes and incubated at room temperature for 5 min. Total RNA from BmN cells was extracted by using Trizol reagent (Sigma). Medium was completely removed from 6 well plate and cells were scraped with 0.2ml of Trizol reagent for each well. Samples were transferred to microcentrifuge tubes and incubated at room temperature for 5 min. 0.2ml of chloroform was added to 1ml of trizol and mixed well before centrifugation. The upper aqueous layer was ethanol precipitated in a fresh tube. The pellet obtained was washed in 70% ethanol and air-dried and dissolved in RNase free water. To remove traces of chromosomal DNA contamination, the RNA solution was treated with RNase free DNase for 30 min. and heat inactivated to inactivate DNase. This solution was further treated once with saturated phenol-chloroform, once with chloroform, and the aqueous layer obtained was ethanol precipitated. The

pellet was washed with 70% ethanol and air-dried before dissolving in water.

First strand cDNA was synthesized in 20µl of reaction mixture containing 4µg of total RNA, 0.5mM dNTPs, 10µg of oligo dT primers, 10 mM dTT, 40units of RNAsin, 10 units of AMV reverse transcriptase and 1x buffer supplied by the manufacturer at 37°C for 3 hrs.. Total RNA, water and oligo dT primers were denatured at 65°C for 10 min. before starting the reaction. One µl of cDNA was subjected to PCR amplification in 20µl of reaction volume containing 10mM Tris-HCl, pH 8.0, 50mM KCl, 1.5mM $MgCl_2$, 100µM dNTP, 1µM of each primer and 1 Unit of Taq Polymerase. The sequences of primers were as follows: BeK sense ccgacagtgacgagttttca, BeK anti-sense tcatcgctgtcagaaacctg, *Bombyx* β-actin sense cactgaggctcccctgaac and β-actin anti-sense ggagtgcgtatccctegtag. For amplifying BeK mRNA, PCR of 26 cycles was performed at 63°C annealing temperature, whereas for *Bombyx* actin amplification, 23 cycles at 60°C annealing temperature was performed. For detecting BeK transcript in total RNA from dsRNA treated BmN cells, ikinF tcaagataccatagagaatttgagga and BK-R tcatcgctgtcagaaacctg primers were used for PCR of 26 cycles at 60°C annealing temperature, as ikinF primer binds outside the non-targeted region of BeK mRNA.

2.10 Antibody preparation

Rabbit polyclonal antibody, which is specific to BeK, was generated using synthetic peptide corresponding to N-terminal 12 amino acids of BeK

(KHSQDKWKALAT). Antibodies were partially purified using MBP-BeK expressed in bacteria. MBP-BeK was resolved on SDS-PAGE and transferred onto Hybond-C (Amersham) membrane. The region containing fusion protein was excised after Ponseau S staining. The membrane was blocked for 1 hr. at room temperature in 1X TBS containing 0.02% Tween-20 and 3% Bovine Serum Albumin (BSA). It was then incubated overnight with 1ml of antiserum in 1X TBS containing 0.02% Tween-20 and 3% BSA at 4°C. Membrane was washed with 150mM NaCl and 1X Phosphate buffered saline for 20 min. each. Finally, antibodies were eluted in elution buffer (0.2M glycine and 1mM EGTA, pH 2.8). After elution, 0.1 volume of 1M Trizma base (Sigma) and 0.1 volume of 10X Phosphate buffered saline was immediately added.

2.11 Immuno-precipitation and kinase assay

Schneider cells transformed with His-tagged BeKw, BeKm and dPEK, were induced with 0.5mM copper sulfate before immuno-precipitation. BeKw and BeKm cells were induced for 48 hrs., whereas dPEK was induced for 24 hrs. These cells were exposed to different stresses as described earlier after respective hrs. of induction. BmN cells were exposed to heat shock at 37°C for 1 hr. before immuno-precipitation for checking auto-phosphorylation. Immediately after stress, cells were washed twice in Tris-buffered saline and suspended in lysis buffer (20 mM HEPES, pH 7.4, 2mM EDTA, 50 mM β-glycerophosphate, 1mM dithiothreitol, 1mM sodium orthovanadate, 1% Triton X-100, 10% glycerol, 25μg/ml aprotinin and 40 μg/ml phenylmethylsulfonyl fluoride). These suspensions were

freeze thawed in liquid nitrogen and 37°C water bath alternatively for two times and solubilized for 30min. on ice and subsequently centrifuged at 14,000 rpm for 15 min. at 4°C. Equal amount of total protein was taken for Immuno-precipitation. The his-epitope tagged protein kinases were immunoprecipitated by incubating for 1 hr. at 4°C with the monoclonal anti-His antibody (Qiagen) prebound to protein G-agarose for 15 min. at room temperature. The supernatant obtained after immuno-binding was used for native eIF2α phosphorylation analysis subsequent to protein concentration estimation (BioRad). In case of BmN cells, pre-immune serum (control) or anti BeK immune serum prebound to protein A-agarose was used for 1 hr. at 4°C for immuno-precipitating the native kinase. The immunoprecipitates were washed twice with RIPA buffer (50mM Tris.Cl, pH 7.5, 150mM NaCl, 1% Nonidet P-40, 0.5% Sodium deoxycholate and 0.1% Sodium dodecyl sulfate) and were finally washed once with kinase assay buffer (25mM HEPES, pH 7.4, 25mM β-glycerophosphate, 25mM MgCl2, 2mM dithiothreitol, 0.1mM sodium orthovanadate). The immunocomplexes were resuspended in 25µl of kinase assay buffer containing 20µM ATP and 1µg of *Drosophila* eIF2α subunit as substrate and incubated at 30°C for 30 min. Reactions were terminated by adding Laemmli sample buffer and denatured in boiling water bath for 5 min. The reactions were then resolved on SDS-PAGE and analyzed by immunoblot.

2.12 Autophosphorylation

BeK from BmN cell lysate was immunoprecipitated with pre-immune serum and anti-BeK antiserum. The isolated immune complexes were incubated with γ^{32}P-ATP in kinase assay buffer. The radiolabeled products were resolved by SDS-PAGE and autoradiographed for detecting auto-phosphorylation.

2.13 Immunoblotting

Drosophila Schneider cells, native (for detecting phosphorylated form of native eIf2α) or transformed (for detecting phosphorylated form of native eIf2α and kinase assay), which were exposed to various stress conditions were lysed using lysis buffer (20 mM HEPES, pH 7.4, 2mM EDTA, 50 mM β glycerophosphate, 1mM dithiothreitol, 1mM sodium orthovanadate, 1% Triton X-100, 10% glycerol, 25μg/ml aprotinin and 40 μg/ml phenylmethylsulfonyl fluoride). BmN cells were lysed with lysis buffer after stress for estimating native eIF2α phosphorylation. BmN cells, which were transfected with dsRNA and incubated for 72 hrs., were exposed to different stress conditions and lysed with lysis buffer. Lysates were prepared immediately after stress and estimated for protein concentration by Bradford's reagent and denatured by adding Laemmli buffer and incubating in boiling water bath for 5min. Equal quantity of protein was separated on SDS-PAGE. After resolving on SDS-PAGE, proteins were transferred onto Hybond C membrane (Amersham) by semidry method

(Sambrook and Russell 2001). The antibody used for recognizing phosphorylated form of eIF2α subunit was bought from Research Genetics. In-house antiserum generated against BeK was used for detecting BeK protein on the blot. Actin antibody (C2) was obtained from SantaCruz biotechnologies. Blots were stained with Ponseau S stain for checking transfer of proteins. The blots were blocked for 1 hr. at room temperature in 1X TBS containing 0.02% Tween-20 (TBST) and 5% skimmed milk at 4°C. After washing 2 times for 10min. each with 1X TBST, blots were incubated with 1X TBST containing 5% skimmed milk and primary antibodies diluted 1:2000 times at 4°C, overnight. After washing 3 times for 10 min. each with TBST, the blots were incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase diluted 1:2000 times. The signal was detected using ECL western blot detection kit (Amersham).

Optical density of the bands thus obtained were estimated and normalized with values obtained for unstressed cells and histogram was plotted.

2.14 Stress

Different stress conditions used on S2 cell lines were as follows-

Heat shock: flasks incubated at 37°C for 1 hr.

Endoplasmic Reticulum stress: 1μM of Thapsigargin for 1 hr.

Osmotic shock: 300mM NaCl for 30 min.

Bacterial stress: 10μg/ml of Lipopolysaccharide (LPS) for 30 min.

Fungal stress: 100µg/ml of β-1,3-glucan for 30 min.

Peroxide stress: 200µM of H_2O_2 for 30 min.

Transformed S2 cells were exposed to these stresses 48 hrs., 48hrs. and 24hrs. after induction with 0.5mM Copper sulfate for BeKw, BeKm and dPEK respectively.

Immediately after stress, cells were lysed in lysis buffer (20 mM HEPES, pH 7.4, 2mM EDTA, 50 mM β-glycerophosphate, 1mM dithiothreitol, 1mM sodium orthovanadate, 1% Triton X-100, 10% glycerol, 25µg/ml aprotinin and 40 µg/ml phenylmethylsulfonyl fluoride) for further use in immuno-precipitation. For estimating the eIF2α phosphorylation status, Laemmli buffer was added to the cleared lysate after total protein estimation and boiled to denature the proteins and loaded onto SDS-PAGE.

For BmN cells, varying levels of stress conditions were used to check for the optimum level of stress inducing maximum level of eIF2α phosphorylation. The stresses used were as follows-

1. Heat shock: flasks of BmN cells were incubated separately at 37°C for 1 hr. and 42°C for one hr. 2. Osmotic shock: 50mM, 150mM, 300mM, 450mM and 600mM NaCl for 30 min. 3. Bacterial stress: 3µg/ml, 6µg/ml, 12µg/ml, 20µg/ml and 30µg/ml of Lipopolysaccharide for 30 min.

The stresses used for double stranded RNA transfected BmN cells were- 1. Heat shock: 37°C for 1 hr., 2. Osmotic shock: 300mM NaCl for 30 min. and 3. bacterial stress: 12µg/ml of Lipopolysaccharide for 30 min.

2.15 *In vitro* RNA synthesis and dsRNA preparation

To create an open end for transcription fall off, pireg (+ and -) and piins (+ and -) plasmids were digested with *Bam*HI for linearisation. Whereas SK*ie1* was digested with *Xba*I and *Hind*III individually to linearise the plasmid with open ends on either side of the *ie1* insert. Digested plasmids were treated with Proteinase K in restriction digestion buffer itself in the presence of 0.5% of SDS at 50°C to remove any trace of RNase. Then, equal volume of 1:1 tris-saturated phenol:chloroform was added and mixed thoroughly followed by centrifugation. Upper aqueous layer was taken in a fresh tube and equal volume of chloroform was added and mixed well before centrifugation. The upper aqueous layer was taken and ethanol precipitated in the presence of 0.1 volume of 3M sodium acetate, pH 5.5, washed once with 70% ethanol, air dried and dissolved in nuclease free water. 3µg of each linearised plasmid was taken in a 100µl reaction mix containing 2.5mM NTPs each, 100 Unit RNaseOUT (Invitrogen), 14mM $MgCl_2$, 40mM Tris-HCl, pH 7.9, 2mM spermidine, 10mM dithiothreitol and 175U of RNA polymerase. T3 RNA polymerase was used for SK*ie1* plasmid digested with *Hind*III, which synthesizes – strand of *ie1* gene, whereas for rest of the plasmids, pireg+ and -, piins+ and -, and SK*ie1* digested with *Xba*I (*ie1* + strand), RNA was synthesized using T7 RNA polymerase at 37°C overnight. DNA was removed by

treating the freshly synthesized RNA with 3 Units of RNase free DNase at 37°C for 30 min. DNase activity was stopped by adding EDTA to a final concentration of 2mM and heat inactivated at 65°C for 10 min. RNA solution was precipitated with 0.1 volume of 3M sodium acetate and 2.5 volumes of ethanol at –20°C for 2 hrs. RNA was pelleted at 14,000 rpm for 15 min., air-dried and dissolved in RNase free water, and quantified using spectrophotometer (260nm).

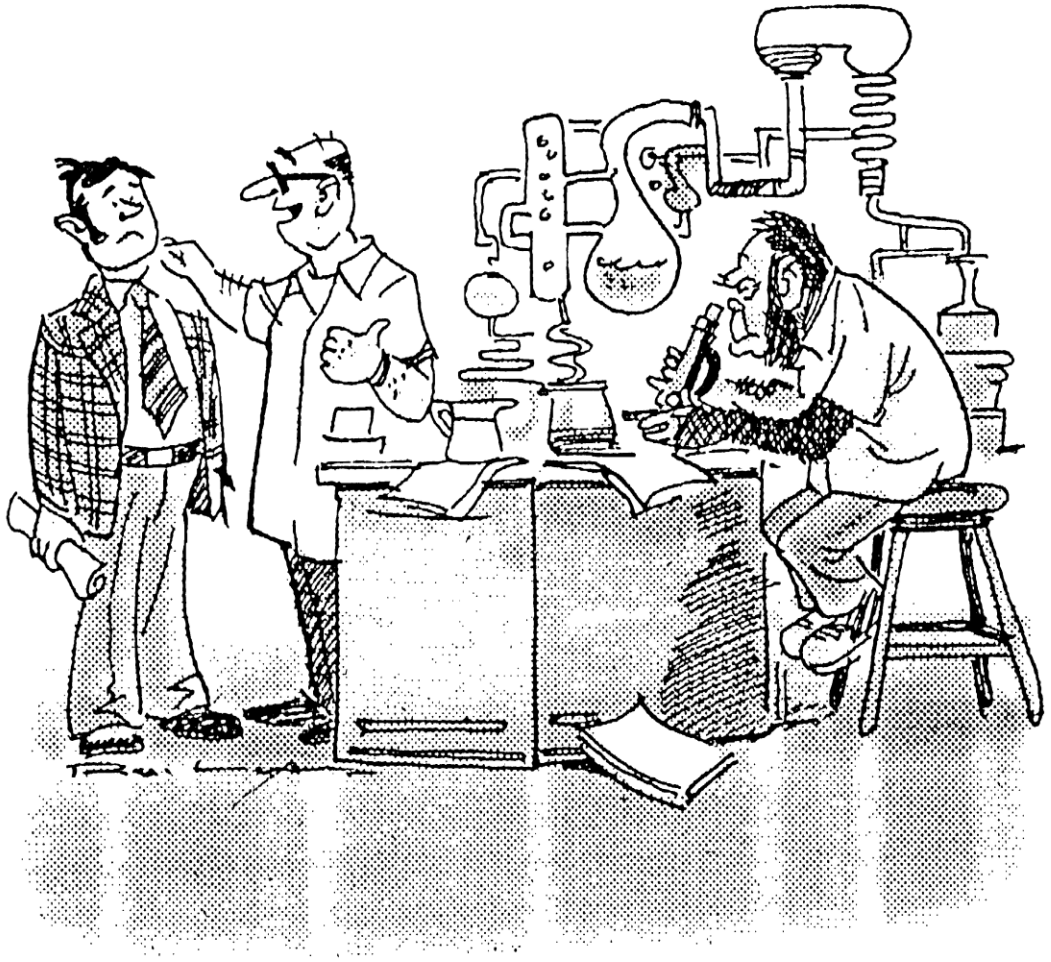
To make double stranded RNA (dsRNA), equal quantity of + and - strand RNA was taken in a tube and heat denatured at 65°C for 10 min., followed by incubation at room temperature for 20 min. and then at 37°C for 8 hrs. To remove single stranded RNA, dsRNA was treated with RNase at 37°C for 2 hrs. and ethanol precipitated and quantified spectrophotometrically. Finally, dsRNA solution was filter sterilized using 0.22μ syringe filter millex (Millipore).

