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UMI
Protein Translation Initiation Factors that interact with TTK

By

SRIKANTH KRISHNAMURTHY

1998

A thesis submitted in conformity with the requirements for the Degree of Master of Science

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Abstract

Protein Translation Initiation Factors that interact with TTK
(threonine tyrosine kinase)

Protein kinases play a role in most basic biological processes including cell growth, gene expression, metabolic regulation, cell cycle control and signal transduction. More than 300 protein kinases from lower eukaryotes to mammals have been identified and characterized (Hoekstra et al., 1991a). A study of novel protein kinases may help us to understand normal and malignant cell growth.

TTK is a serine-threonine-tyrosine kinase that was isolated and characterized in my host laboratory. As the role of TTK in mammalian cells is unknown, we used the yeast two-hybrid system to identify interacting proteins. Using the N-terminal domain of TTK as bait, we isolated several clones, two of which encoded the eukaryotic translation initiation factors-eIF4G-1 (p220/eIF4γ) and eIF4G-3 (DAP-5/NAT-1). My project involved the characterization and interaction of these proteins with TTK. In this thesis, I describe the isolation and characterization of the full-length cDNA for eIF4G-3 and the study of its interaction with TTK.

The full-length cDNA of the clone eIF4G-3 was isolated through cDNA library screening and 5' RACE. Manual and automated sequencing yielded the complete sequence of the clone, predicting a protein of molecular mass 97 kDa. Northern blotting experiments showed that eIF4G-3 mRNA is ubiquitously expressed among the cell
lines and tissues used. To confirm that TTK associates with the eIF4G-1 and eIF4G-3 initiation factors in mammalian cells, we carried out a series of co-immunoprecipitation experiments. I expressed eIF4G-1, eIF4G-3, eIF4G-3', TTK and TTK\textsuperscript{m} (kinase-dead mutant) in COS cells using a vaccinia virus transient expression system. Co-immunoprecipitation experiments demonstrated that TTK interacts with both eIF4G-1 and eIF4G-3. In addition, using untransfected T98G cells we were able to demonstrate that endogenous TTK interacts with eIF4G-1 and (probably eIF4G-3). \textit{In vitro} kinase assays of TTK-eIF4G-1 precipitated complex showed strong phosphorylation of a band co-migrating with eIF4G-1; the same band was also detected by anti-phosphotyrosine antibody in western blotting experiments.

In light of the results from co-immunoprecipitation and \textit{in vitro} kinase assays, we conclude that TTK interacts with and phosphorylates both eIF4G-1 and eIF4G-3 in mammalian cells. We are now addressing the hypothesis that TTK plays an important role in the control of mRNA translation in mammalian cells in a cell cycle dependent manner.
Acknowledgements

I sincerely acknowledge here with gratitude the following people who have made the completion of my project possible:

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<th>Definition</th>
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<tr>
<td>aa</td>
<td>Amino acids</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>A-site</td>
<td>Amino-acyl tRNA binding site</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>β-gal</td>
<td>Beta galactosidase</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>Co-IP</td>
<td>Co-immunoprecipitation</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleoside triphosphate</td>
</tr>
<tr>
<td>dpc</td>
<td>Days post coitum</td>
</tr>
<tr>
<td>D-MEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>eIF</td>
<td>Eukaryotic initiation factor</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-thio-beta-D-galactoside</td>
</tr>
<tr>
<td>IVK</td>
<td>In vitro kinase</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilo base</td>
</tr>
<tr>
<td>LB medium</td>
<td>Luria-Bertani medium</td>
</tr>
<tr>
<td>MAb</td>
<td>Monoclonal Antibody</td>
</tr>
<tr>
<td>MPF</td>
<td>Maturation progression factor</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>nt</td>
<td>Nucleotides</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBL</td>
<td>Peripheral blood lymphocytes</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate-buffered saline tween</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>pser</td>
<td>Phospho-serine</td>
</tr>
<tr>
<td>P-site</td>
<td>Peptidyl-tRNA binding site</td>
</tr>
<tr>
<td>pthr</td>
<td>Phospho-threonine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td>ptyr</td>
<td>Phospho-tyrosine</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>ser/thr</td>
<td>Serine/Threonine</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
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<tr>
<td>TBST</td>
<td>Tris-buffered saline tween</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer ribonucleic acid</td>
</tr>
<tr>
<td>tyr</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
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1. Introduction

1.1 A brief overview of the cell cycle

Protein kinases play a role in most basic biological processes including cell growth and development, gene expression, metabolic regulation, cell cycle control, signal transduction and cellular differentiation. More than 300 protein kinases from lower eukaryotes to mammals have been identified and characterized (Hoekstra et al., 1991a). Hence, a study of novel protein kinases may help us to understand normal and malignant cell growth.

To coordinate and order correctly the various stages of the cell cycle, checkpoints are set up at each step of the proliferation process. Otherwise, genetic damage can occur if cells progress to the next phase of the cycle before the previous phase is properly completed. The primary cell cycle checkpoints are,

a) Entry of mammalian cells into the cell cycle (G1-S progression) is controlled at the 'restriction point' (‘START’ in yeast). Mammalian cells cultured in the absence of growth factors arrest with a diploid complement of chromosomes in Go-G1 phase. If growth factors are added to the culture medium, the cells pass through the ‘restriction point’ 14-16 hrs later, enter the S-phase 6-8 hrs after that, and complete the remainder of the cell cycle.

b) An S phase checkpoint that prevents DNA replication initiation in the presence of damaged DNA.
c) A mitotic checkpoint that prevents entry into mitosis until the DNA has been duplicated and both sets of replicated chromosomes are poised to segregate.

d) Finally, exit from mitosis and cleavage into daughter cells does not take place until the chromosomes have segregated to separate poles of the cell (Hartwell and Weinert, 1989).

Cell cycle control systems have been studied in yeast, frog oocytes and mammalian cells. Much of our current understanding of this process has emerged from studies of temperature sensitive mutants (ts) in the budding yeast - *Saccharomyces cerevisiae* and the fission yeast - *Schizosaccharomyces pombe*. Mutagenesis of yeast, followed by screening for ts strains that failed to grow at the non-permissive temperature provided large numbers of mutants that arrested at specific points in the cell cycle (designated as cell division cycle - cdc mutants).

The wild type alleles of recessive cdc-ts mutant alleles can be isolated by transforming haploid mutant cells with a plasmid library prepared from wild-type cells and then plating at the non-permissive temperature. Complementation of the recessive mutation by the wild type allele on one of the plasmids in the library allows a transformed mutant cell to form a colony; the plasmid bearing the wild-type allele is then by recovered from those cells. Because many of the proteins that regulate the cell cycle are highly conserved, human cDNAs cloned into yeast expression vectors often complement yeast cell cycle mutants, leading to the rapid isolation of human genes encoding cell-cycle control proteins. (Hartwell *et al.*, 1974; Forsberg and Nurse, 1991).

Cell cycle studies in the frog oocytes (*Xenopus laevis*) took advantage of the fact that the cells are arrested in G2 prior to fertilization. Stimulation by progesterone
induces cells to enter meiosis I, progress through interphase, and arrest again at the second meiotic metaphase. Fertilization allows cells to complete metaphase, form a zygote and enter mitosis (Murray and Kirschner, 1989; Norbury and Nurse, 1992). The early stages of cell cycle in X. laevis oocytes are very fast, due to the absence of the G1 and G2 phases. Therefore, the mass of the embryo does not increase during this period. Progesterone treatment of eggs activates a substance, which triggers cell cycle progression in unfertilized eggs, designated maturation progression factor (MPF). Injection of MPF from progesterone stimulated eggs induces meiotic progression in naive eggs. (Dunphy et al., 1988; Gautier et al., 1988). Frog MPF is a heterodimer composed of a catalytic protein kinase subunit and a regulatory subunit, cyclin B.

New protein synthesis is required for the increase in MPF during the mitotic phase of each cell cycle in early frog embryos. Following this clue, studies on sea urchin embryos in which initial cell cycles occur synchronously (as in frog embryos), helped to identify the protein cyclin B. The cyclin B detected in sea urchin embryos peaked in intensity at each mitosis and then fell abruptly during anaphase of each mitosis, slowly accumulating during the next interphase to peak again at the beginning of the following mitosis. Subsequently, a cDNA clone for sea urchin cyclin B was used to isolate a homologous cyclin B cDNA from Xenopus laevis. Similar mitotic cyclins have now been found in all eukaryotic cells analyzed. The cyclin B protein was then shown to comprise one of the two subunits of the MPF.

Identification of the catalytic subunit of MPF and further insight into its regulation came from genetic analysis of the cell cycle in the fission yeast S. pombe. The discovery that Cdc2 from S. pombe is a protein kinase was one of the important clues that led
investigators to demonstrate that MPF has protein kinase activity. Moreover, the
catalytic MPF subunit from Xenopus reacts with antibody prepared against the region
of Cdc2 that is most highly conserved between the human and yeast Cdc2 proteins, and
is about the same size as the Cdc2 protein. Sequencing demonstrated that the MPF
catalytic subunit encodes a protein kinase similar to S. pombe Cdc2.

Most of the proteins identified in cell cycle control and progression are protein
kinases or phosphatases that are regulated by their phosphorylation and
dehphosphorylation states. Cyclins, along with their partners, the cyclin dependent
kinases (cdks) - a family of serine-threonine kinases- together regulate the progression
through the cell cycle. Each cdk is partnered with one or more cyclins, and by acting
together, the specificity of kinase action is achieved.

Activation of the cdks requires both association with their cyclin partners as well as
phosphorylation or dephosphorylation on specific serine and threonine residues
(Hunter, 1991). For instance, activation of cdc2 and subsequent entry into mitosis
requires the synthesis of cyclin B, phosphorylation of cdc2 on threonine161 by cdc2
activating kinase (CAK) and dephosphorylation on tyrosine 15. This regulatory
phosphorylation of cdc2 occurs after the mitotic cyclin B (cdc13) binds to cdc2. Cdc2 is
active only when it is monophosphorylated at threonine 161. The inhibitory
phosphorylation of cdc2 is attributed to Wee1 (and Mik1) along with the simultaneous
inactivity of the phosphatase cdc25.

The most common pathways to inactivate Cdk-cyclin complexes are to destroy the
cyclin or to phosphorylate on substrate or dephosphorylate the cdk. Cdk-cyclin
complexes can also be inhibited by phosphorylation at two sites near the amino
terminus, thr14 and tyr15 in human Cdc2 and Cdk2, respectively. Phosphorylation of these two residues is particularly important in the control of Cdc2 activation at mitosis. Cdc2-cyclinB complexes are maintained in an inactive state, until thr14, tyr15 dephosphorylation at the end of G2 activates Cdc2. Wee1, originally identified in *S. pombe*, phosphorylates Cdc2 *in vitro* at tyr15 but not thr14, supporting the existence of a separate thr14 kinase, possibly the Chk1 kinase (Parker and Piwnica, 1992; Mcgowan and Russell, 1993). Wee1 activity declines during mitosis and concomitantly there is a decrease in the inhibitory phosphorylation. In fission yeast, the protein kinase Nim1 inhibits Wee1 by phosphorylating its carboxy-terminal catalytic domain (Wu and Russell, 1993). The thr14 and tyr15 residues are both dephosphorylated by the phosphatase Cdc25, while activity increases during mitosis, largely because of increased phosphorylation in its N-terminal half (Dunphy, 1994).

Wee1 tyrosine kinase activity is high and cdc25 phosphatase activity is low during interphase, holding the cdc2 and the newly synthesized cyclin B in an inactive state with the cdc2 tyr15 phosphorylated. When mitosis is initiated, Wee1 activity diminishes and cdc25 activity increases, converting cdc2 into its active form. In its active form, cdc25 is phosphorylated, suggesting that one or more additional protein kinases and phosphatases control its activity.

Recent studies have shown that cdc25 becomes phosphorylated *in vivo* on ser 216 after DNA damage in mammalian cells, which pauses progression through the cell cycle either before DNA replication in G1 (G1 checkpoint) or before mitosis in G2 (G2-M checkpoint). The phosphorylation of ser 216 by Chk1 was shown to be functionally important, as non-phosphorylable mutants of cdc25 are defective for G2-M arrest.
14-3-3 proteins sequester cdc25 phosphorylated on ser 216 (Peng et al., 1997) and thereby prevent it from activating cdc2 by dephosphorylation. The inactivation of cdc25 by 14-3-3 proteins is supported by the fact that 14-3-3 proteins encoded by rad24 and rad25 genes are involved in cell cycle arrest after DNA damage in fission yeast (Weinert, 1997).

The early events of mitosis - chromosome condensation and nuclear envelope breakdown occur when MPF (cdc2) protein kinase activity reaches its highest levels in parallel with the rise in cyclin B concentration. Conversely, cyclin B degradation is required for exit from mitosis. Animal cells contain three cyclins that function in a similar manner to cyclin B - cyclin A and two closely related cyclin Bs: All contain a homologous sequence near their N-termini called the 'destruction box'. In intact cells, cyclin degradation begins at the onset of anaphase, while mutant forms of cyclin B that lack the destruction box are not degraded. This degradation occurs by the covalent attachment of multiple copies of a 76-residue protein called ubiquitin (in a process called polyubiquitination), that marks proteins for rapid degradation. Polyubiquitination in turn requires two proteins: a recognition protein that binds the substrate to be degraded; and the enzyme ubiquitin ligase, which adds ubiquitin to the side-chain amino group of lysine residues in the substrate protein. Polyubiquitinated proteins are degraded rapidly by multiprotein complexes of proteolytic enzymes called proteosomes.

In mammalian cells progression through S-phase requires activation of cdk2 paired with cyclins E and A. The synthesis and destruction of cyclin A oscillate in advance of cyclin B during the cell cycle. The A and B type cyclins appear not to be essential for the onset of S-phase, but disruption of cyclin A function can nonetheless inhibit
chromosomal DNA replication. When mammalian cells enter S-phase, cdk2 becomes associated with cyclin A, the synthesis of which begins as cells approach the G1-S transition. This cdk2-cyclin A complex is crucial for progression through the S-phase.

The S-phase checkpoint assesses DNA damage, as replication of damaged chromosomes may lead to cell death or cancer. Detection of DNA damage leads to an increase in p53, which block cell cycle progression by increasing p21 expression and activates DNA repair (through PCNA, GADD45 and other repair components).