2.16 Transfection of dsRNA

BmN cells were allowed to grow up to 2 x 10^6 cells in each well of a 6 well plate. Media was removed and replaced with 0.75 ml of fresh TC 100 medium containing 10% FBS and 50μg/ml kanamycin. dsRNA was taken in 1.5ml polypropylene tube to which 0.75 ml of transfection buffer (25mM HEPES, pH 7.1, 140mM NaCl, 125mM $CaCl_2$) was added and mixed well. dsRNA solution with transfection buffer was added dropwise to the medium in the well during which white calcium phosphate

precipitate would form. Plates were then incubated at 27°C for 8 hr. After incubation, calcium phosphate with medium was removed, washed once with fresh medium and then the cells were incubated with 2 ml of fresh medium in each well at 27°C for 72 hrs.

3 Results



Conditions are excellent here, young man — he was brought here for experimental purpose and today he heads the department!

3.1 BeK gene identification and analysis

PCR amplification of bacteria-challenged λgt-10 *Bombyx* fat body cDNA library using anti-sense degenerate primer P1 and λgt 10 forward or reverse primer as sense primer resulted in seven specific products of approximately 200, 650, 750, 1100, 1150, 1200 and 1300 base pairs (bp). These fragments were sub-cloned and sequenced. The 1300 bp fragment, which showed 30% similarity to human PKR, was used for screening *Bombyx* fat body cDNA library. The tertiary screening yielded six positive clones out of which two were sub-cloned and sequenced.

The 1938bp sequence obtained from a sub-cloned insert consists of a 5′ non-coding region of 144bp, an open reading frame of 1741 nucleotides corresponding to 579 amino acids and a 3′ untranslated region of 54bp (Fig. 1). The deduced amino acid sequence has a amino-terminal regulatory domain of 138 amino acid (a.a.) and a kinase domain of 441 a.a. A characteristic insert sequence of 115 amino acids is present between the kinase subdomains IV and V (Fig.2).

Sequence comparison of catalytic kinase domain with other known eIF2α kinases showed 29% identity to Yeast GCN2, 24% identity to *Drosophila* GCN2, 23% identity to *Drosophila* PEK, 30% identity with all known HRI (human, mouse, rat and rabbit) and 26% identity to mammalian PKR at protein level (Fig. 2). NH_2 terminal regulatory domain of 138 amino acids did not show any significant similarity to known sequences when homology search was done against Genbank sequences. Kinase insertion

```
                                                    ggt acc atg gag cga agg atg gca
aaa taa aaa tgc att ttg cga atc gtg tga atg cat cag taa aat ggc att ggc ctg act
tct acg aat tga aaa tct taa aaa cat caa att ttg att gta gtt aac aat taa taa aaa
145/1                                           175/11
atg gat aaa cat agc caa gac aaa tgg aaa gca ttg gcg aca gtg aaa tcc ttc gat tta
 M   D   K   H   S   Q   D   K   W   K   A   L   A   T   V   K   S   F   D   L
205/21                                          235/31
ggc ata tcg gct agt cac cat gag tca ttc gta cag cag agt aga caa cag att gat gtc
 G   I   S   A   S   H   H   E   S   F   V   Q   Q   S   R   Q   Q   I   D   V
265/41                                          295/51
atc aat gcc cca acc aca acc cca atc agc ctc cta gtt caa tca ctc gtt aaa caa ttg
 I   N   A   P   T   T   T   P   I   S   L   L   V   Q   S   L   V   K   Q   L
325/61                                          355/71
tgt tca ttg tta caa aaa gac agt att ata gcc aat cag ctt tac aac aaa ata tgt gag
 C   S   L   L   Q   K   D   S   I   I   A   N   Q   L   Y   N   K   I   C   E
385/81                                          415/91
aaa ctt cat agt atg aac ttg att gac aat tcg tat gct atg gga gag ttt gaa gct atg
 K   L   H   S   M   N   L   I   D   N   S   Y   A   M   G   E   F   E   A   M
445/101                                         475/111
aga agt caa tat cag aga gcc ttg tat cag ctt gtg acg gtc gcc agc gga aca gag ata
 R   S   Q   Y   Q   R   A   L   Y   Q   L   V   T   V   A   S   G   T   E   I
505/121                                         535/131
ccg ata ata ctg cca gca act tgg cct ata gtt cag ccg tct gga ctt gaa tgg tca aga
 P   I   I   L   P   A   T   W   P   I   V   Q   P   S   G   L   E   W   S   R
565/141                                         595/151
tac cat aga gaa ttt gag gag ctc tac ttc ata gct ggt gga ggc ttc ggg agc gtt ttc
 Y   H   R   E   F   E   E   L   Y   F   I   A   G   G   G   F   G   S   V   F
625/161                                         655/171
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685/181                                         715/191
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 S   D   V   D   S   I   M   S   H   L   S   E   V   K   T   I   A   S   L   N
745/201                                         775/211
cac ccg aac ata gta aac tat aag gca gcg tgg ctc gaa ccc atg ata gag tct aca gtt
 H   P   N   I   V   N   Y   K   A   A   W   L   E   P   M   I   E   S   T   V
805/221                                         835/231
aaa aag aaa ggc aaa tat caa atg gac acc gac agt gac gag ttt tca tta agc tct gac
 K   K   K   G   K   Y   Q   M   D   T   D   S   D   E   F   S   L   S   S   D
865/241                                         895/251
ctc ata tca tca gca cat ccc aac gta ata aat tca ttc aag act cac aac tcc aag gag
 L   I   S   S   A   H   P   N   V   I   N   S   F   K   T   H   N   S   K   E
925/261                                         955/271
ttg acc aaa aac aag agc ctg tcc gac ttc att att tcc ttc aag aac tcc aac agc ttc
 L   T   K   N   K   S   L   S   D   F   I   I   S   F   K   N   S   N   S   F
985/281                                         1015/291
gag aac ttg aac agt tcg aac gaa gaa ctg cag gtt tct gac agc gat gac gag tcc gtt
 E   N   L   N   S   S   N   E   E   L   Q   V   S   D   S   D   D   E   S   V
```

Fig. 1 continued

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1045/301                                                    1075/311
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 S   Q   E   E   N   A   V   C   N   L   F   S   S   K   E   Y   E   N   C   S
1105/321                                                    1135/331
cgt ata aac ctt aaa tgg gcc acc ttg ttc atc cag atg acg ttc tgc cag caa acc ttg
 R   I   N   L   K   W   A   T   L   F   I   Q   M   T   F   C   Q   Q   T   L
1165/341                                                    1195/351
aag cag tgg ctt gac gag cgc aac aac cat atg tca gtg tcg cga aaa ggt tcc gac gat
 K   Q   W   L   D   E   R   N   N   H   M   S   V   S   R   K   G   S   D   D
1225/361                                                    1255/371
ttc act ctg ccg tta cat gtg tgt gaa tct cca att gag gct aaa gat atc acg ttc cca
 F   T   L   P   L   H   V   C   E   S   P   I   E   A   K   D   I   T   F   P
1285/381                                                    1315/391
gcg agt att aat cac att gac ctg ctg ata gac atg ttc acg cag ctg gtg cgt ggt ctc
 A   S   I   N   H   I   D   L   L   I   D   M   F   T   Q   L   V   R   G   L
1345/401                                                    1375/411
cat tat ata cat tcc cgt ggt att atc cac cac gac ata aag ccg agt aat gta ttc gtc
 H   Y   I   H   S   R   G   I   I   H   H   D   I   K   P   S   N   V   F   V
1405/421                                                    1435/431
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 A   P   H   E   G   G   L   L   V   Q   L   G   D   F   G   L   A   C   P   L
1465/441                                                    1495/451
cag cag tcc cat agt gga ttg gca ctc ggt aca cat atg tat gct gca ccg gag caa ctg
 Q   Q   S   H   S   G   L   A   L   G   T   H   M   Y   A   A   P   E   Q   L
1525/461                                                    1555/471
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 D   G   Q   C   N   P   K   S   D   M   Y   S   L   G   I   I   L   L   E   L
1585/481                                                    1615/491
gta gaa cca ttc gtg act gat atg gaa cga gtg aaa act atc acc gac ctc cgc aaa ggt
 V   E   P   F   V   T   D   M   E   R   V   K   T   I   T   D   L   R   K   G
1645/501                                                    1675/511
cag att cca gct cac ctc act gcc aac tac cca aaa att gct cat atc atc ggc aaa ctg
 Q   I   P   A   H   L   T   A   N   Y   P   K   I   A   H   I   I   G   K   L
1705/521                                                    1735/531
gtg caa agg aag ccc agc aag aga ctg gac acg gcc cag ctg ctg gag gaa ctc aag acc
 V   Q   R   K   P   S   K   R   L   D   T   A   Q   L   L   E   E   L   K   T
1765/541                                                    1795/551
ctg gcc gag aat aaa gat gac acg atc aga tcg ttg cga gag gag ctc gct gcg aaa gat
 L   A   E   N   K   D   D   T   I   R   S   L   R   E   E   L   A   A   K   D
1825/561                                                    1855/571
gac gaa ata gct aaa ctc aag atg atg ctg gcg aac ttg aat ttt aaa tct tca gtg tga
 D   E   I   A   K   L   K   M   M   L   A   N   L   N   F   K   S   S   V   *
ctg agt gtg tgt gtg act act taa taa att cta gta tta act ttt cca tgg tac
```

Fig. 1 Translated sequence of *Bombyx* eIF2α Kinase (BeK) showing complete ORF with 5' untranslated region and 3' tail. Lysine amino acid at 174th position mutated to arginine (aga) to generate BeKm is underlined. Rectangular box indicates the region used for designing degenerate primer P1, with orientation in the direction of the arrow. * indicates the stop codon. This sequence is submitted to Genbank under the accession number U87236

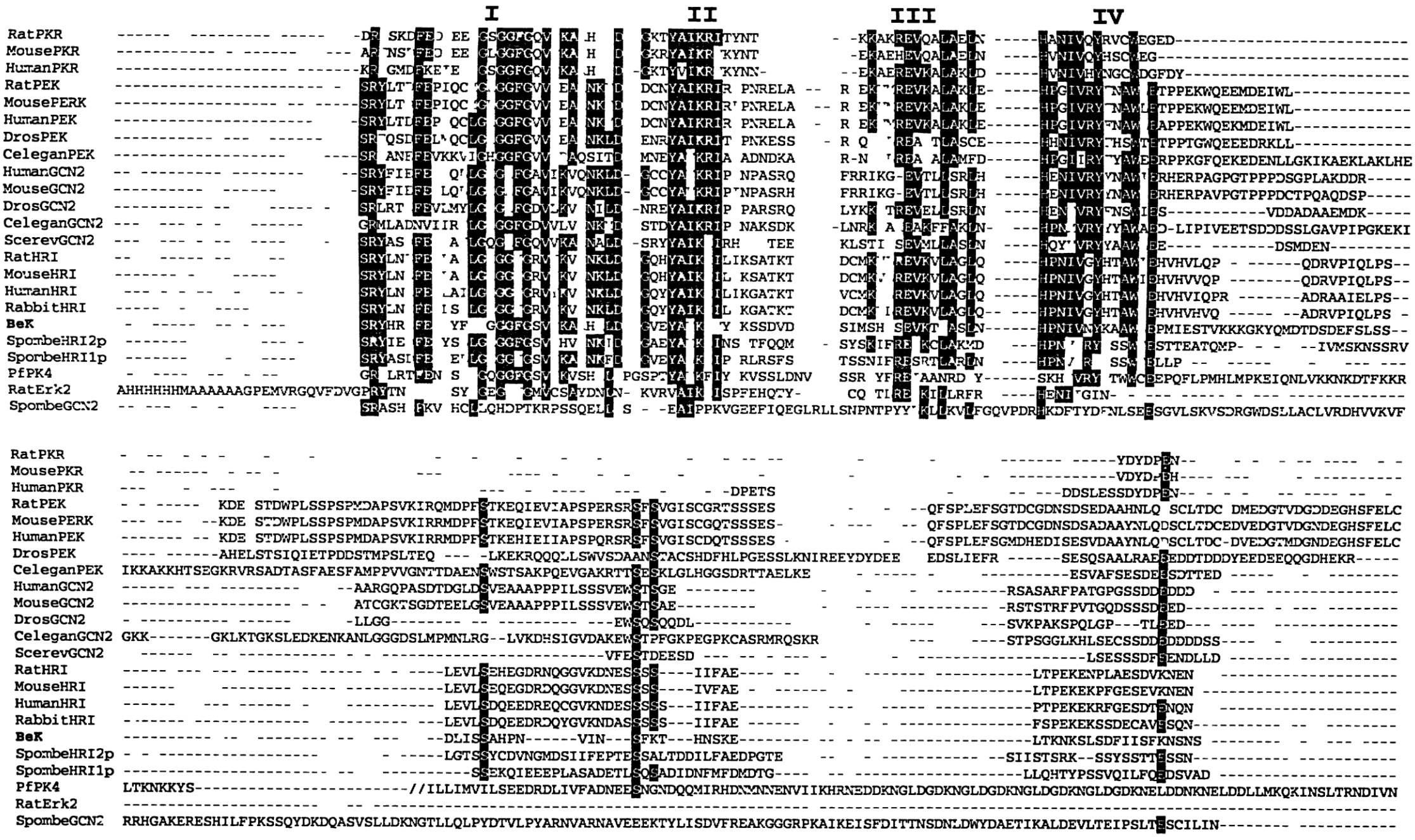


Figure 2 continued...

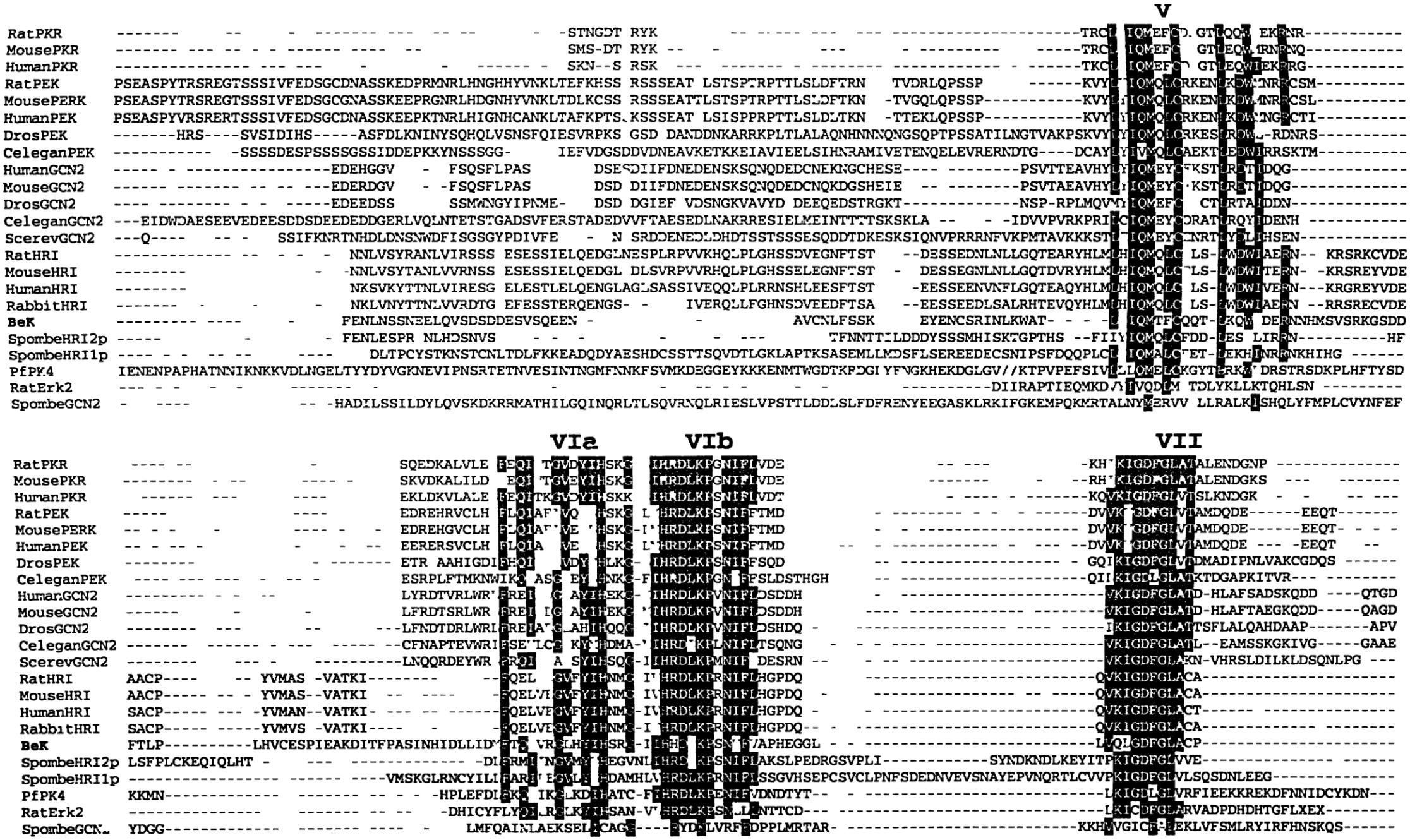


Figure 2 continued...

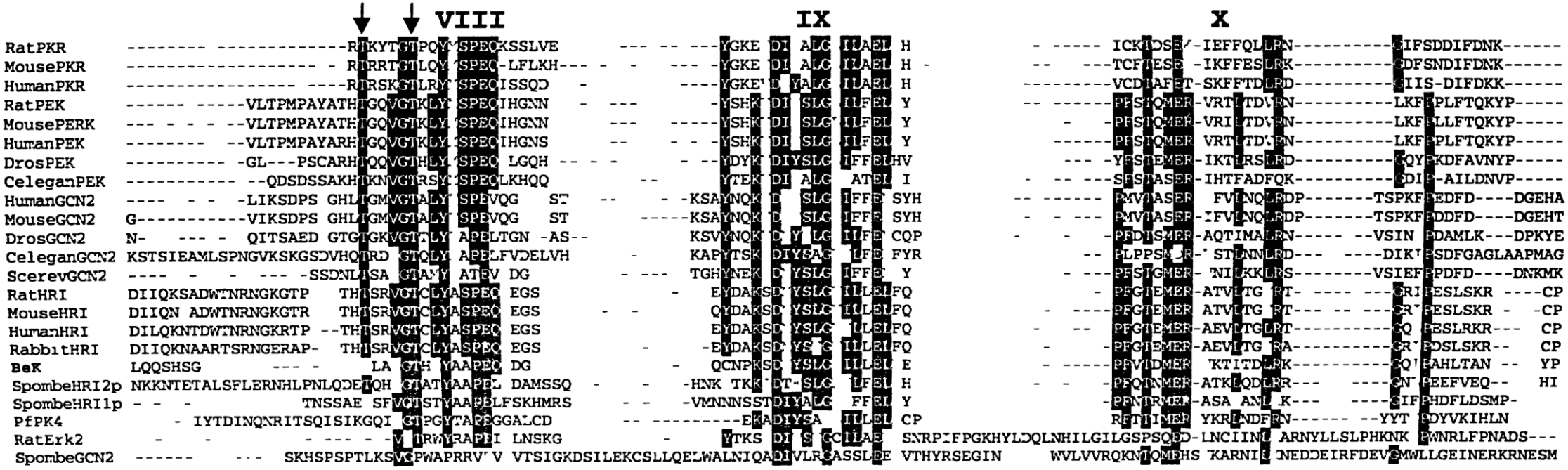


Figure 2 continued...

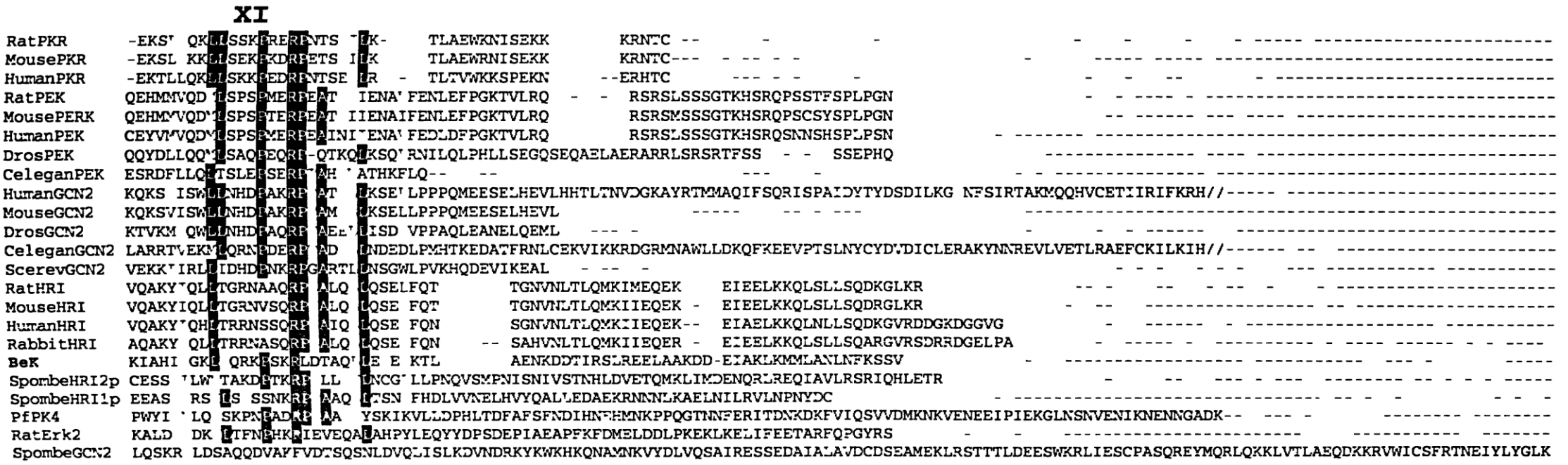


Figure 2. Alignment showing kinase domains of all the eIF2α kinases reported. Alignment is generated by CLUSTAL W and blocked using BOXSHADE. – indicates gaps introduced for global arrangements. Dros is *Drosophila*, Celegan is *C. elegans*, Scerev is *S. cereviseae* and Spombe is *S. pombe*. PfPK4, Human GCN2 and *C. elegans* GCN2 had long stretches of non-overlapping sequences with other kinases, so the regions where there were no overlaps were removed from the alignment for a better view, which is indicated by //. Roman numerals above the alignment indicate respective subdomains. Arrows above the alignment indicate the autophosphorylation sites, Thr-446 and Thr-451 of Human PKR (Thr-882 and Thr-887 of yeast GCN2) which is necessary for kinase activity.

domain of 115 amino acids was also unique, which along with regulatory domain may suggest a distinct function for this kinase.

When kinase domains of all known eIF2α kinases were aligned, many similar residues shared by the members of this protein family were found. The LF/Y/HHQMQ/EL/F/YC, necessary for eIF2α phosphorylation was conserved in all the sequences. In this motif, all PEK proteins in addition to GCN2 (except *S. cerevisiae* and *C. elegans* GCN2) possess Y after L, whereas all PKR possess F residue. All PKR and GCN2 proteins have E residue after M, whereas PEK and HRI except for *S. pombe* Hri1p and PfPK4 have Q. BeK protein has Phenylalanine (F) residue after Leucine (L) and a unique residue Threonine (T) after Methionine (M). In addition BeK does not have Threonine residue corresponding to Thr-446 of human PKR and Thr-882 of yeast GCN2 which are shown to be necessary for auto-phosphorylation and complete kinase activity (Fig. 2). Insert domains are conserved within different classes of eIF2α kinase but not between the classes. The length of insert domain is also conserved among different class, the shortest being the PKR and longest being PEK. PfPK4 possess exceptionally long insert, the largest among eIF2α kinases. BeK had a unique stretch of 21 amino acids between V and VIa subdomains (Fig. 2). The sequence of *S. pombe* GCN2 was completely different from other sequences. This protein did not have the conserved LXIQMXXC domain indicating that this might be a pseudogene or possess different function. A single ACPLQQ HRM sequence exist in VII subdomain of BeK.

We constructed a phylogenetic tree using Rat Erk2 protein as outgroup to check the phylogenetic distribution of eIF2α kinases. BeK gets clustered with the HRI class of proteins (Fig. 3). As it can be seen in the tree, each class of kinases gets clustered under a separate group, PEK goes into a separate cluster, similarly, HRI, PKR and GCN2. PKR and PEK cluster together under a larger group indicating that they share some amount of common lineage. PfPK4 from *Plasmodium falciparum* comes out as a basal element out of all the clusters (Fig. 3). *S. pombe* GCN2 that is different from other sequences as evident by lack of the conserved LXIQMXXC also comes out as a basal element.

3.2 BeK expression in different tissues and developmental stages

To check for tissue specific expression of BeK, RT-PCR of total RNA from different tissues was done. BeK was found to be constitutively transcribed in all the tissues during different stages of development we tested (Fig. 4a). The tissues we tested were fatbody, midgut, gonad, muscle, silkgland, epidermal tissue, malpighian tubule, cardiac system and 4 day old 4th instar larvae and 3 day old pupae. Significantly higher level of BeK expression was seen in gonads as compared to all other 5th instar larval tissues. As gonadal cells undergo extensive cell division and differentiation into ovary or sperm, it prompted us to test BeK expression in embryonic stages. Interestingly, we found transcripts in embryonic stages of 40, 56, 67 and 78 hr. post oviposition and also in freshly hatched larvae (Fig. 4b).

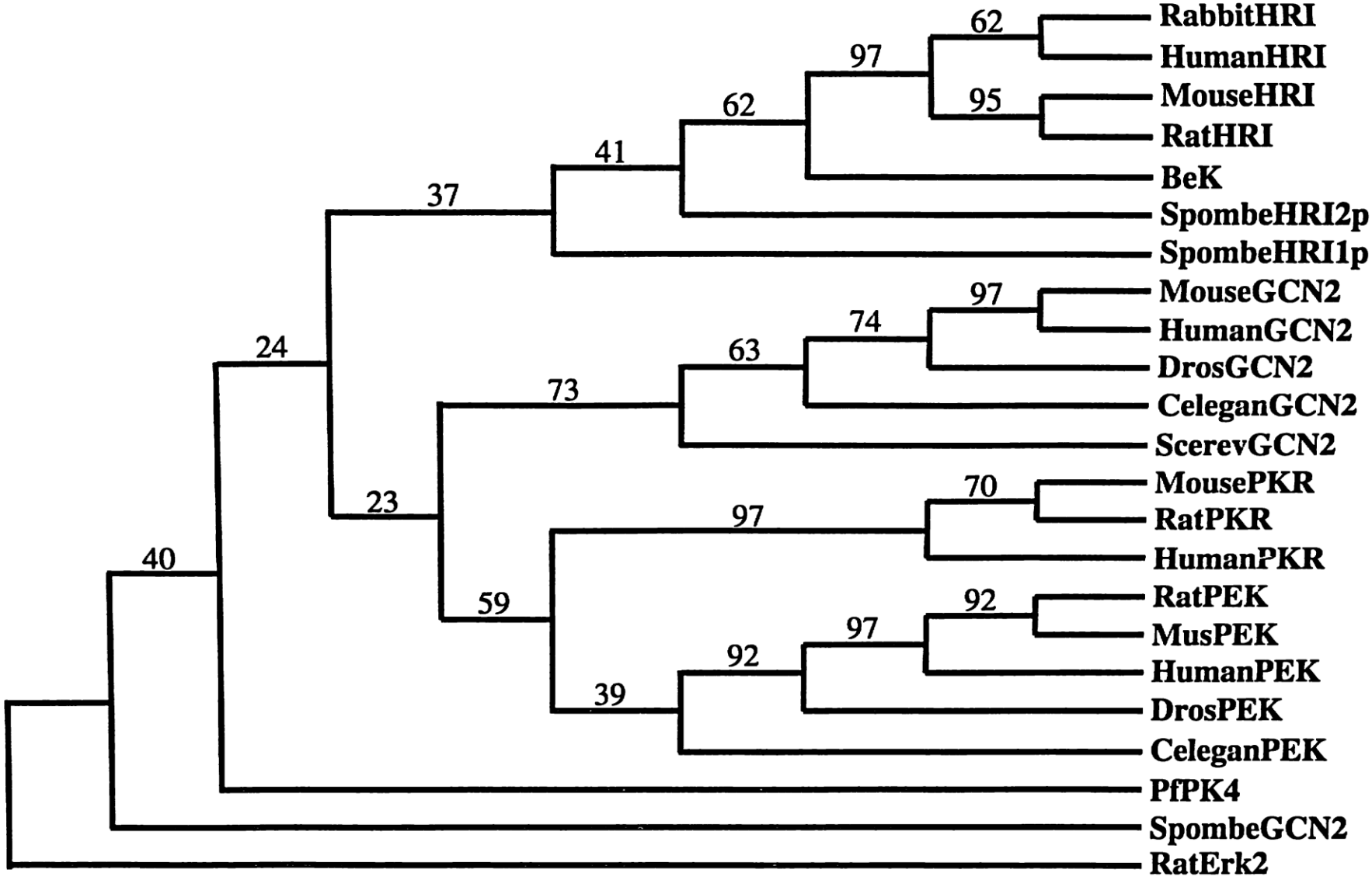


Figure 3. Phylogenetic tree generated using protein parsimony of PHYLIP. This tree is rooted by an outgroup RatErk2. The numbers above branching points show the bootstrap values indicating the number of occurrences of that particular branch out of 100 trees.

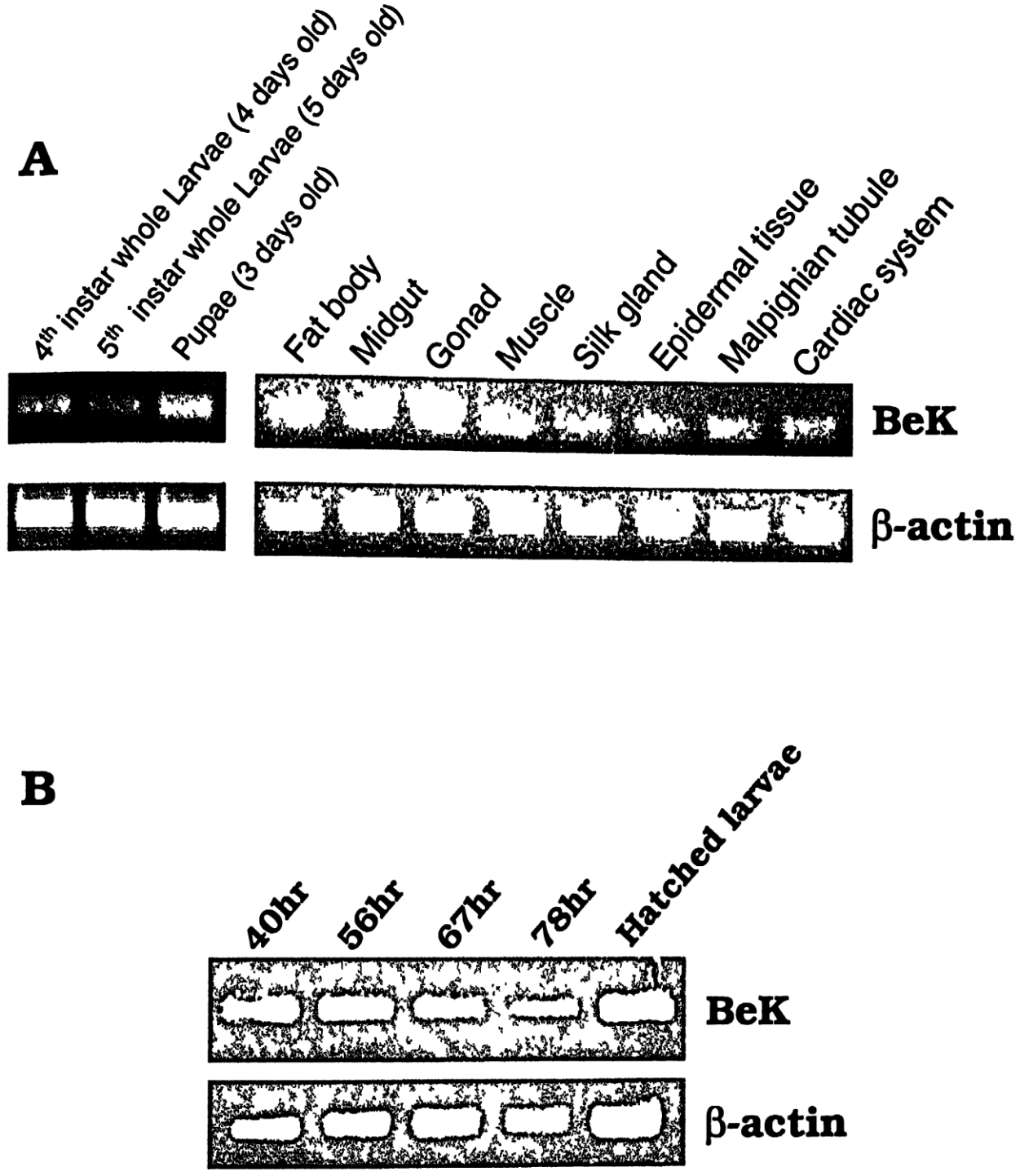


Figure 4. RT-PCR analysis of BeK transcript. (A) From whole larvae and pupae, and from different 5 days old fifth instar larval tissues (fat body, midgut, gonad, muscle, silk gland, epidermal tissue, malpighian tubules and cardiac system). (B) From various time points of embryo development.

Expressions in embryonic stages were higher than the levels expressed in the larval tissues.

3.3 ORF confirmation and immunoblot