The major control element for passage through the S-phase is the retinoblastoma protein (Rb). The ability of Rb to control the G1/S transition is mediated largely through its interactions with E2F. This protein belongs to the E2F/DRTF1 family, a group of transcriptional activators whose target genes are involved in DNA replication and control of cell growth. Upon exposure to proliferative signal activation of the G1 cyclin-dependent kinases CDK4 and CDK6 with their regulatory cyclins phosphorylate the Rb protein of Rb/E2F. At mid-to-late G1, Rb becomes hyperphosphorylated and is released from the promoter-bound E2F, allowing transcription of E2F-regulated genes, such as cyclin E, irreversibly committing the cell to enter S-phase. Recently it has been shown that Rb is associated with yet another family of proteins designated HDAC1-3 (histone deacetylase), which modulate the architecture of chromatin. Chromatin regulation is implicated in cell-cycle control by the Rb/HDAC1-3 complex. Thus, Rb/HDAC1-3 complex is a potent repressor when anchored to E2F-regulated promoters (eg., cyclin E). This repression is alleviated by CDK-directed phosphorylation of Rb, enabling the expression of genes that are essential for cell-cycle progression (Depinho, 1998; Brehm et al., 1998; Magnaghi-jaulin et al., 1998)
Additional regulatory subunits for the Cdns have been identified recently. These proteins bind to the Cdk-cyclin complex, inhibit their activity, and have been designated cdk inhibitory proteins (CKIs). There are at present seven mammalian CKIs that bind to and inactivate CDK-cyclin complexes. Two CKIs from *S.cerevisiae* FAR1 and p40 (SIC1/SDB25), inhibit CDC28-CLN and CDC28-CLB complexes, respectively. A third yeast CKI (PHO81) inhibits the activity of PHO85-PHO80, a CDK-cyclin complex involved in phosphatase gene expression.

The seven mammalian CKIs fall into two classes, the p21 family comprising p21, p27 and p57kip1, and the ankyrin family - p15, p16, p18 and p19. p21 (CIP1/WAF1/CAP20/SDI 1) and p27 are related proteins with a preference for CDK2 and CDK4-cyclin complexes. P16INK4 and p15INK4B are closely related CKIs specific for CDK4 and CDK6-cyclin complexes (Mathias and Ira, 1994).

Disregulation of CDK activity may lead to cancer. For instance, overexpression of cyclin D contributes to testicular cancer development and cyclin E to breast cancer (Wang et al, 1994) while germline mutations of p16 is associated with familial melanoma (Bonetta, 1994; Wainwright, 1994). The role of protein kinases in cell cycle control has been well established. Therefore, the identification of novel cell cycle regulated protein kinases should shed additional light on the control of proliferation in normal and malignant cells.

1.2 **TTK, a novel kinase and its characteristics**

Threonine tyrosine kinase (TTK) is a novel human kinase that is expressed in rapidly proliferating cells of several different cell lineages (Mills *et al.*, 1992). Affinity
purified polyclonal anti-phosphotyrosine (anti-ptyr) antibodies were used to screen a lambda ZAP expression library from YT2C2, a natural killer (NK)-like human cell line. Two clones (31BG and 31CF) encoding identical partial cDNA sequences for TTK were isolated. Subsequent screening with a fragment of this clone of a thymus λgt10 cDNA library and a third cDNA library constructed from the ovarian cancer cell line HEY in lambda ZAPII yielded the 5', 3' and non-coding sequences of the TTK cDNA.

The open reading frame of TTK encompasses 2573 base pairs from nucleotides 978-3550 and initiates at an ATG. The predicted protein sequence comprises 857 amino acids of molecular mass 96,995 daltons, which can be divided into 3 domains, an amino terminal domain (524 aa), a kinase domain (266 aa) and a short carboxy terminal domain (67 aa). The amino terminal domain is unique in that it does not show homology to any known nucleotide or amino acid sequences in respective databases. The amino terminal domain also contains six potential N-linked glycosylation sites and is rich in serine, threonine and cysteine residues (cys) (Fig. 3). The cysteines are arranged in two clusters with 10-15 aa separating each cys residue.

Southern blotting and cloning experiments combined with cloning of the TTK genomic sequence demonstrated that this kinase exists as a single copy gene with no closely related sequences in the human genome. Southern blotting experiments using a 1.7 kb EcoRI fragment of the cDNA as probe revealed hybridizing bands in human, mouse, hamster, bovine, salmon, and yeast DNA. These data indicate that TTK or related sequences have been highly conserved during evolution. PCR analysis of human-hamster hybrid cell lines demonstrated that TTK is on human chromosome 6;
fluorescent in-situ hybridization (FISH) further localized the TTK gene to human chromosome 6q14.

Two polyclonal antibodies (K and L) raised against TTK react with a 97 kDa protein in all proliferating human cells following immunoprecipitation and western blotting. Both antisera immunoprecipitate a 97 kDa protein from $^{35}$S-methionine labeled Jurkat T cells in agreement with the calculated protein mass of TTK.

TTK does not contain the HRDL (his-arg-asp-leu) sequence which is invariably present in members of the tyrosine (tyr) kinase family but rather contains the DLK (asp-leu-lys) motif which is found in members of the serine/threonine (ser/thr) kinase family and in kinases which phosphorylate ser, thr and tyr residues (Hanks and Quinn, 1988; Hanks et al., 1991).

To determine whether TTK phosphorylates tyr, three E.coli expression constructs were used: trpE-R2, which contains the entire kinase domain of TTK; trpE-R14, which is a truncated kinase dead form of trpE-R2; and trpE-fps/fes which contains the entire tyrosine kinase domain of the fps/fes kinase. Western blotting of E.coli lysates with the monoclonal anti-phosphotyrosine (ptyr) antibody PY20 identified a single reactive band in trpE-R2 expressing bacteria, while no reactivity was observed in E.coli expressing trpE-R14. In lysates of E.coli expressing trpE-fps/fes, anti-ptyr antibody reacted with the expressed protein as well as additional proteins native to E.coli. Furthermore, reactivity of the anti-ptyr antibodies with E.coli proteins is blocked by addition of ptyr but not pser or pthr to the western blots. These data suggest that TTK phosphorylates tyr residues.
To demonstrate directly which hydroxyamino acid residues TTK phosphorylates, transformed *E.coli* were loaded with $^{32}$PO$_4$, lysed and blotted to membranes. Bands were cut and hydrolyzed. Phosphoamino acids were separated by two dimensional phosphoamino acid analyses on a cellulose plate. Labeled phosphoamino acids were detected by autoradiography and identified by comparison to ninhydrin stained standards. Pser, pthr, and a small amount of ptyr were detected in *E.coli* transformed with trpE-R2 but not with trpE-R14. Ptyr but not pser or pthr is present in *E.coli* transformed with trpE-fps/fes. This suggests that TTK as expressed in *E.coli* phosphorylates ser, thr and tyr residues, which may explain the ability to detect TTK with anti-ptyr antibodies. The reactivity of the anti-ptyr antibodies with *E.coli* proteins was blocked by addition of ptyr but not pser or pthr, confirming that TTK could phosphorylate tyr. An alternative explanation is that the anti-ptyr antibodies were reacting aberrantly with proteins having ser/thr residues.