To confirm the putative coding region, BeK was expressed in *E. coli* as a maltose binding protein (MBP) fusion protein. The resulting recombinant protein was purified using amylose resin and a single protein band with molecular mass of 110 kDa on SDS-PAGE was obtained (Fig. 5a). As MBP is of 42 kDa size, the apparent molecular mass of BeK is about 68 kDa. This result is in good agreement with the theoretically calculated BeK molecular mass of 65,238 Da. The recombinant MBP-BeK was specifically recognized by antiserum directed against NH_2 terminal peptide of BeK, whereas pre-immune serum did not recognize the recombinant BeK (Fig. 5b), confirming the BeK protein expression and ORF integrity.

3.4 Over-expression of BeK in S2 cells and kinase activity

S2 cells were used for biochemical study as plasmid based stable expression system was not available for BmN cells. We established transformed *Drosophila* Schneider S2 cell line for wild (BeKw) and mutant BeK (BeKm) under the control of metal inducible promoter, which over-expressed a 65 kDa protein as recognized by partially purified anti BeK antiserum. BeK expression was detected only in $CuSO_4$ induced cells, whereas no protein was detected in cells that were not induced for expression (Fig. 6a). Out of the 4 different transformed cell lines,

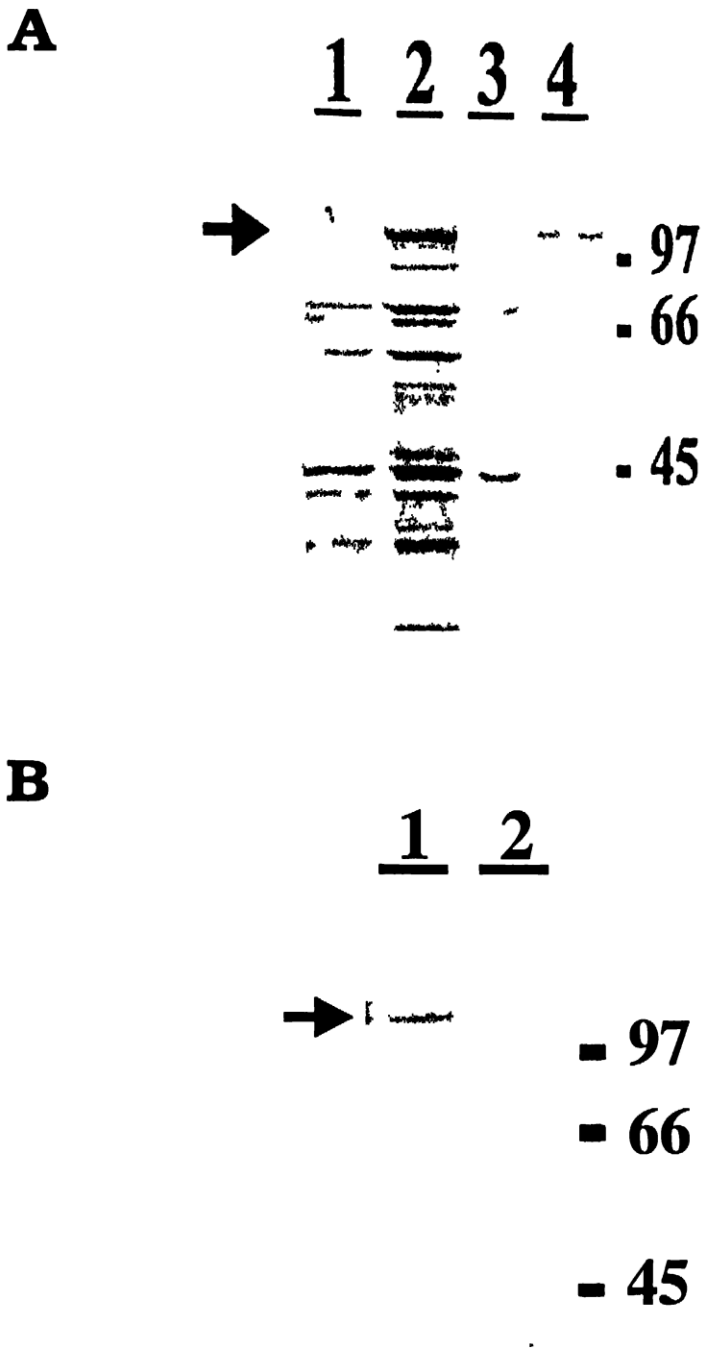


Figure 5. MBP-BeK fusion protein purification and detection. (A) Coomassie blue stained PAGE showing MBP-BeK protein purification. Lanes 1 to 4 show uninduced sample, induced sample, wash eluate and purified protein respectively. Arrow indicates MBP-BeK of 110 kDa. (B) Blot showing MBP-BeK fusion protein detection by anti BeK antiserum. Lane 1 is hybridized with anti BeK antiserum and lane 2 with preimmune serum. Arrow indicates the fusion protein of 110 kDa.

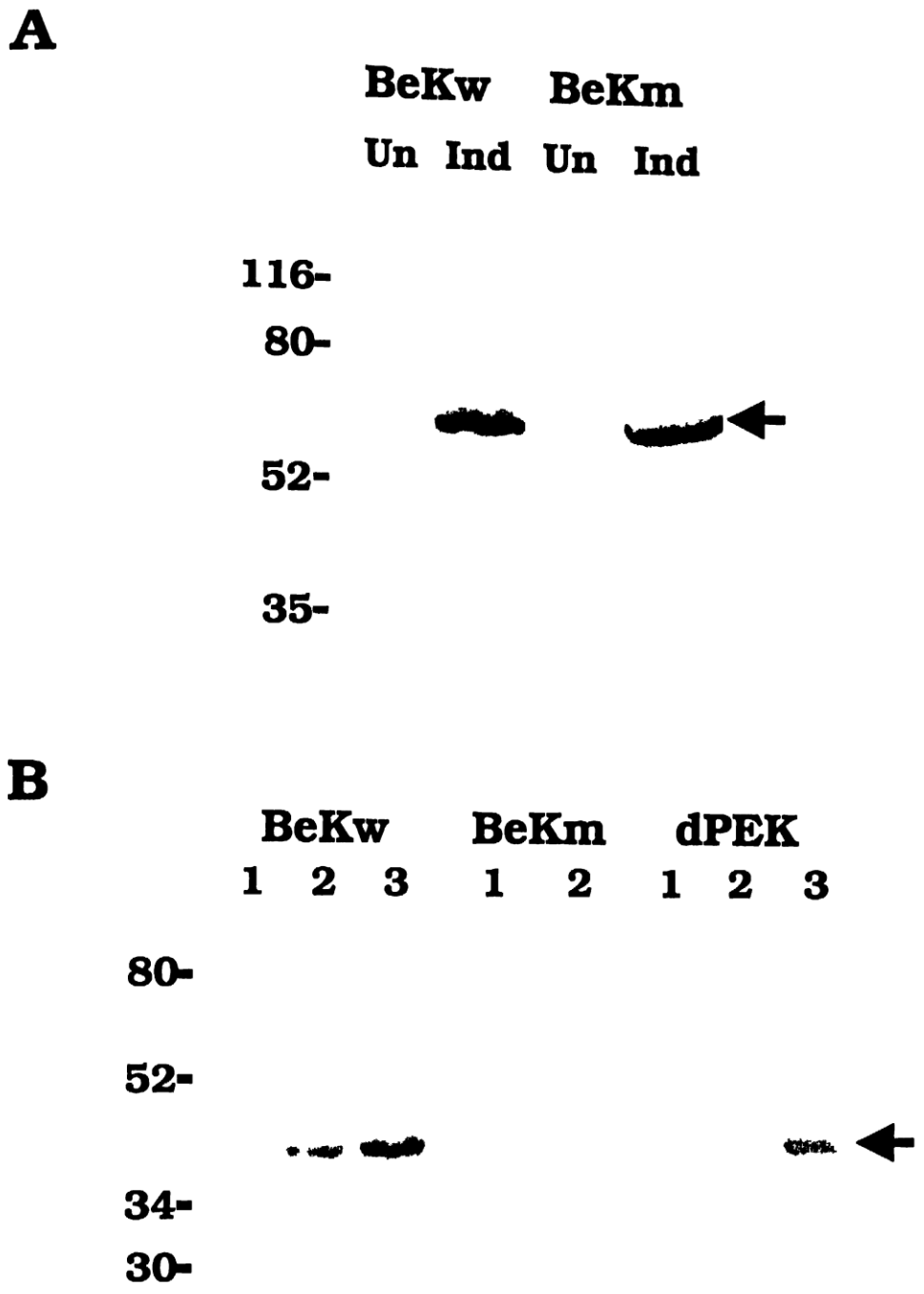


Figure 6. Detection of over-expression and kinase activity of BeKw and BeKm. (A) Detection of BeKw and BeKm protein expression in transformed *Drosophila* Schneider cells. Un is uninduced and In is induced for expression by 0.5mM CuSO4. Arrow indicates 65 kDa BeKw and BeKm protein. (B) Kinase activity of BeK wild (BeKw), BeK mutant (BeKm) and *Drosophila* PEK (dPEK) was performed using eIF2α as substrate. Lane 1 is uninduced cells, 2 and 3 are induced cells while lane 3 cells were exposed to heat shock. Arrow indicates the phosphorylated form of eIF2α subunit detected by phosphospecific eIF2α antibody.

established for BeKw, only one cell line showed protein expression, which was used for further experiments. BeKw and BeKm transformed cells were induced for 48 hrs. for optimum expression. We also transformed S2 cells with *Drosophila* PEK (dPEK) to obtain stable over-expression of dPEK, which was used as a positive control in *in vitro* kinase reactions. dPEK transformed cells were induced for 24 hrs. but prolonging time of expression did not produce detectable quantity of dPEK expression. After inducing with $CuSO_4$ dPEK over-expression could not be detected on western blot.

To determine whether BeK can phosphorylate the substrate, eIF2α subunit, which is the most common substrate of eIF2α family kinases, *in vitro* kinase reaction was done with hexa histidine tagged recombinant *Drosophila* eIF2α subunit as substrate. BeKw, BeKm and dPEK was immunoprecipitated from the respective transformed *Drosophila* S2 cells. One flask each of BeKw and dPEK were treated with heat shock before immuno-precipitation and the Kinase reactions were performed using the immunoprecipitates. Phosphorylation at Ser-50 (Same as Ser-51 in mammals) of eIF2α subunit was detected using phospho-specific antibody that recognizes only eIF2α subunit phosphorylated at Ser-50. Phosphorylation of eIF2α was seen only in immunoprecipitate from induced BeKw and dPEK, whereas immunoprecipitates from uninduced cells did not show any phosphorylation (Fig. 6b). As mutant BeK is a non-functional kinase, phosphorylation was not detected in reactions where

immunoprecipitate from mutant BeK cells was used. A slight increase in phosphorylation levels of cells exposed to heat shock was observed indicating increased activity of the kinase under stress (Fig. 6b).

3.5 Autophosphorylation

Autophosphorylation, which is an essential step for activation of eIF2α kinase family members, was checked using BmN cells. BeK was immunoprecipitated with anti BeK immune serum and pre-immune serum and used for autophosphorylation reaction. Immune complex with BeK antiserum gave a single phosphorylated band whereas no specific band was observed in immune complex with pre-immune serum (Fig. 7). Autophosphorylated BeK migrated slower than the transfected BeK with a molecular mass of 80 kDa.

3.6 Native eIF2α phosphorylation in S2 and BmN cells under normal and stressed conditions

To identify the influence of native eIF2α phosphorylation under different stress inducers like temperature, salt, bacterial cell wall, Thapsigargin, β-1,3-glucan and H_2O_2. After applying stress, equal amount of total protein was taken and resolved by SDS-PAGE and immunoblotted for detecting the phosphorylated form of eIF2α subunit. A basic level of phosphorylation was found in control cells, which were unexposed to stress (Fig. 8a, lane 1). Except heat shock and osmotic stresses, others did not result in increased eIF2α phosphorylation (Fig. 8a).

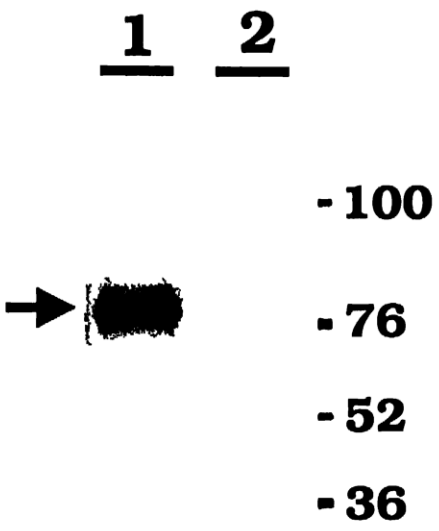


Figure 7. Autokinase and kinase activity of endogenous BeK from *Bombyx* BmN cell line. Lane 1 is the immunoprecipitate from anti BeK immune serum and lane 2 is immunoprecipitate from pre-immune serum. Arrow indicates 80 kDa autophosphorylated BeK.

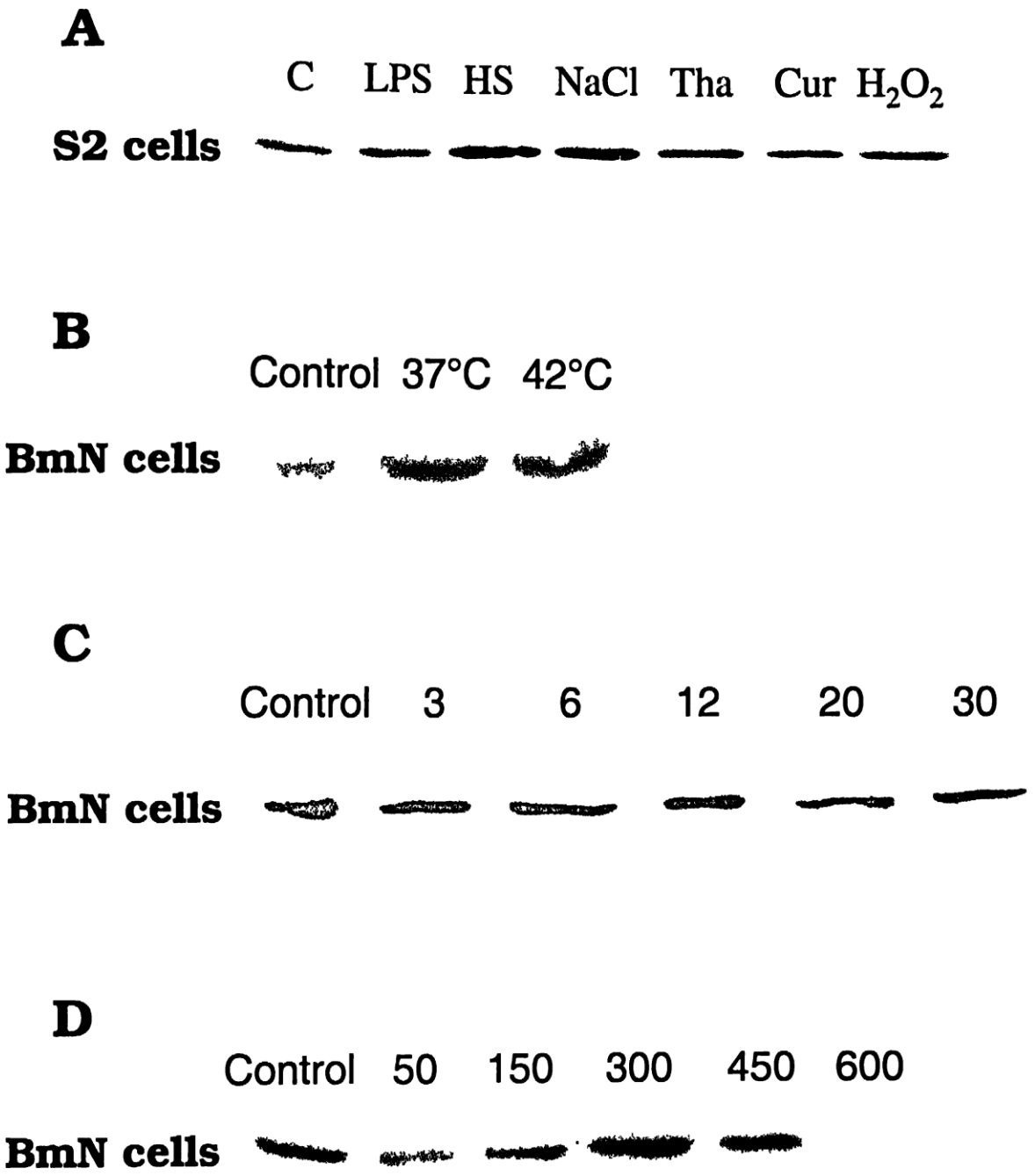


Figure 8. Native eIF2α phosphorylation status under different stresses in *Drosophila* S2 and BmN cells. (A) S2 cells under different stress conditions. C is total protein from control cells, other lanes are cells treated with Lipopolysaccharide (LPS), heat shock (HS), NaCl, thapsigargin (Tha), curdlan/β, 1-3, glucan (Cur) and H_2O_2. (B) BmN cells exposed to 37°C and 42°C (C) BmN cells exposed to bacterial cell wall stress with varying amounts of LPS shown in μgs. (D) BmN cells exposed to osmotic stress with the varying concentration of NaCl shown in mM.

This indicates that these two stresses were very efficient in activating eIF2α kinases and preventing protein translation initiation.

There is no report in the literature on estimation of eIF2α phosphorylation levels under different stress conditions. Thus, for standardizing the levels of stress and duration of exposure, we exposed BmN cells to different stresses and checked for phospho-eIF2α levels in relation to control. Significant increases in the phosphorylation levels were observed in cells exposed to 37°C and 42°C for 1 hr (Fig. 8b). For further experiments, treatment at 37°C for 1 hr. was used as standard for heat shock stress induction. When cells were exposed to varying concentrations of LPS for 30 min. there was a moderate increase in phosphorylation levels with 6 and 12 µg compared to the control (Fig. 8c). For further experiments 10 µg of LPS for 30 min. was used as standard for inducing bacterial cell wall stress. In a similar way when cells were exposed to osmotic stress with varying concentrations of sodium chloride, a significant increase in phosphorylation was observed only when 300 mM NaCl was used. Lower concentrations failed to induce high phosphorylation, whereas higher concentrations induced extensive cell lysis (Fig. 8d). For further experiments, 300 mM NaCl for 30 min. was used as standard to induce osmotic stress.

3.7 Influence of stress on Kinase activity

To identify activation of BeK under different stress conditions, S2 cells transformed with BeKw were exposed to different stress inducers.

Following stress induction, BeKw was immunoprecipitated and kinase assay was done to estimate its activity. To avoid relative difference in lysed protein, we started with equal concentration of protein. As shown earlier (Fig. 6b), over-expression of BeKw without any stress causes eIF2α phosphorylation which is obtained in this experiment also (Fig. 9a, lane 2). BeKw showed 5-fold higher activity under heat shock, 3-fold higher activity under osmotic stress and a 2-fold higher activity under bacterial cell wall stress (Fig. 9b). Other stress conditions like Thapsigargin, β-1,3-glucan and H_2O_2 used did not show any significant increase in activity. This increase in activity also corresponds to the increase in phosphorylation of native eIF2α subunit, as shown on the lower blot (Fig. 9a, BeKw-eIf*). Densitometric readings of each band from three independent experiments were taken and plotted on a histogram (Fig. 9b). The average value of the three independent experiments was plotted on Y-axis in fold increase of activity and only the stress factors showing significant increase were plotted on X-axis. The percentage activity was expressed in terms of the optical density of the bands obtained as compared to the control band, which was considered as one fold. Control was obtained by not exposing the cells to any stress (Fig. 9b).

3.8 Inhibition of kinase activity by baculoviral PK2

The inhibitory effect of baculoviral protein, PK2 was checked *in vitro* using the immunoprecipitated BeKw protein. To the immunoprecipitates from the transformed *Drosophila* S2 cells different concentrations of BmPK2 and AcPK2 proteins were added separately in addition to other

components of normal kinase reaction. Initially kinase reactions were done using following controls: AcPK2 and eIF2α subunit, BmPK2 and eIF2α subunit, immunoprecipitate without eIF2α subunit. The results showed that PK2 protein (truncated eIF2α kinase consisting of V to XI subdomains) alone could not phosphorylate eIF2α subunit. Kinase reactions done using 10μg of PK2 protein showed that PK2 reduced BeK activity by 50% (Fig. 10a). To check for the concentration dependent inhibition of BeK activity, we used different amount of PK2 protein in *in vitro* kinase reaction. The concentrations used were 1, 3, 8 and 15μg of recombinant PK2 protein. 15μg of BSA was used as a control to maintain the protein load. The bands obtained showed a clear inhibition of *in vitro* kinase activity (Fig. 10). Control (normal kinase reaction) and control plus BSA had same activity (Lanes 1 and 2, Fig. 10b), whereas the reactions with PK2 proteins showed a dose dependent inhibition of kinase activity (Lanes 3 to 10, Fig. 10b). The average optical density measurements of these bands from two independent experiments are shown in the graph (Fig. 10b). The density of band obtained in control plus BSA reaction was considered as 100% activity and the other bands were compared to this value to derive the percentage activity. This result showed a concentration-based inhibition of kinase activity. We observed that, increasing concentration of PK2 from 8 to 15μg did not increase the inhibitory activity implicating saturation (Fig. 10c).

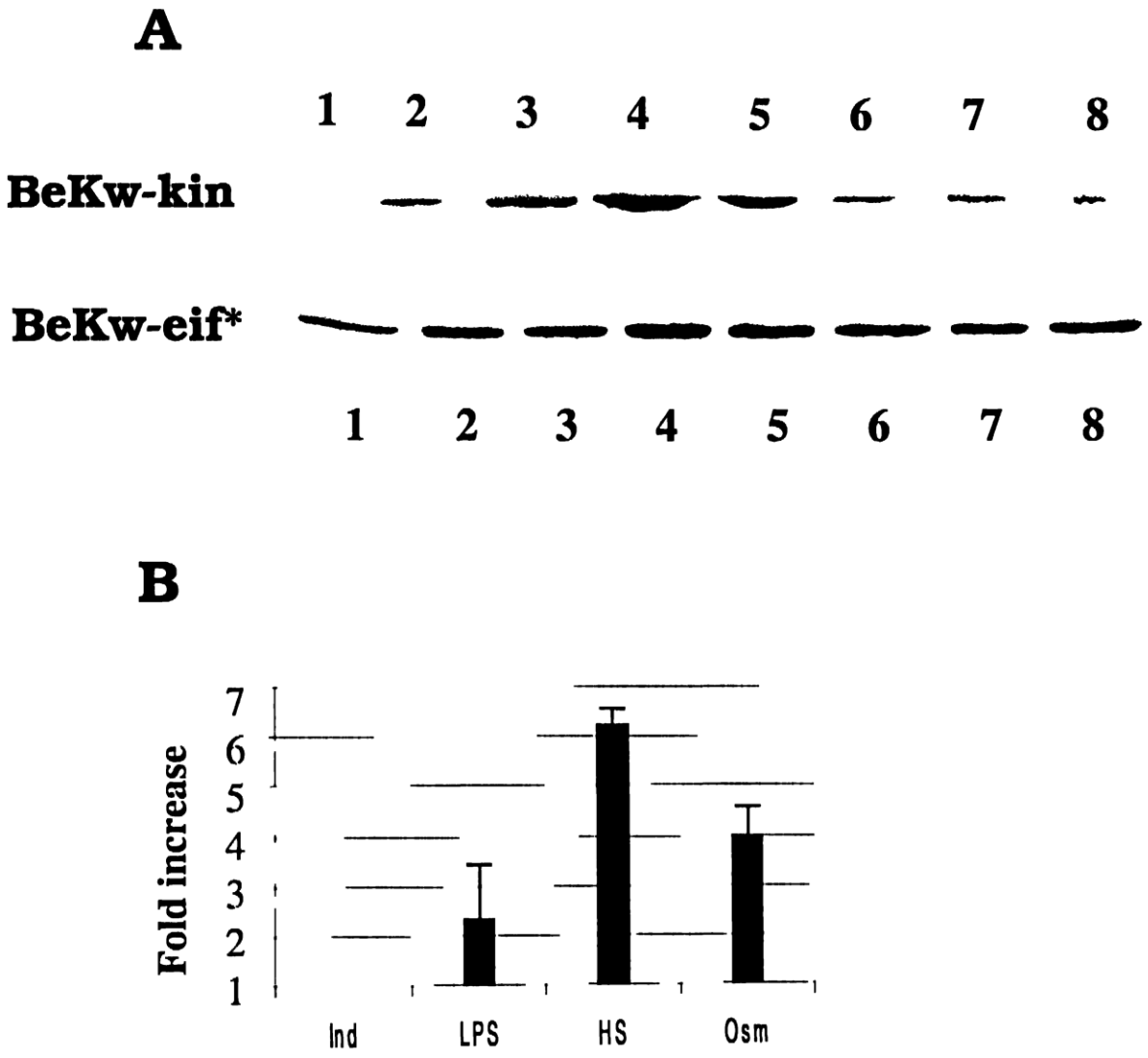


Figure 9. Kinase activity under different stress conditions. (A) BeKw-Kin indicates the kinase activity of BeKw protein under different stress conditions in S2 cells. BeKw-eif* blot indicates the native eIF2α subunit phosphorylation in the same cells as above. Lane 1 indicates cells which were not induced for expression and Lane 2 is from induced cells but not stressed. Lanes 3 to 8 are cells treated with LPS, Heat shock, NaCl, Thapsigargin, Curdlan/β, 1-3, glucan and H_2O_2. (B) Histogram showing the fold increase in activity of BeKw (mean of three independent experiments are plotted) under LPS, Heat shock (HS) and Osmotic stress (Osm).

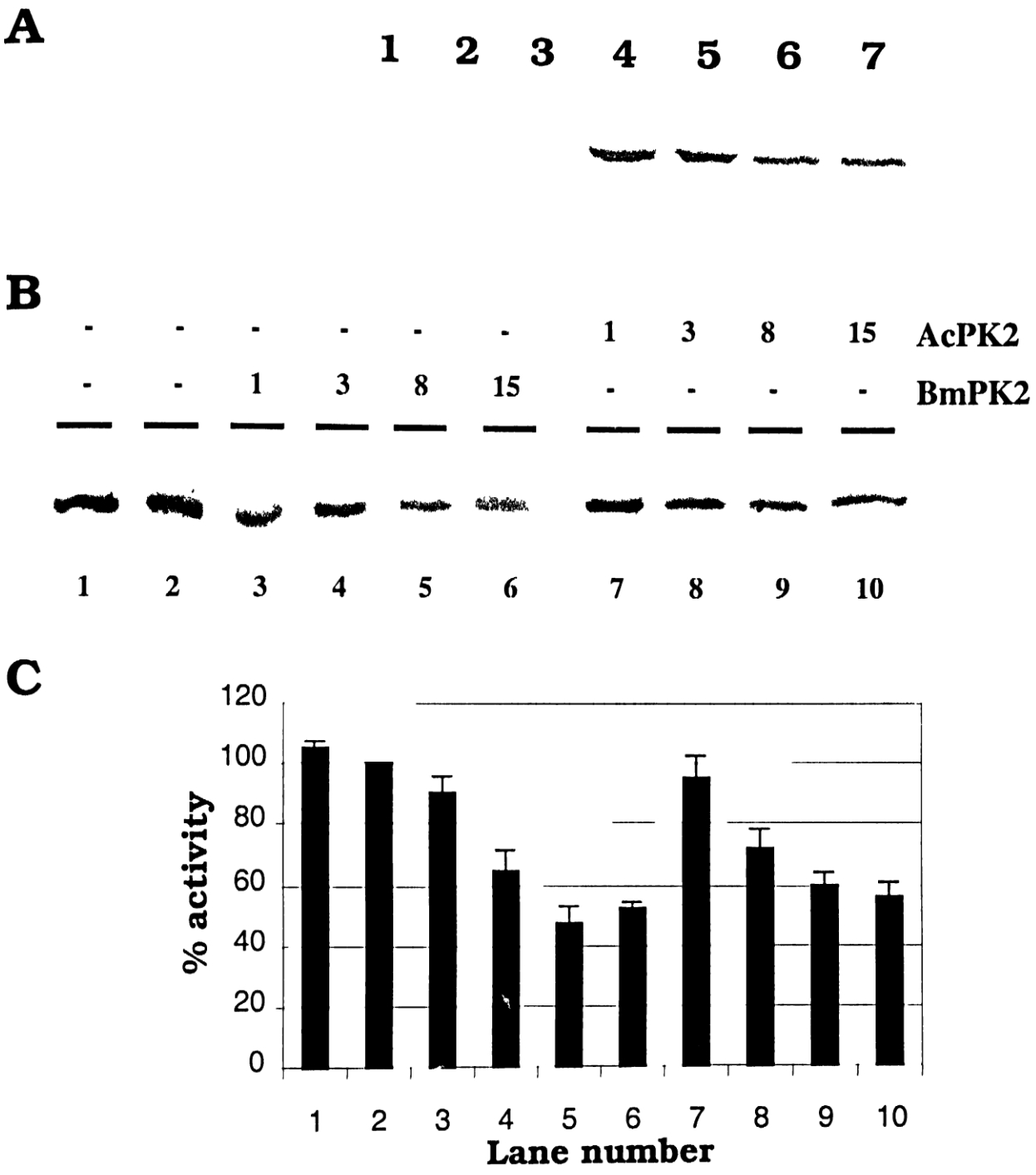


Figure 10. Inhibitory activity of baculoviral PK2 protein on BeK activity. (A) Different controls and standard recombinant PK2 concentrations. Lane 1: AcPK2 and substrate; lane 2: BmPK2 and substrate; lane 3: immunoprecipitate without substrate; lane 4: normal kinase reaction without PK2 protein; lane 5: kinase reaction with 10μg of BSA, and lanes 6 and 7: kinase reactions with 10μg of BmPK2 and AcPK2 respectively. (B) The activity of BeKw in the presence or absence of PK2 protein in *in vitro* kinase assay. Lane 1, control without PK2; lane 2, control with 15μg BSA; lanes 3-6 and 7-10, are with increasing concentration as indicated above the blot, of BmPK2 and AcPK2 respectively. (C) The histogram indicate % activity of BeKw based on the optical density of the bands represented in panel A. Lane numbers are as in panel 'B'.

3.9 RNAi based BeK knockdown

Regulatory and insert domains that are unique were used for RNAi experiments. Kinase domain that is highly conserved was not used for knockdown as it may bind to other kinases. Regulatory (reg) and insert (ins) domains that are 398 bp and 323 bp respectively were cloned, recombinant plasmid was linearised and RNA was synthesized using T7 RNA polymerase as represented in Fig. 11. Double stranded RNA was prepared by annealing the + and – strands. RNA synthesized *in vitro* as + and – strand and the double stranded RNA of insert, regulatory and *ie1* control are separated on agarose gel to check the quality (Fig. 12a). Single stranded RNA was always obtained as two species of different sizes, one being the lower than expected size. But, after making double stranded RNA only single diffused band of corresponding size was obtained.

To measure the effect of dsRNA on BmN cells, dsRNA was transfected using calcium phosphate method. Initially we transfected different amounts of dsRNA to identify the concentration at which maximum knockdown of the target gene expression occurs. After 72 hr. of incubation cells were normal without any visible change in morphology. Total RNA was isolated and BeK transcript was detected by RT-PCR. We used one primer ikinF, located between the regulatory and insert domains and the other, BK-R located in the insert domain for RT-PCR so that there will not be any amplification of transfected dsRNA. The results showed that transcript could be detected in the control without dsRNA, non specific dsRNA control with 100 µg *dsie1* and 20 µg of *dsins*.

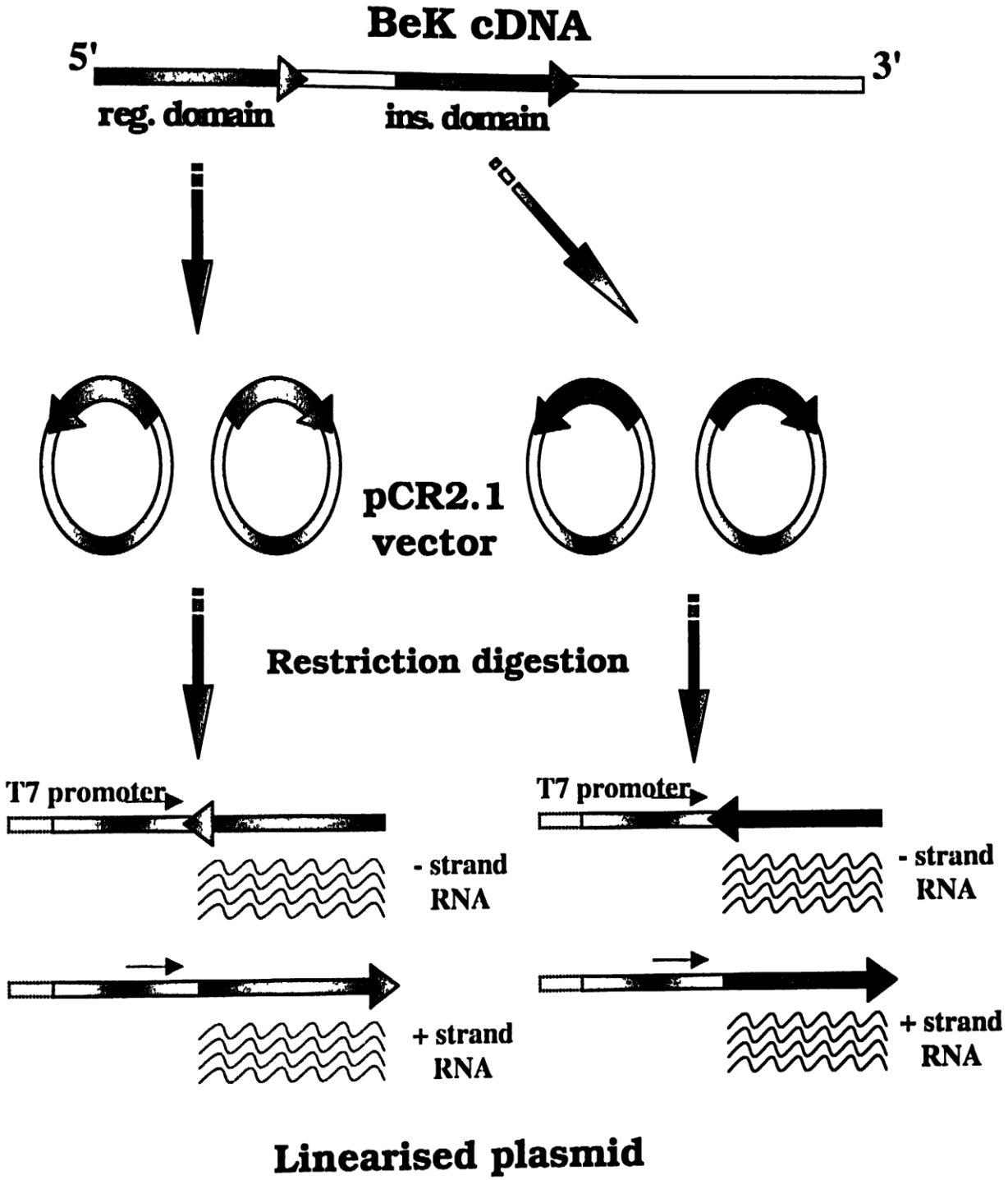


Figure 11. Strategy adapted for *in vitro* RNA synthesis of regulatory (reg) and insert (ins) domain for preparing double stranded RNA. Regulatory and insert domains were PCR amplified and cloned in pCR2.1 vector in both orientation. Direction of arrow represents 5′-3′ orientation. Plasmids were linearised at one end resulting with insert in both orientation under T7 promoter. RNA was synthesized using T7 RNA polymerase.

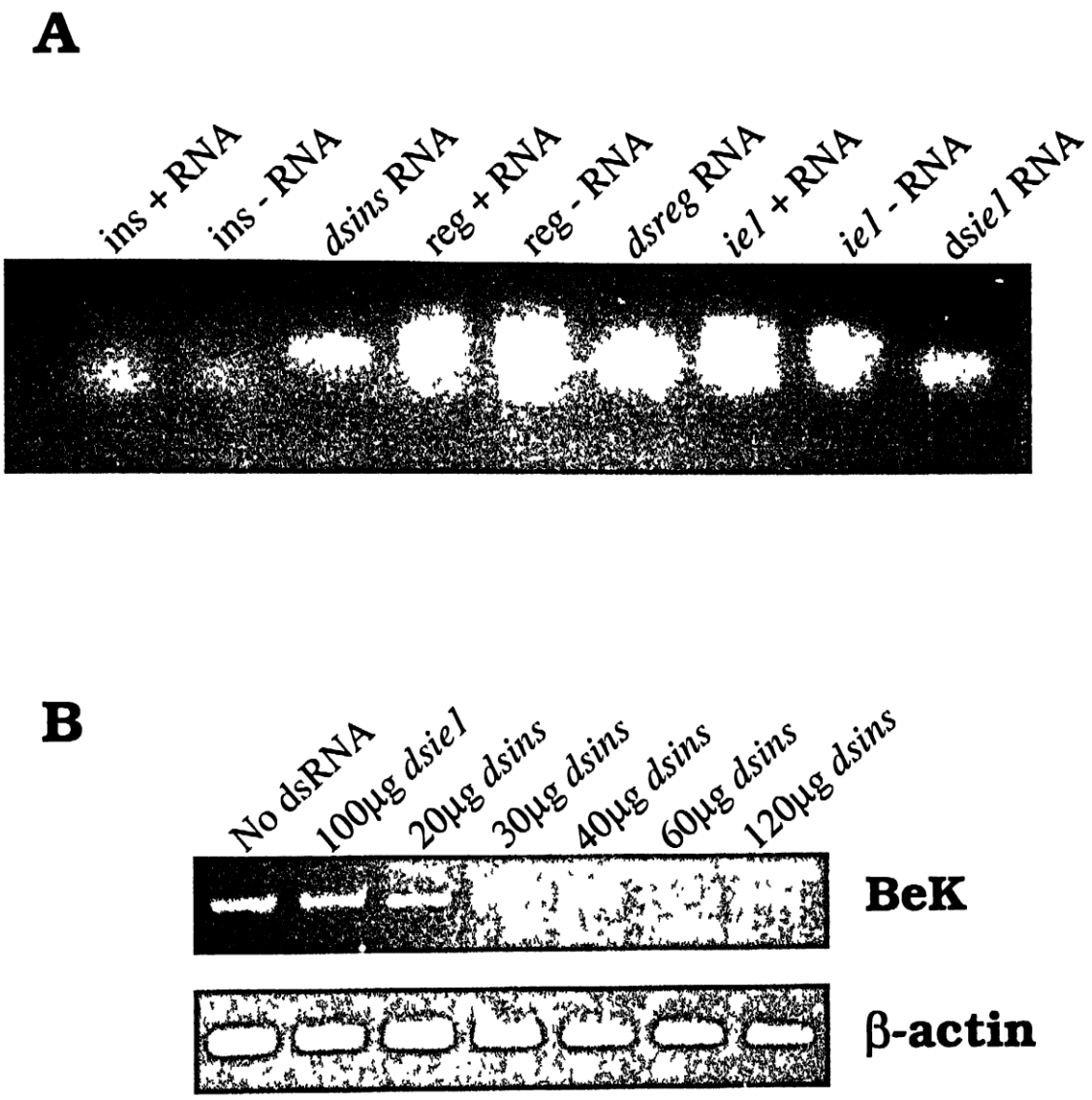


Figure 12. *In vitro* RNA synthesis and BeK transcript degradation in BmN cells by RNA interference. (A) Non denaturing agarose gel picture showing insert domain (ins) + strand, - strand and double stranded (ds) RNA, regulatory domain (reg) + strand, - strand and double stranded RNA and AcMNPV Immediate Early 1 (*ie1*) gene + strand, - strand and double stranded RNA. (B) BeK transcript and β-actin control transcript PCR amplified from BmN cells treated with respective quantity per million cells of dsRNA as mentioned above the gel picture.

Faint amplification was found in 30 and 40 µg of *dsins*, showing there is degradation but not enough to completely knockdown BeK. But in cells transfected with 60 and 120 µg of *dsins* per million cells more than 95% degradation was observed (Fig. 12b). Similar results were observed for *dsreg* also. 60 µg of *dsins* per million cells was used as standard for further RNAi experiments.

3.10 eIF2α phosphorylation levels under gene knockdown

To test the function of BeK, the strategy of checking native eIF2α phosphorylation levels after stress under gene knockdown condition was used. BeK knockdown in BmN cells using RNAi strategy was achieved by transfecting *dsins*. dsRNA transfected cells were incubated for 72 hrs. to allow complete degradation of target mRNA and its translation product before exposing to different stresses like heat shock, bacterial cell wall stress and osmotic shock. Cell lysates were prepared immediately after stress and checked for the status of eIF2α phosphorylation by immunoblotting. There was a significant decrease in the phosphorylation levels under heat shock stress and osmotic stress compared to the two experimental controls and actin loading control (Fig. 13a and c), whereas, there was no difference in the phosphorylation levels in cells exposed to LPS (Fig. 13b).

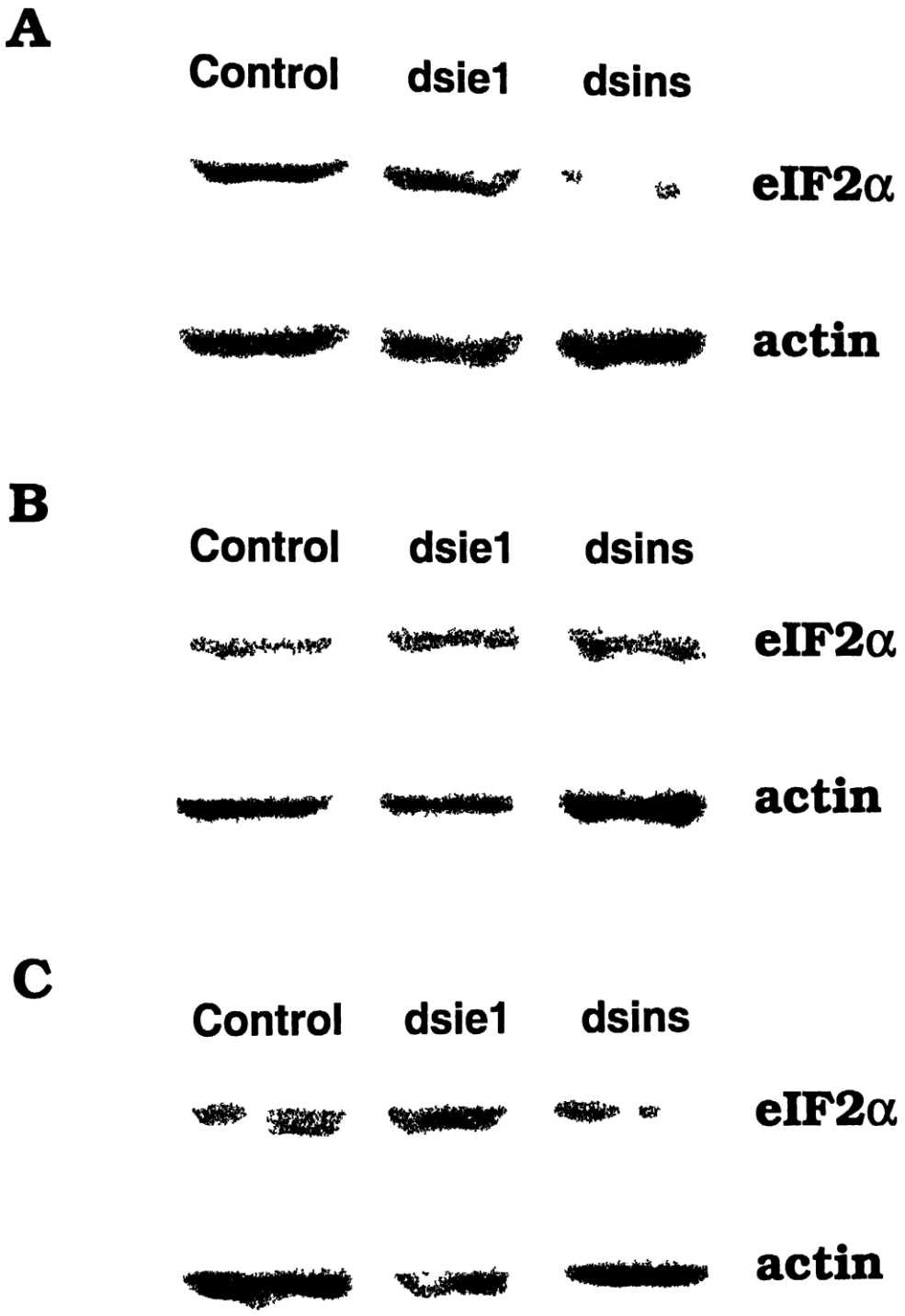
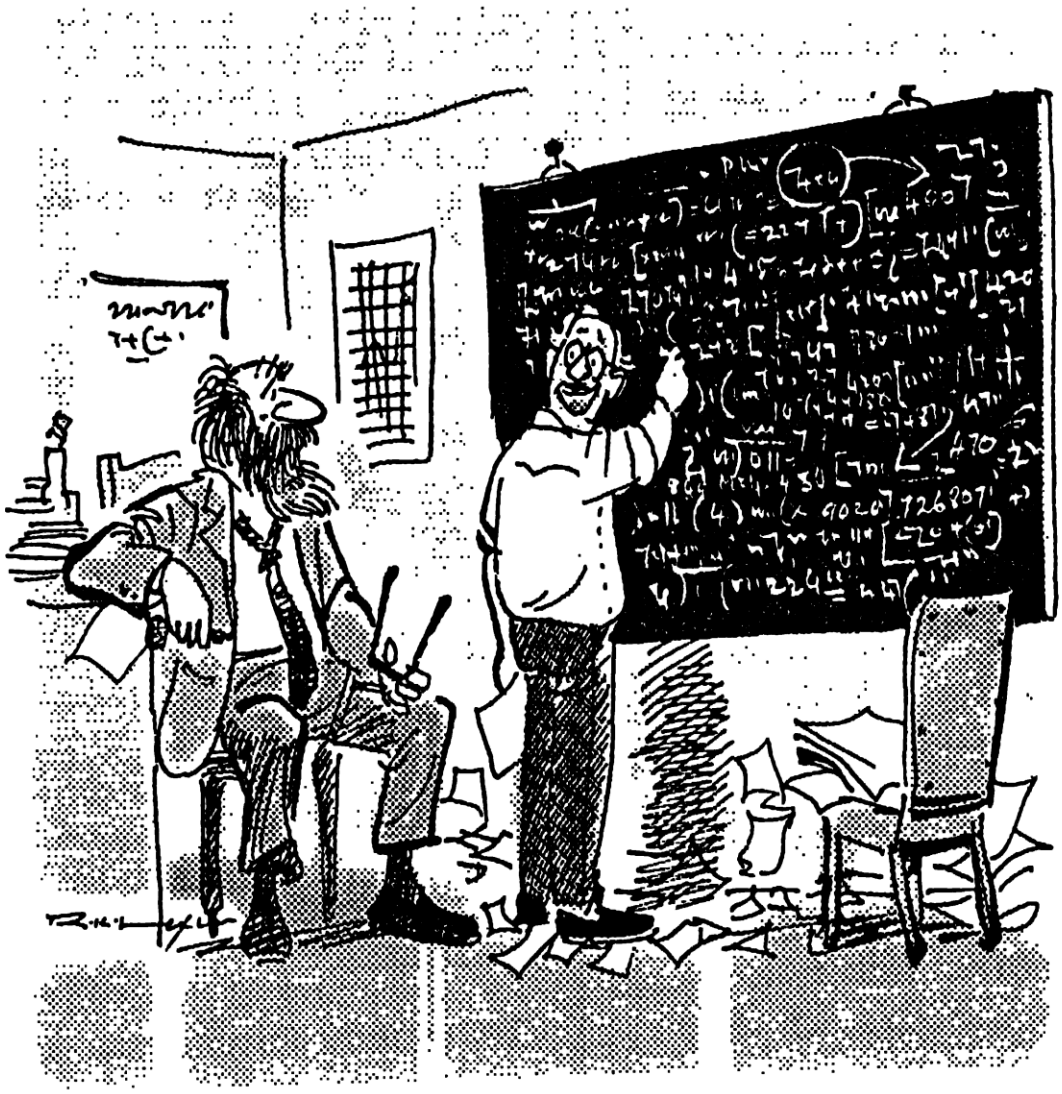


Figure 13. The effect of RNAi based BeK knockdown on the phosphorylation of native eIF2α subunit in BmN cells. In all the sections upper blot is the phosphorylated eIF2α subunit and the lower blot shows actin control. Control lane is transfected with water without dsRNA, *dsie1* is nonspecific control where *dsie1* dsRNA is transfected and *dsins* is specific BeK knockdown. Each blot shows the phorphorylation levels of eIF2α subunit under heat shock stress (A), Bacterial cell wall stress (B) and osmotic stress (C).

4 Discussion



Ah, I have traced the mistake, Professor. Instead of "1+2=3", you have written 4!

4.1 Sequence characteristics

Stress signals are transferred to the effector molecules directly or indirectly through protein phosphorylation. Cells respond to the stress based on the activation/inactivation of these effector molecules. This intricate mechanism of activation and inactivation works through a number of kinases and phosphatases. To identify these stress regulated proteins we used bacterially challenged fat body cDNA library because fat body is the site for the production of host defense molecules and hence will show increased expression of anti-stress transcripts. Using degenerate primers, we identified a 1.3 kb product, which showed 30% similarity to human PKR. This product was further used to screen cDNA library for identifying the full length cDNA. Sequencing analysis showed that BeK clone contained a 1939 bp insert composed of a 5′ non-coding region, an open reading frame of 1741 nucleotides, corresponding to 579 a.a., and a 3′ untranslated region (Fig. 1). BeK, consisting of 579 a.a. has an N terminal regulatory domain and C terminal catalytic domain (Fig. 1). The regulatory domain of 138 a. a. does not show significant similarity to any of the known proteins submitted in the Genbank database. The C terminal catalytic kinase domain possesses 115 a. a. insert between IV and V subdomains. The characteristic insert region does not share any significant homology to any of the eIF2α kinase family of proteins. The unique primary structure of kinase insert domain along with regulatory domain may suggest a distinct function for BeK.