Database (TIGR, Genbank and EMBL) searches using TTK sequences identify serine/threonine kinases or multifunctional kinases such as RPK1 (Poch *et al.*, 1991), which phosphorylates serine, threonine and tyrosine residues. RPK1 gene inactivation is lethal, and leads to unscheduled mitosis relative to DNA replication. RPK1 is thus hypothesized to function in a checkpoint control, which couples DNA replication to mitosis (Poch *et al.*, 1994).

### 1.2.1 TTK in cell cycle control

Northern blot analysis of RNA from freshly isolated human tissues, and using a TTK cDNA probe, demonstrated a 4.0 kb message in concordance with the TTK cDNA
size of 3.9 kb. TTK mRNA appears to be present in rapidly proliferating cells both in vivo and in vitro. In normal tissues, steady state levels of TTK mRNA were found to correlate with the extent of proliferation, being highest in the rapidly dividing tissues including bone marrow, testis and thymus. TTK mRNA was also expressed in freshly isolated malignant tissues. TTK mRNA was found to be present in all proliferating cell lines derived from ovarian cancer, choriocarcinoma, melanoma, cervical carcinoma, neuroblastoma, and hematopoietic cells (Mills et al., 1992). Finally, peripheral blood lymphocytes (PBL) that were induced to proliferate by mitogens expressed TTK mRNA, whereas quiescent PBL did not.

Because TTK expression was invariably associated with normal and malignant cell proliferation, it is hypothesized that it might be regulated during the cell cycle. To address this possibility, TTK mRNA, protein and kinase activities throughout the cell cycle in synchronized populations of the neuroblastoma cell line T98G were examined. Indeed, both TTK mRNA levels and kinase activity peaked during the G2/M and fell in early G1, while TTK protein levels remained relatively constant (Hogg et al., 1994). These data are similar to previous observations of other human cell cycle regulated kinases such as p34cdc2 and p33cdk2 which show little change in protein levels throughout the cell cycle, while their mRNA levels and kinase activity vary widely. (Rosenblatt et al., 1992; Welch and Wang, 1992; Dalton, 1993). In fact, TTK mRNA expression and kinase activity closely resemble that of p33cdk2.

It is hypothesized that TTK plays a role in cell cycle control. In support of this notion, the catalytic domain of the S.cerevisiae - RPK1 (regulatory cell proliferation kinase) dual-specificity kinase has strong homology to TTK (Oliver et al, 1994). Null
alleles of this gene are lethal in yeast due to mitotic catastrophe that gives rise to the accumulation of non-viable cells with less than 1N DNA content. Thus, RPK1 may control entry into mitosis in yeast.

In summary, the identification of RPK1 as a homologous member of the family of dual-specificity kinases and its likely role in yeast cell cycle control lends further credence to the hypothesis that TTK also plays a role in mammalian cell cycle control.

1.2.2 Determining the functional role of TTK

To determine which proteins interact with TTK we carried out a protein-protein yeast two-hybrid (Y2H) interaction screen. Protein-protein interactions are essential in all biological processes, including transcription, replication, secretion, signal transduction and metabolism. We hoped that the Y2H screen would provide us with a clearer understanding of the relationship of TTK to one or more signal transduction networks.

The Y2H system is based on the modular nature of many eukaryotic transcriptional activators, such as the yeast GAL4 and lexA proteins (Fig. 2) Many of these transcriptional activators contain separable domains for DNA-binding and transcriptional activation (Ma and Ptashne, 1987). The DNA-binding domain localizes the transcription factor to specific DNA sequences present in the upstream region of genes that are regulated by this factor, while the activation domain contacts other components of the transcription machinery and initiates the transcription process (Fig. 2). Other activators such as herpes virus protein VP16 interact with the DNA indirectly
by binding to other proteins, which in turn directly contact DNA (Mcknight et al., 1987; Ma and Ptashne, 1988).

In the Y2H system, plasmids are constructed that encode two hybrid proteins: one consists of the DNA-binding domain of lex A fused in frame to a bait protein and is selected by the TRPI gene. The other fusion vector consists of the herpes virus VP16 activation domain fused to a cDNA library and carries the LEU2 gene for selection. These plasmids are transformed into a strain of the yeast S.cerevisiae that contains a reporter gene (eg. lac Z) whose regulatory region contains lexA binding sites. The L40 yeast strain can detect weak lexA activators as histidine prototrophs. Positive colonies are detected by the expression of both the lacZ and HIS3 coding sequences driven by GAL1 promoters. Either hybrid protein alone will not activate transcription of the reporter gene, as the DNA binding domain lacks a transactivation function and conversely, the activation domain lacks the lexA binding site. Interaction of the two test proteins reconstitutes the function of lexA, resulting in expression of the reporter gene, which is detected by an assay for the corresponding gene product (for a detailed overview see the Materials and Methods section 2.2). We chose a region of TTK for the bait that lies between codons 135-366, amino terminal to the kinase domain (expression of which is lethal in yeast). This region has no homology to known kinases or other proteins and lacking the kinase domain, does not interfere with growth of yeast colonies. We used a 9.5-10.5 dpc murine embryo library as a source for the input prey cDNA library.
By histidine selection we obtained 100 His (+) yeast colonies of which 20-30 also expressed lacZ. Further mating experiments revealed that only 9 clones expressed both reporter genes only in the presence of both plasmids.

Two Y2H screenings were performed. In the first screening, eIF4G-1 and eIF4G-3 as well as 7 other clones were obtained. In the second Y2H screening, eIF4G-3 and 4 additional clones were obtained. Both eIF4G-1 and eIF4G-3 are eukaryotic protein translation initiation factors.

1.3 Introduction to protein translation

The expression of many eukaryotic genes is regulated largely at the translational level. The mechanisms responsible include phosphorylation of initiation factors, secondary and /or tertiary mRNA structures, cap-independent initiation, and repressor proteins that mask mRNAs (Thach, 1992).

Eukaryotic mRNA is first processed in the nucleus and then transported to the cytoplasm for translation. This processing stabilizes the mRNA by removing the introns, adding a methylated cap at the 5' end and synthesizing a poly (A) tract at the 3' terminus.