The alignment with all the known eIF2α kinases, revealed a significant homologous region, LF/Y/HIQMQ/EL/F/YCE/R/D (Fig. 2). This region falls under subdomain V (Hanks and Hunter, 1995), connecting two lobes of the kinase domain, and is known to be very important for substrate binding (Cai and Williams, 1998). This conservation is not found in non eIF2α kinase proteins (Hanks and Hunter, 1995). Mutants of this conserved region are shown to require hydrophobicity for substrate binding (Cai and Williams, 1998). Thr-446 and Thr-451 in the activation loop of human PKR located between VII and VIII subdomains are proved to be the phosphorylation sites necessary for activation (Romano *et al.*, 1998). Thr-446 is the primary auto-phosphorylation site, and together with Thr-451 influences the kinase activity. Thr-882 and Thr-887 of yeast GCN2, Thr-980 and Thr-985 of human PEK corresponding to the above mentioned Threonine residues of human PKR are found to be auto-phosphorylated, which is an essential step for activation of eIF2α family proteins (Romano *et al.*, 1998; Ma *et al.*, 2002). Only BeK, PfPK4 and Spombe1ri2p lack the first Threonine, corresponding to Thr-446 of human PKR (shown by arrows in Fig. 2), suggesting the possibility of other auto-phosphorylation sites and possibly a different mode of regulation. The uniqueness in the N-terminal regulatory domain, conserved LXIQMXXC motif, lack of Threonine corresponding to Thr-446 of human PKR, suggests a novel regulation of this protein.

4.2 Phylogenetic relationship

The phylogenetic tree clusters all the kinases into separate groups based on their function except PfPK4 and *S. pombe* GCN2, which come out as basal elements (Fig. 3). This separation might be because of the divergent sequence in these two elements. *S. pombe* GCN2, predicted by a software based on homology to other eIF2α kinases, does not possess the conserved LXIQMXXC sequence raising doubts of its function as an eIF2α kinase. BeK was earlier found not to cluster with any of the known class of kinases when selected eIF2α kinases were used (Prasad *et al.*, 2003). In our present phylogenetic tree where we used all the known eIF2α kinases, BeK clusters with HRI class of kinases. This kind of systematic clustering of different class of elements based on the function is a very good way to predict the function of eIF2α kinases.

4.3 Distribution

To test the tissue distribution of BeK, RT-PCR analysis was performed using different tissues from 5th instar larvae, whole pupae and 4th instar larvae. The result showed that BeK was ubiquitously expressed in all tissues including fat body, midgut, gonads, muscle, silk gland, epidermal tissue, malpighian tubule and cardiac system (Fig. 4a). Interestingly we found a significantly higher level of expression in the gonads, which is one of the highly differentiating tissues in 5th instar larvae. A similar increased expression was found in mouse testis (Berlanga *et al.*, 1998). Previous work on eIF2α phosphorylation indicated its role in cell growth

and differentiation (Reviewed in De Haro *et al.*, 1996; Williams, 1999). In a study on myogenic cell line L8, growth factor TGF-β and the chelating agent EGTA reduced the level of muscle specific proteins to a significant level, correlated with decrease in PKR activity during differentiation linking the level of PKR with the degree of cellular differentiation (Salzberg *et al.*, 1995). Based on some of the above observations we speculate that BeK might help in cell differentiation. This result prompted us to check BeK expression in embryo, where GCN2 has been proposed to help in differentiating three different germ layers and determining neural cell identity (Santoyo *et al.*, 1997). BeK transcript was identified in embryo of different stages and hatched larvae (Fig. 4b). To explain the involvement of BeK as stress regulated protein at embryonic stage looks little far-fetched. So, we propose that it might be involved in developmental regulation. We are further trying to understand the function of BeK in embryonic stage by knocking down this gene by RNAi technology to see developmental deformity, if any.

4.4 Gene and function validation

The predicted ORF was tested for its complete coding by using MBP as a fusion protein. The results confirmed the theoretically estimated size of 65 kDa (Fig. 5a). Purified MBP-BeK was also identified by the antiserum we generated against the N-terminal domain proving that antisera was specific towards BeK (Fig. 5b).

Phosphorylation of eIF2α is the central signaling process of eIF2α kinases. To test the kinase activity of BeK we first generated a BeKw construct and a catalytically inactive BeKm construct with Lys^{174} replaced with Arg. S2 cells transformed stably with BeKw and BeKm constructs expressed a 65 kDa protein suggesting that continuos BeK ORF is functional in heterologous system (Fig. 6a). Whereas, S2 cells transformed with *Drosophila* PEK for the purpose of positive control did not show a detectable amount of protein. One possible explanation for this low expression of dPEK might be a regulatory control on over-expression because of homologous system used (Fig. 6a). This kind of low expression and extensive eIF2α phosphorylation was observed while expressing mammalian PEK and HRI in Sf9 cells which was not the case when mutants were expressed, wherein over-expression of mutants was detected (Shi *et al.*, 1999; Chefalo *et al.*, 1994). Kinase activity was checked by doing *in vitro* kinase reaction and western blot. BeKw showed kinase activity whereas BeKm did not show any activity (Fig. 6b) although it was expressed in equivalent amounts in cell lysate (Fig. 6a). Positive control, dPEK, also showed kinase activity. This proved that BeK functionally belongs to eIF2α kinase family and is involved in general translation control through eIF2α phosphorylation.

Autophosphorylation is known to be an essential step for the activation of other eIF2α kinases (Galabru and Hovanessian, 1987; Harding *et al.*, 1999). BeK immunocomplex with immuneserum showed autophosphorylation activity (Fig. 7) suggesting that it is an autokinase.

The autophosphorylated band ran at a higher size of 80 kDa that is normal to phosphoproteins (Harding *et al.*, 1999).

4.5 Stress and eIF2α phosphorylation

In insects other than *Drosophila*, very little is known about eIF2α phosphorylation under different stress conditions. In *Drosophila* it is well known that protein synthesis is dramatically inhibited in response to heat shock at 37°C (Lindquist, 1986) although the specific signaling molecules are not known. Recently *Drosophila* GCN2 and PEK were cloned and GCN2 was found to functionally substitute yeast GCN2 in *S. cerevisiae* (Olsen *et al.*, 1998; Sood *et al.*, 2000b). Another report discusses about the stress inducibility of eIF2α phosphorylation, specifically by serum starvation and ER stress in *Drosophila* cells (Williams *et al.*, 2001). To check for native eIF2α phosphorylation status before proceeding to analyzing stress induced activity of BeK, *Drosophila* and *Bombyx* cells were exposed to different stress conditions. S2 cells showed significant increase in eIF2α phosphorylation under heat shock and osmotic shock, whereas LPS, Thapsigargin, β-1,3-glucan and H_2O_2 did not show a significant increase although there seems to be a slight increase compared to nonstressed cells (Fig. 8a). The time of exposure used for Thapsigargin stress is lesser compared to that used by Williams *et al.*, 2001, which may be the reason for not getting a significant increase in eIF2α phosphorylation. Both S2 and BmN cells showed a basal level of eIF2α phosphorylation (Fig. 8). There might be an equilibrium maintained

between the phosphorylated and non-phosphorylated forms of eIF2α phosphorylation. To determine the optimum level of stress which induces eIF2α phosphorylation BmN cells were exposed to varying levels of heat shock, osmotic stress and LPS stress. eIF2α phosphorylation was found to be high after 1hr. at 37°C and 42°C (Fig. 8b), as 37°C being the standard used in other insect cells like *Drosophila* (Lindquist, 1986), we used it as the standard in our experiments. Similarly, 10 μg of LPS for 30 min. (Fig. 8c) and 300mM NaCl for 30 min. (Fig. 8d) were found to be optimum to induce eIF2α phosphorylation.