Eukaryotic mRNAs are monocistronic and exist as a cytoplasmic ribonucleoprotein particle. The process of protein translation is divided into three stages, a) initiation, b) elongation and c) termination. A brief overview of these three stages is described here.

a) Initiation

An initiation factor designated eIF2 in eukaryotes binds a molecule of GTP (guanosine 5'-triphosphate) and a molecule of Met-tRNA^met to form a ternary complex.
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hydrolyzed, and the cycle is repeated. After the incoming aminoacyl-tRNA is correctly placed in the 'A' site (correct codon-anticodon pairing) the peptide chain is transferred to the amino group of the newly arrived aminoacyl-tRNA, generating a peptidyl-tRNA that has acquired an additional amino acid. At this stage, the peptidyl-tRNA is bound to the ribosome at the 'A' site, and the ribosome moves one codon down the mRNA chain. The translocation reaction is catalyzed by the elongation factor EF₂ in eukaryotes using energy from the hydrolysis of GTP. With this movement, the empty tRNA is released from the 'P' site and the peptidyl-tRNA is shifted to the 'P' site. This sequence of events is repeated for every amino acid added to the growing chain. Thus, two molecules of GTP are used: one to position the tRNA and one to add an amino acid during translocation.

c) Termination

When the ribosome arrives at the UAG codon, the translation is completed with the aid of termination factors. Hydrolysis of the peptidyl-tRNA on the ribosome, with the release of the completed polypeptide and the last tRNA, is followed by dissociation of the two ribosomal subunits. This final step also requires GTP hydrolysis (Lodish et al., 1995).
1.3.1 A brief overview of protein translation initiation

There are five major steps in the pathway that influence the process of translation initiation:

i) Dissociation of ribosomes into subunits

ii) Binding of Met-tRNA to the 40S ribosomal subunit

iii) Binding of mRNA

iv) Recognition of the initiator codon and

v) Junction with the 60S subunit

The process of protein synthesis initiation begins with the small ribosomal subunit by the dissociation of the 80S ribosomes into 60S and 40S subunits. This process is aided by initiation factors, which influence the equilibrium of ribosome subunit dissociation. Specifically, eIF1A and eIF3 bind to the 40S ribosomal subunit and prevent its association with the 60S subunit (Goumans et al., 1980), while eIF6 binds to the 60S ribosomal subunit and inhibits its association with the smaller subunit.

The second step in the initiation process is an interaction between the mRNA cap and a 25 kDa cap recognition protein known as eIF4E. This protein is either already, or subsequently becomes associated with two other subunits, eIF4A and eIF4F, which is a complex of eIF4E, 4A and 4G (Merrick and Hershey, 1996).

Third, helix unwinding is carried out by the ATP-dependent binding of the eIF4A helicase subunit to the first 15 nucleotides of mRNA. This step can be rate limiting for translation if the binding on the mRNA site is masked by a stable secondary structure. EIF4B then dissociates eIF4E and eIF4G-1 subunits from the eIF4F complex. The
released components can recruit free eIF4A to reconstitute active eIF4F. Alternatively
eIF4A and 4B may remain on the mRNA and contribute to further unwinding of the
mRNA (see Fig. 1).

1.3.2 Regulation by mRNA structural elements

The translational efficiency of a mRNA and the rate of initiation are determined by
several structural elements present in a mRNA. These include the M7cap; the initiator
AUG codon and the surrounding Kozak consensus sequences PuCCAUGG; the length
of the 5’ untranslated region (5’UTR); the amount and stability of secondary structure
in the 5’UTR; open reading frames (ORFs) upstream of the initiator AUG; and the
length and structure of the 3’UTR and the polyA tail. Alterations of each of these
structural elements influence both the efficiency and rate of mRNA translation.

The M7cap structure variably affects translational efficiency of the mRNA (Gallie,
1991). Inhibition of cap structure by M7G analogs decreases the rate of protein
translation due to a reduction in binding of eIF4E to the cap (Lawson et al., 1988)

Most mRNAs initiate translation at an AUG codon, as non-AUG codons such as
GUG are less efficient in directing initiation in mammalian cells (Kozak 1989a).
Typically, the first AUG initiates translation, providing it lies within an acceptable
Kozak sequences, comprising a purine at -3 and a G at +4, if the surrounding sequences
do not correspond to the consensus, the ribosome may skip over the initial AUG in a
process called leaky scanning.

An AUG located upstream of the authentic start codon can inhibit translation
initiation. If a stop codon is present immediately after this, the ribosome resumes
scanning and reinitiates at the correct AUG start codon. The longer the distance
between this upstream stop codon and the authentic start codon, the smaller the
inhibitory effect of the upstream AUG. If the stop codon is located within the coding
sequence, i.e. downstream of the authentic AUG, inhibitory effects of the upstream
AUG are maximal, since only ribosomes that ignore the upstream AUG initiate at the
correct start codon (Wera et al., 1995).

A cap-proximal regulatory sequence, designated 5’ terminal oligopyrimidine tract
(5’TOP), mediates the growth dependent translational stimulation of a family of
mRNAs. These mRNAs encode several ribosomal proteins, eukaryotic translation
elongation factors 1A and w, and the poly (A)-binding protein (Sachs et al., 1997;
Meyuhas et al., 1996). Substitution of a purine at the 5’TOP sequences abolishes
translational regulation (Avni et al., 1994).

Another rate limiting element is the 5’UTR. Long 5’UTR secondary structures are
important as they facilitate initiation at the correct site by promoting pausing of the
scanning 40S (Sonenberg, 1996). Currently nothing is known about the tertiary
structure of the whole mRNA (Merrick and Hershey, 1996).

The 3’UTR may contain potential RNA-destabilizing sequences in short lived
transcripts such as c-myc transcript which contains a 3’ UTR AUUUA sequence that
mediates rapid mRNA turnover (Wera et al., 1995).
1.4 The process of eukaryotic protein synthesis

i) Dissociation of ribosomes into subunits

At physiological Mg$^{2+}$ concentrations (~1-2mM), the predominant species is the 80S ribosome; dissociation into 40S and 60S subunits is facilitated by three initiation factors: eIF1A and eIF3 bind to the 40S ribosomal subunit and prevent its association with the 60S ribosomal subunit. EIF6 binds to the 60S ribosomal subunit and inhibits its association with the smaller subunit.

ii) Binding of Met-tRNA to the 40S ribosomal subunit

Prior to binding to the 40S ribosomal subunit, Met-tRNA forms a ternary complex with eIF2 and GTP, stabilized by two initiation factors, eIF2C and eIF3 which are present in low concentrations. When eIF2 completes a round of initiation, it is released from the ribosome as a binary complex with GDP. For eIF2 to function in another round of initiation, eIF2 must exchange the GDP for GTP prior to ternary complex formation catalyzed by eIF2B. It is thought that the ternary complex formation may be partly influenced by the GTP/GDP ratio. The ternary complex binds to 40S ribosomal subunits to form a 43S preinitiation complex. Both eIF1A and eIF3 stabilize ternary complex binding and are present in the 43S preinitiation complex. The binding of Met-tRNA to 40S ribosomes is one of the most important sites of translational control.

iii) Binding of mRNA and recognition of the initiator codon

Two mechanisms exist for the ribosomes to bind to mRNA in eukaryotes. They are,

a) To form a preinitiation complex binding to the 5' terminus, followed by ‘scanning’ down the mRNA until the initiation codon is recognized
b) To bind to an internal site in the mRNA at or upstream of the initiator codon

The first step in the scanning pathway is the recognition of the m⁷G cap structure by eIF4E. Together with eIF4A and eIF4G-1, eIF4E occurs in a cap-binding protein complex designated eIF4F. The affinity of eIF4F for capped mRNA is about 15-fold greater than that of eIF4E alone, suggesting that eIF4F may interact with other regions of the mRNA. Bound eIF4F together with eIF4B possesses ATP-dependent RNA helicase activity which melts secondary structure near the 5' terminus of the mRNA. Next, the 40S preinitiation complex binds to the unfolded mRNA through an interaction of the eIF4G-1 subunit with eIF3, the latter already bound to the 40S subunit.

Alternative models have been suggested to the simple scanning mechanism. It is not certain that scanning involves a linear movement of the 40S complex along the mRNA until the AUG is recognized. In a model suggested by Joshi et al. (1994), eIF4E alone binds the cap and then complexes with eIF4G-1 which already is bound to the 40S ribosome through its interaction with eIF3. Another model called the 'looping' model suggests that the 5' terminus is bound by eIF4F on the ribosome and the AUG simultaneously interacts with ribosome-bound Met-tRNA.

The scanning model predicts that the 5'-proximal AUG serves as the initiator codon for more than 90% of mRNAs. Exceptions to the rule are found if the AUG is less than about ten nucleotides from the 5' terminus (Kozak 1991b). The purine at -3 of the consensus sequence PuCCAUGG appears to have the most impact of any of the AUG context nucleotides. However, the components of the translational apparatus that actually recognize the consensus sequence have not been identified.
v) Junction with the 60S subunit

After the formation of the 40S preinitiation complex, eIF5 binds to the 40S complex promoting the hydrolysis of the GTP carried by eIF2. eIF5 likely functions as a fidelity factor, assessing the correctness of the Met-tRNA-initiator codon interaction. When GTP hydrolysis occurs, the bound initiation factors show reduced affinity for the 40S ribosome. EIF2 is released as a binary complex with GDP. After the initiation factors have dissociated, the 60S ribosomal subunit joins the 40S-initiation complex to form the 80S-initiation complex, which is then competent to enter the elongation phase of translation (Merrick and Hershey, 1996). Evidence from *S. cerevisiae* indicates that the poly (A)-binding protein (PABP) may be involved in the 60S-junction step (Sachs and Davis, 1989).

1.5 eIF4G-1 and its characteristics

We isolated 9 and 7 clones that interacted with TTK in two yeast two hybridization (Y2H) screening. Two of these clones belong to the family of eukaryotic translation initiation factors (eIFs) and are designated *eIF4G-1* (p220; eIF4γ: screen #1) and *eIF4G-3* (p97; NAT-1; DAP-5: screens #1 and 2). Examination of the corresponding full length coding sequences demonstrated that *eIF4G-3* is an isolog of *eIF4G-1*. Since, there was no anticipation of TTK interacting with eukaryotic translation initiation factors, this finding proved to be interesting enough to concentrate on these two proteins for further studies.

*EIF4G-1* was formerly called p220 or eIF4γ. The mammalian *eIF4G-1* cDNA encodes a protein of 154 kDa that migrates as a cluster of polypeptides with mobilities
corresponding to 185-220 kDa due to post translational modifications and partial proteolysis (Joshi et al., 1994; Lamphear and Panniers, 1990). EIF4G-1 is rich in PEST (pro-glu-ser-thr) regions that lead to rapid in vivo degradation in other proteins; stretches of polyglutamic acid; and numerous potential phosphorylation sites. EIF4G-1 also contains a sequence motif AGLGPR (ala-gly-leu-gly-pro-arg) that is common to the substrate recognition sequence of protease 2A from rhinovirus serotype 14 and poliovirus (Yan, et al., 1992).

Recently eIF4G-1 has been shown to possess two separate and independent binding sites for eIF4A (Imataka, et al., 1997). In the translation initiation complex, eIF4G-1 serves as an adapter or assembly protein by binding its amino terminal half to eIF4E and its carboxy terminal half to eIF4A and eIF3 (Mader et al., 1995, Lamphear et al., 1995). It has been recently demonstrated that eIF4G-1 binds simultaneously to Pablp and eIF4E, and these interactions result in the formation of a complex that circularizes mRNA (Tarun et al., 1997; Sachs et al., 1998). Kessler et al. (1998) recently showed that RNA recognition motif 2 of yeast Pablp is required for its functional interaction with eukaryotic initiation factor 4G. Some viral proteases disrupt the entire complex by targeting the adapter molecule, eIF4G-1. For example, cleavage by poliovirus protease 2A (and other picornavirus proteases) separates the eIF4E and eIF3 binding regions of eIF4G-1 (Lamphear et al., 1995). Like eIF4E, eIF4G-1 has been shown to be a translational oncogene whose over production leads to malignant transformation (Fukuchi-Shimogori et al., 1997). EIF4G-1 maps to chromosome 3q27-qter, a region that is amplified in some cases of squamous cells cancer of the lung (Yan et al., 1995).
Using sucrose gradient sedimentation Joshi et al. (1992) demonstrated that eIF4G-1 is present in both 43 and 48S initiation complexes, but not in 80S complexes. This observation supports a model in which free eIF4E binds to mRNA followed by binding of the eIF4E-mRNA complex to a 43S initiation complex already containing eIF4G-1 (Joshi et al., 1992).

The eIF4G-1 homologues in yeast, TIF4631 and TIF4632 (Goyer et al., 1993) show functionally different eIF4E binding sites. Disruption of TIF4631 in yeast leads to a slow growth phenotype, while disruption of TIF4632 fails to show any phenotype. Double gene disruption is lethal in yeast (Goyer et al., 1993). It is possible that eIF4G-1 disruption in mammalian cells will have a similar effect.

Plants contain two different eIF4F complexes. Complex I consist of a factor analogous to eIF4G-1 and p26, equivalent to mammalian eIF4G-1 and eIF4E. Complex II designated eIF (iso) 4F, consists of p28, another homologue of mammalian eIF4E and p82 (Browning et al., 1990; Allen et al., 1992). P82 exhibits significant sequence similarity to human eIF4G-1 (Allen et al., 1992) and the binding site for eIF4E is conserved in the amino-terminus (Mader et al., 1995).

EIF4G-1 is an in vitro substrate for protein kinase A, protein kinase C and S6 kinase. As expected, the amino acid sequence of eIF4G-1 has consensus recognition sites for each of these kinases (McMullin et al., 1988; Tuazon et al., 1989, Yan et al., 1992).

Using heat shock experiments Tarun et al (1997) demonstrated that eIF4G-1 is required for the translation of capped mRNAs, but cannot be synthesized until there are sufficient amounts of active eIF4G-1. To resolve this paradox, Tarun et al showed that
eIF4G-1 mRNA is translated in heat shocked cells under non-cap dependent conditions by means of an IRES sequence.

1.6 eIF4G-3 and its characteristics

EIF4G-3 was cloned by Imataka et al., (1997), Levy-Strumpf et al., (1997; DAP-5), Yamanaka et al., (1997; NAT-1) and our lab (eIF4G-3). EIF4G-3 exhibits 28% identity at the protein level to the C-terminal two thirds of eIF4G-1. The N-terminal part of eIF4G-3 has 63% similarity and 39% identity to the central region of mammalian eIF4G-1.

Translation initiates at a GUG codon in the eIF4G-3 mRNA producing a protein product of 97 kDa (Imataka et al., 1997). EIF4G-3 binds to eIF4A and eIF3, but not to eIF4E as it lacks the amino terminal sequences homologous to eIF4G-1 and hence cannot bind eIF4E. EIF4G-1 binds to eIF4E.

Overexpression of eIF4G-3 represses both cap-dependent and independent translation, and thus reduces overall protein synthesis (Imataka et al., 1997). A mouse cDNA homologue of eIF4G-3 was recently cloned and the sequence deposited into GenBank (U63323). The murine cDNA is shorter by 24 nucleotides at the 5' end compared to the human clone.