4.6 BeK activation by stress

Except for heat shock and viral infection which induce eIF2α phosphorylation in lepidopteran cells (Lindquist, 1986; Du and Thiem, 1997; Dever *et al.*, 1998), other stresses are largely unknown. To investigate this and the involvement of BeK in this process, kinase assay was performed after exposing cells to different stress conditions. Over-expression of BeKw in the absence of stress causes a basal level of kinase activity, which might be caused by presence of high concentration of BeK in cells inducing protein-protein interactions. This phenomenon was also observed by over-expression of PERK in Cos1 cells (Harding *et al.*, 1999). BeK showed a significant level of activity under heat shock and osmotic shock accounting for 5 and 3 fold increase respectively and doubling of activity under LPS. Other stress activity could not induce a significant BeK activity (Fig. 9). Heat shock and osmotic stresses are general

stresses, where the activators of eIF2α kinases are not known. Whereas, LPS is a specific stress acting through membrane receptors although downstream signaling molecules to eIF2α kinases are not known (Kim *et al.*, 2000; Tanlal and Tomita, 2000).

4.7 BeK inhibition by viral protein

The phosphorylation of eIF2α by eIF2α kinase results in the inhibition of general translational machinery, which is a common cellular mechanism for limiting protein synthesis under stress conditions. Many viruses have developed a number of strategies to down regulate eIF2α kinase to successfully proliferate in host cells (reviewed in Tan and Katze, 1999). AcMNPV infection induces protein synthesis shut down in *Lymantria dispar* Ld652Y cells. Host range factor 1 of LdMNPV and p35 of AcMNPV were proposed to inhibit and activate protein synthesis shutdown respectively in Ld652Y cells (Du and Thiem, 1997). Baculovirus PK2, resembling to V to XII subdomains of eIF2α kinase domain, was identified in AcMNPV and shown to inhibit eIF2α phosphorylation in Sf9 cells. PK2 was specifically found to inhibit human PKR and yeast GCN2 (Dever *et al.*, 1998). However, it is unknown whether PK2 can inhibit the enzymatic activity of purified insect eIF2α kinase by direct interaction. To investigate if PK2 viral protein can inhibit the kinase activity of BeK, we performed *in vitro* kinase reaction in the presence of recombinant PK2 protein. AcMNPV PK2 and BmNPV PK2 that share about 90% homology were used separately in kinase reactions. BeK kinase activity showed a dose

dependent inhibition. Recombinant PK2 inhibited BeK *in vitro* kinase activity by 50% and did not increase further with the increase in PK2 concentration (Fig. 10), suggesting that inhibition reaches saturation at 8 µg of PK2 protein. PK2 is proposed to be a dimerization inhibitor, it requires to form heterodimer with BeK for its inhibitory activity (Dever *et al.*, 1998). eIF2α kinases form dimers for their complete activity, thus BeK molecules, which are already dimerized during the process of over-expression and immunoprecipitation, will not be inhibited by PK2, which explains the saturation in its inhibitory capacity. This gives a direct evidence on the strategy employed by baculovirus to inhibit protein synthesis shutdown during its replication in insect cells. This is also supported by the experiments where eIF2α phosphorylation was shown to increase when Sf9 cells were infected with AcMNPV lacking PK2 was used as compared to that in wild type virus infected cells. And, the low level of occluded virus production when Sf21 cells over-expressing PKR was infected with PK2 mutant virus (Dever *et al.*, 1998). These observations show that eIF2α kinase similar to BeK must be existing in *S. frugiperda* and that over-expression of eIF2α kinase may probably confer antiviral property to the cells.

4.8 eIF2α phosphorylation under BeK knockdown

To study the behavior of cells under gene knockdown, we developed a strategy for generating dsRNA targeting regulatory and insert domains of BeK (Fig. 11). The transcript degradation was monitored by RT PCR of

total RNA. The results showed that, there was greater than 60% BeK transcript degradation at 30 and 40 µg of dsRNA per million cells. This increased to greater than 95% at 60 µg., but complete degradation was not observed even at 120 µg concentration (Fig. 12b). Lack of complete obliteration of target gene expression by using dsRNA is a natural feature of RNAi (McManus and Sharp, 2002) because of which it is known as knockdown and not knockout. Just bathing the cells with dsRNA up to 150 µg per million cells failed to result in BeK transcript degradation. This indicates that "bathing" strategy used in *Drosophila* cells and *C. elegans* (McManus and Sharp, 2002) does not work in BmN cells. Lipofectin, a common transfection reagent used for Sf9 cells did not result in transcript degradation, probably because it was inefficient in transfecting ds RNA into BmN cells.

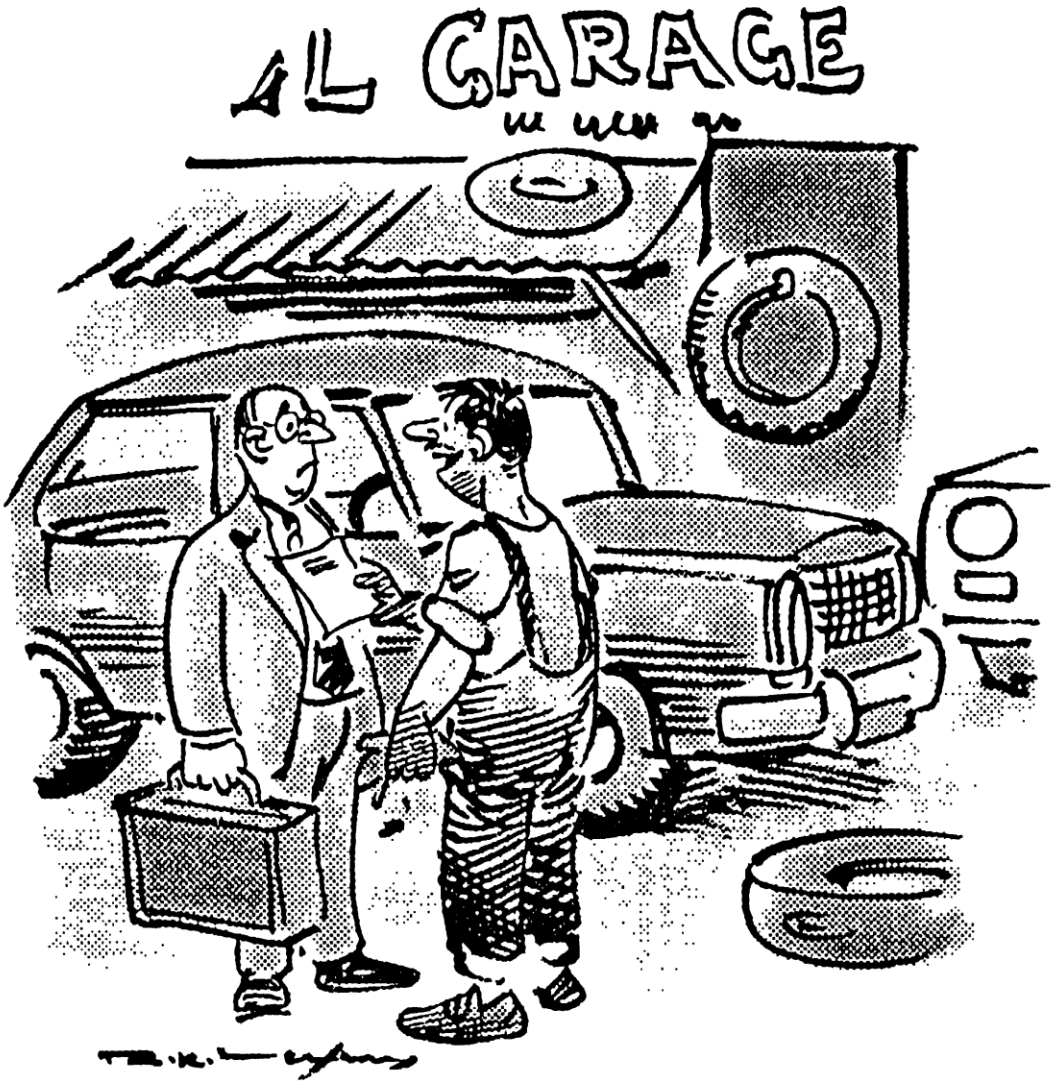
As it has been shown earlier BeK activity increased under heat shock, osmotic and bacterial cell wall stress but not the others (Fig. 9), thus we tested native eIF2α phosphorylation under BeK knockdown using these stress conditions. BeK knockdown cells showed significant decrease in eIF2α phosphorylation under heat shock and osmotic stresses, whereas LPS stress did not bring about any significant change in the phosphorylation status (Fig. 13). BeK is one of the major eIF2α kinases, which respond to general stresses like osmotic and heat shock stresses, whereas, it is not a major transducer of signal under LPS stress. This

suggests that there may be other eIF2α kinases, which might overtake the function of BeK under LPS stress.

The physiological role of members of this class of kinases may be to act in coordination as homeostatic guardians against environmental stress by ultimately regulating protein synthesis in response to specific exogenous signals thus ensuring cell survival.

We conclude that BeK cloned from *B. mori* is a novel eIF2α kinase family member having unique features in primary structure. The *in vitro* kinase activity of BeK expressed in S2 cells increases under bacterial cell wall stress, heat shock and osmotic stress. Baculoviral PK2 protein inhibits the *in vitro* kinase activity by forming heterodimer with BeK. BeK is one of the major eIF2α phosphorylating kinases under heat shock and osmotic stress although it may not be the only one in *B. mori*.

5 References



There was nothing wrong, Sir. It wouldn't start because the petrol tank was empty! Here is the bill for Rs 2,471-00 for finding it out.

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6 Summary


I'VE DECIPHERED THE HUMAN GENOME...
BUT I STILL DON'T UNDERSTAND WOMEN!

The primary task of cell under stress is to defend itself to overcome the stress. To orient itself towards defense, one of the first and rapid responses is to shut down global protein synthesis machinery or selectively translate a few useful transcription factors. Eukaryotic cells use translational control system as a rapid response to various types of environmental stresses, such as, amino acid (aa.) starvation, heat shock, viral infection, heme deprivation and endoplasmic reticulum (ER) stress. Under these conditions, Eukaryotic Initiation Factor 2α (eIF2α) kinase phosphorylates the α subunit of eIF2 and plays an important role in the cellular defense system operating under unfavorable conditions. The phosphorylated form of eIF2α sequesters eIF2B, which is necessary for the exchange of GDP to GTP in the recycling of eIF2. Because eIF2B is a quantitatively limiting factor, phosphorylated eIF2α is sufficient to sequester all available eIF2B and stop the initiation of translation.

Among the eIF2α kinase family members, four eIF2α kinases have been extensively studied. They are heme-regulated eIF2α kinase (HRI), double-stranded RNA-dependent eIF2α kinase (PKR), yeast GCN2 protein kinase and ER resident kinase (PERK or PEK). HRI is activated under conditions of heme deficiency of reticulocytes, and inhibits protein synthesis. PKR is induced by interferons and is activated by low concentrations of double-stranded RNA produced during viral infections. Yeast GCN2 kinase is activated by aa. starvation, and generates a signal that leads to the enhanced expression of genes involved in aa. biosynthesis. PERK is a type

I transmembrane protein and is activated in response to a stress signal from the ER. All these kinases use eIF2α as a common substrate, which suggests that eIF2α phosphorylation by eIF2α kinase is a central signaling event for the inhibition of general translation in response to various stresses. However, the identity of the eIF2α kinases involved in eIF2α phosphorylation under specific stress conditions is largely unknown. Identification of additional eIF2α kinases and the elucidation of their functions are necessary to understand how different eIF2α kinases can specifically respond to distinct stimuli.