EIF4G-3 may inhibit translation by competing with eIF4G-1 for eIF4A, eIF3 and pabp1 or all the three proteins. Conversely, eIF4G-3 may have evolved from eIF4G-1, lost the binding site for eIF4E and hence become a translational repressor (Imataka et al., 1997). While eIF4G-3 clearly represses protein translation, its biological role(s)
may be more widespread. In support of this conclusion, several groups isolated the gene by employing different functional approaches.

Levy-Strumpf et al. (1997) isolated a number of expression clones that conferred resistance to interferon-γ (IFNγ) induced apoptosis. One clone containing the c-terminal portion of eIF4G-3 was expressed at low levels; high level expression was incompatible with continuous cell growth. This clone was designated death associated protein (DAP-5).

Yamanaka et al. (1997) examined transgenic mice expressing the apoB mRNA-editing enzyme (APOBEC-1). These animals developed hepatomas at high frequency, and the tumor cells contained NAT-1 (novel APOBEC-1 target 1) mRNA that was extensively edited to contain missense and nonsense mutations, which reduced the levels of the corresponding protein. NAT1 mRNA is ubiquitously expressed. NAT-1 has homology to the carboxy-terminal portion of the eIF4G-I that binds to eIF4A and eIF4E to form eIF4F. NAT1 bound to eIF4A but not eIF4E and inhibited both cap-dependent and cap-independent translation.

1.7 Control mechanisms of protein translation

The process of protein translation is complex. Instead of merely verifying the end product, a series of regulatory checkpoints verify the fidelity of the ongoing translation process. Aberrant proteins are terminated at any one of these control points. Some of the mechanisms are as follows.
1.8 Growth factor dependent signaling pathways regulating protein translation

Signaling pathways may modulate protein translation by phosphorylating initiation and elongation factors. Initial evidence for regulation of phosphorylation of initiation factors comes from studies on the inhibition of protein synthesis in rabbit reticulocytes by heme deprivation via phosphorylation of eIF2 on the α-subunit (ser-51) (Tuazon et al., 1989). Phosphopeptide mapping of tryptic digests of the phosphorylated subunit show that the individual protein kinases (Protein Kinase C, S6, protease-activated kinase I, cAMP-dependent protein kinase, casein kinase II, casein Kinase I and mitogen-stimulated s6 kinase) modify specific sites, both in vitro and in vivo. For example, treatment of reticulocytes with tumor promoting phorbol esters proved that phosphorylation of translational components in vitro reflects those modified in vivo (Morely and Traugh, 1989). The rate and extent of phosphorylation and dephosphorylation of the four initiation factors, eIF-2; eIF-3, eIF-4B and eIF-4F modify their interactions. These interactions differ in response to different physiological signals that alter the activities of specific protein kinases and consequently the sites and extent of phosphorylation. Four of the initiation factors, eIF2, 3, 4B and 4F are phosphorylated in vitro by protein kinase C and the S6 kinase from liver, an active form of protease activated kinase II. Casein kinase II and protease activated kinase I significantly modify three (eIF3, 4B and 4F) of the four factors. The cAMP-dependent protein kinase phosphorylates eIF4B and to a lesser extent, eIF3. Casein kinase I
modifies only eIF4B, while the mitogen stimulated S6 kinase, isolated from 3T3-L1 cells stimulated with insulin, is specific for ribosomal protein S6 (Tuazon et al., 1989). Specific phosphorylation of eIF2 by the heme controlled repressor and the double stranded RNA activated inhibitor results in formation of an inactive eIF2 P-GDP-guanine nucleotide exchange factor complex (Pain, 1986). In most other cases, phosphorylation of initiation factors regulates translation positively. For instance, phosphorylation of eIF4E (p28) increases translation efficiency, while heat shock of HeLa cells leads to a rapid inhibition of protein synthesis, due in part to dephosphorylation of eIF4E followed by disaggregation of the eIF4F. In addition, in serum starved cells, the readdition of serum causes a stimulation of eIF4B (Duncan and Hershey, 1985). These data are indicative of the importance of phosphorylation of translation initiation factors in translation control (Duncan, et al., 1987).

Additional experiments have further refined the mechanism of eIF4E/4F modification. Following treatment of rabbit reticulocytes with phorbol12-myristate 13-acetate (PMA) for 30 min, increased phosphorylation of both the eIF4E and 4G subunits occurs. Only the more acidic of the two variants of eIF4E becomes phosphorylated, with the level of phosphorylation increasing upon PMA treatment. Two-dimensional phosphopeptide mapping of labeled eIF4E identifies a single phosphopeptide, the intensity of which increases upon PMA treatment of the cells. The phosphorylation site from this was localized to ser 53. Phosphorylation of eIF4E does not appear to alter the apparent recognition of the cap structure. However, overexpression of wild type eIF4E-ser 53, but not mutated eIF4E-ala 53, results in malignant transformation of NIH 3T3 cells. This observation suggests a growth
controlling function for eIF4F independent of m⁷GTP binding and indicates that phosphorylation of ser 53 is required for eIF4F function (Karatzas, 1990). A more complex phosphopeptide map is observed in the case of eIF4G-1. The maps for both subunits contain the same phosphopeptides as those obtained when eIF4F is phosphorylated in vitro by the Ca²⁺/phospholipid-dependent protein kinase, suggesting that protein kinase directly modulates eIF4F in response to PMA (Morley and Traugh, 1989).

The overall rate of protein synthesis in a cell is a function of the number of actively translating ribosomes and the rate at which they incorporate amino acids into new proteins. Different mRNAs are translated at varying rates, possibly due to control at the level of translation initiation. That eIF4E may play a role at this point is suggested by the observation that there is a cytoplasmic redistribution of ribosomal protein L32 mRNA and phosphorylation of eIF4E after mitogenic stimulation of Swiss 3T3 cells. These data are consistent with a model in which mitogenically induced phosphorylation increases the pool of active eIF4E molecules, which in turn increases the recruitment of translationally controlled mRNAs to actively synthesizing ribosomes (Kaspar et al., 1990). Chemical cross-linking of eIF4F to cap labeled mRNA, further demonstrates that phosphorylation increases the interaction of eIF4E with the 5' end of mRNA, resulting in an increased rate of initiation complex formation (as measured by an increase in the association of labeled mRNA with 40S ribosomal subunits in the translation complex) (Morley et al., 1991). The interaction of eIF4E with the mRNA 5’-cap structure is dramatically enhanced by eIF4G-1, as determined by an UV-induced cross-linking assay. Furthermore, assembly of the eIF4F complex at the cap structure, as well as
ATP hydrolysis, is shown as a requisite for the cross-linking of another initiation factor, eIF4B, to the cap structure (Haghighat and Sonenberg, 1997). Phosphorylation of both eIF4E and 4G subunits are perhaps required for maximal activity of the factors. Interaction between the subunits of the eIF4F complex alters their conformation and influence phosphorylation effect and binding to mRNA cap. EIF4F is more effective in binding to the mRNA cap than eIF4E alone. But, it is not known whether eIF4G-1 becomes associated with 4E before mRNA binding to 40S ribosomal subunit, after mRNA binding, or following transfer of 4E and mRNA to the 43S initiation complex (Rhoads, 1988).