To identify eIF2α kinase in *Bombyx mori*, an anti-sense degenerate primer was synthesized based on the a.a. (KPSNV/IF) in conserved kinase subdomain VI. We used this primer in combination with λgt-10 forward or reverse primer and performed Polymerase chain reaction (PCR) using λgt-10 Bombyx fat body library as template. The 1300 bp fragment obtained showed 30 % homology to human PKR gene and was used to screen a cDNA library from bacterially challenged *Bombyx* fat body. Two clones thus obtained were sub-cloned and sequenced. Sequencing analysis showed that it contained a 1959 bp insert composed of a 5' non-coding region, an open reading frame of 1737 nucleotides, corresponding to 579 a.a. and a 3' untranslated region. The deduced a.a. sequence has a NH_2 terminal regulatory domain and a kinase insert domain of 115 a.a. between kinase subdomain IV and V. Sequence comparisons of the catalytic kinase domain with other known eIF2α kinases showed 23% to

30% identity with other eIF2α kinases at protein level. The NH_2 terminal regulatory domain of 138 a.a. did not show any significant similarity to known sequences by homology search of GenBank sequences. The kinase insertion domain of 115 a.a. was also unique, which along with the regulatory domain suggests a distinct function for this kinase. Sequence alignment analysis showed that the "Leu-Xaa-Ile-Gln-Met-Xaa-Xaa-Cys" motif, known to be necessary for eIF2α phosphorylation, was conserved among all sequences. When we constructed a phylogenetic tree using Rat Erk2 protein as an outgroup, **B**ombyx mori **e**IF2α **K**inase (BeK) protein got clustered under HRI kinases.

RT-PCR results showed that BeK was ubiquitously expressed in all the tissues examined, including fat body, midgut, gonads, muscle, silk gland, epidermal tissue, malpighian tubules and the cardiac tissue from day 5 of 5^{th} instar larvae. Interestingly, we found a significantly higher level of expression in the gonads. BeK was also detected in early embryonic stages of *B. mori* indicating that it might play a role in development.

To examine the enzymatic activity of BeK, we first generated a BeK wild type (BeKw) construct and a BeK catalytically inactive mutant (BeKm) construct that replaced Lys174 with Arg by PCR based mutagenesis. Using these constructs, Drosophila Schneider cells stably expressing hexahistidine-tagged BeKw or hexahistidine-tagged BeKm under the control of metallothionine promoter were generated. In these cell lines, we could detect BeKw and BeKm with molecular mass of 65kDa following

copper induction. To investigate whether BeK can directly phosphorylate the alpha subunit of eIF2 as substrate, a common substrate for eIF2α kinase family, cell lysate from Schneider cells expressing His-BeKw was immunoprecipitated with monoclonal anti-His antibody, and a kinase assay was performed using the recombinant Drosophila eIF2α subunit. The phosphorylation of the recombinant eIF2α subunit was detected by Western blot analysis using antibody specifically recognizing the eIF2α subunit phosphorylated at Ser50 (which corresponds to Ser51 in mammalian eIF2α). We found that BeKw can indeed phosphorylate the Ser50 site of eIF2α subunit. In contrast to BeKw, BeKn showed no kinase activity although it was expressed in equivalent amounts in cell lysate. These results strongly support our contention that BeK is involved in the general translational control system and that it acts by phosphorylating the Ser50 site of the eIF2α subunit.

In insects, it is well known that protein synthesis is dramatically inhibited in response to heat shock and viral infection. However, other stress stimuli leading to eIF2α phosphorylation are largely unknown. Drosophila Schneider cells stably expressing BeKw were subjected to various eIF2α phosphorylating forms of stress (immune stress, osmotic stress, heat stress, ER stress, and oxidative stress). BeKw was then immunoprecipitated and *in vitro* kinase assay was performed. Of the different stress conditions, BeK is mainly activated in Drosophila Schneider cells following heat shock and osmotic stress and a slight

increase under bacterial cell wall stress. Activated-BeK was shown to phosphorylate the eIF2α subunit at the Ser 50 site. However, other stress conditions, such as fungal cell wall stress, ER stress and oxidative stress, could not significantly activate BeK activity. Densitometric analysis showed that heat stress and osmotic stress induced 5-fold and 3-fold increase in BeK activity respectively, whereas LPS increased 1-fold activity.

The inhibition of general translational machinery by eIF2α kinase is a common cellular mechanism for limiting protein synthesis under stress conditions. Many viruses have developed a number of strategies to down-regulate eIF2α kinase to successfully propagate in host cells. Insect viruses, such as *B. mori* Nuclear Polyhedrosis Virus (BmNPV) and *Autographa californica* Multiple Nuclear Polyhedrosis Virus (AcMNPV), possess a truncated kinase referred to as PK2, which has been shown to inhibit eIF2α phosphorylation in insect SF9 cells. However, it is not known whether PK2 can inhibit the enzymatic activity of purified insect eIF2α kinase. To investigate whether the insect viral gene product, PK2, inhibits the enzymatic activity of insect eIF2α kinase, we performed an immunocomplex kinase assay in the presence of recombinant PK2. PK2 from BmNPV and from AcMNPV, which share about 90% homology, were chosen for this investigation. It was found that BeK enzymatic activity is significantly reduced in the presence of either BmNPV-PK2 or AcMNPV-PK2 versus BeK activity in the absence of viral PK2. No kinase activity

was detected with viral PK2 alone. This result suggests that BeK may be one of the host targets of the viral eIF2α kinase inhibitor, PK2. Further studies are necessary to elucidate the exact role of BeK in stress response and insect viral replication.

RNA interference is a new technique to achieve gene knockdown that utilizes double stranded RNA (dsRNA). BeK knockdown in BmN cells was achieved by using dsRNA against the insert region of BeK. A gradual degradation of BeK RNA was observed with increasing concentration of dsRNA from 20 to 120 μg reaching the maximum at 60 μg per million cells. Under the knockdown condition we observed a significant reduction in native eIF2α phosphorylation when cells were exposed to heat shock and osmotic stress. But, we did not find a significant effect by LPS, confirming that BeK is the main eIF2α kinase activated under heat shock and osmotic stress but not under LPS. There might be other eIF2α kinases, which might substitute the function of BeK.

Here, we report a novel eIF2α kinase, termed BeK, from the silkworm, *B. mori*. This gene encodes 579 aa. and contains all 11 catalytic domains of protein-serine/threonine kinases. Most notably, it contains a "Leu-Xaa-Ile-Gln Met-Xaa-Xaa-Cys" motif, which is highly conserved from yeast to mammalian eIF2α kinases. BeK does not show any significant homology in the NH2 terminal regulatory domain, suggesting a distinct regulatory mechanism of this novel eIF2α kinase. BeK is ubiquitously expressed in

the various tissues throughout the final larval stage. It is also found to express during early embryonic stages. Importantly, BeK is activated in *Drosophila* Schneider cells following heat shock and osmotic stress, and activated-BeK has been shown to phosphorylate an eIF2α subunit at the Ser50 site. However, other forms of stress, such as immune stress, ER stress and oxidative stress, cannot significantly elicit BeK activity. Interestingly, the baculovirus gene product, PK2 can inhibit BeK enzymatic activity suggesting that BeK may be an endogenous target for a viral gene product. The effect of eIF2α phosphorylation under heat shock and osmotic stress was significantly reduced under BeK knockdown indicating that this kinase is the major eIF2α kinase active under these stresses. Taken together, these data indicate that BeK is a novel eIF2α kinase involved in the stress response in *B. mori*.

7 List of Publications

Smile, Your Honour!

Prasad M D, Han S-J, Nagaraju J, Lee W-J and Brey P T (2003)
Cloning and Characterization of an eukaryotic Initiation factor 2 alpha kinase, from the silkworm, *Bombyx mori*.
Biochim Biophys Acta. 1628(1):56-63.

Prasad M D and Nagaraju J (2003)
A comparative phylogenetic analysis of full-length *mariner* elements isolated from Indian tasar silkmoth, *Antheraea mylitta* (Lepidoptera: saturniidae).
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Prasad M D, Nurminsky D I and Nagaraju J (2002)
Characterization and molecular phylogenetic analysis of mariner transposon elements from wild and domesticated species of silkmoths.
Mol. Phyl. Evol. 25(1):210-217.