Experiments using okadaic acid, which results in the phosphorylation of eIF4E and 4G, further demonstrated that phosphorylation stabilizes interaction between the eIF4E and 4G subunits. Treatment of HepG2 cells with okadaic acid results in phosphorylation of as much as 20% of eIF4E on threonine residues; tryptic phosphopeptide maps identify several previously unrecognized phosphopeptides. In addition, hyperphosphorylation of eIF4E and 4G in response to okadaic acid increases the quantity of the eIF4E- eIF4G-1 complex that binds to the mRNA cap structure (Bu et al., 1993). The observation that dephosphorylation of eIF4E in heat shocked Ehrlich ascites tumor cells correlates with decreased binding of eIF4G-1 but not 4E to m7GTP-Sepharose is likely due to the observation that only phosphorylated eIF4E associates with 4G (Lamphear and Panniers, 1991). As of this time, not all protein kinases that phosphorylate eIF4E and eIF4G response to growth regulatory signals have been identified (Bu et al., 1993). Moreover, because okadaic acid treatment results in
hyperphosphorylation of both eIF4E and eIF4G-1, specific phosphatases may also play a role in the control of protein translation.

1.9 Rapamycin regulated control of protein translation (FRAP)

The process of translation initiation is effected by the efficient assembly of the complex of initiation factors on the mRNA that unwind secondary structures in the 5'UTR (Sonenberg, 1996). Recently two regulatory phosphoproteins that interact with eIF4E have been identified and are designated 4E-BP1 and 4E-BP2. These compete with eIF4G-1 for binding to eIF4E; unphosphorylated 4E-BP1 and BP2 bind directly to 4E thereby preventing eIF4G-1 from associating with the complex. Growth factors stimulate the phosphorylation of 4E-BP1 and cause its dissociation from eIF4E (Haghighat et al., 1995; Feigenblum and Schneider, 1996). Rapamycin inhibits the serum-induced phosphorylation of 4E-BP1 (Beretta et al., 1996) suggesting that translational events dependent on the rapamycin-sensitive signaling pathway be regulated in part through the phosphorylation of 4E-BP1.

The serine/threonine kinase p70s6k, which is stimulated by growth factors, is another rapamycin-sensitive mediator in signaling cascade. For a long time, this kinase was known to phosphorylate the S6 protein in the 40S ribosomal subunit (Chou and Blenis, 1995), and phosphorylated S6 protein is found in active polyribosomes compared to inactive ones. Rapamycin causes rapid dephosphorylation of p70s6k and results in its inactivation. The exact nature of the interaction between S6 phosphorylation and 4E-BP1 is not well established.
The inhibitory effects of rapamycin on cell cycle progression and translation are dependent upon an association of rapamycin with the intracellular protein FK506-binding protein (FKBP12). The FKBP12-rapamycin complex is a high affinity inhibitor of a 228 kDa protein termed FRAP/RAFT1 (for FKBP12-rapamycin associated protein/rapamycin and FKBP12 target, respectively). FRAP belongs to the newly discovered family of phosphatidylinositol kinases-related kinases (PIK-related kinases) involved in events ranging from cell cycle regulation to DNA recombination (Keith and Schreiber, 1995). FRAP has intrinsic protein kinase activity as it autophosphorylates on a serine residue in vitro (Brown et al., 1995) and regulates the activity of p70^{65k} and hence 4E-BP1 phosphorylation. Treatment of cells with wortmannin (an inhibitor of phosphatidylinositol-3-kinase-pI3K) prevents the growth factor-regulated increase in p70^{65k} activity (Chou and Blenis, 1995) and 4E-BP1 phosphorylation (von Manteuffel et al., 1996). Currently it is not clear how signals are propagated from pI3K to p70^{65k} and 4E-BP1, as FRAP does not appear to be upstream of pI3K (Chou and Blenis, 1995). In addition, phospholipase-C gamma (PLCγ) protein kinases PKC alpha and Akt/PKB may play a role(s) in this pathway (Chou and Blenis, 1995).

Recent studies show that regulated translation modulates the activity of cyclin-dependent kinases in mammalian cells. The expression of the cdk inhibitor p27^{kip1} changes after treatment with PDGF or with lovastatin through an altered rate of mRNA translation (Agrawal, et al., 1996; Hengst and Reed, 1996). Since mitogen stimulated activity of cyclin-dependent kinases is sensitive to rapamycin treatment, FRAP might play a role in p27^{kip1} expression. Treatment of T-lymphocytes with IL-2 modulates the levels of cdk inhibitor p21^{cip1} and p27^{kip1} in a rapamycin sensitive fashion (Nourse et al.,
1994), and increasing levels of p27kip1 inhibit cyclinE/cdk2 in non-proliferating cells. IL-2 mediated removal of p27kip1 is blocked significantly by rapamycin but not by FK506, indicating that alteration of p27kip1 is FRAP/RAFT-dependent. Cdk inhibitory activity associated with p27kip1 is induced by the drug lovastatin or upon density mediated growth arrest in G1 by altering the amount of p27kip1 protein, but not the mRNA. This post transcriptional control of p27kip1 protein levels is partly due to translational control, although in density arrested fibroblasts and thymidine arrested HeLa cells the half life of the protein also changes (Hengst and Reed, 1996).

Autophosphorylation of FRAP is inhibited by FKBP12-rapamycin, consistent with an essential role for FRAP kinase activity in vivo. Deletion studies in FRAP indicate that the kinase activity of FRAP alone is not sufficient for control of p70^s6k and that an amino-terminal domain in FRAP is also required (Brown, et al., 1995). A rapamycin-sensitive pathway translationally controls the insulin-like growth factor (IGF-II) mRNA. (Nielsen, et al., 1995). The human IGF-II gene generates two transcripts; comprising a minor 4.8 kb mRNA and a 6.0 kb mRNA, having different 5' untranslated regions (5'UTRs), but identical coding regions and 3'UTRs. The 4.8 kb minor transcript directs the synthesis of pre-pro IGF-II, while the major 6.0 kb mRNA is stored in a 100S ribonucleoprotein particle. In dispersed exponentially growing cells the 6.0 kb mRNA is selectively mobilized and translated. The translation activation is inhibited by rapamycin and by anisomycin, suggesting that translation of the 6.0 kb mRNA is regulated by the p70^s6k/85^s6k kinase signaling pathway (Nielsen et al., 1995).

Present data indicate that rapamycin exerts its effect on translation by inhibiting p70^s6k/p85^s6k, causing the dephosphorylation of S6. This implicates S6 phosphorylation
in a selective translational control mechanism that up-regulates the translation of the 5'TOP mRNA family after mitogenic stimulation (Jeffereies and Thomas, 1996).

Although both the cytoplasmic p70^S6K and the nuclear p85^S6K isoforms share a common signaling pathway, the upstream kinases and mediators have not yet been identified. Recent studies demonstrate that p70^S6K/p85^S6K activation is controlled by the phosphorylation of two sets of amino acid residues, implying that at least two signaling pathways lead to its activation (Pearson, et al., 1995). Identification of these sites is critical for the evaluation of the regulatory pathways involved in the activation of these kinases.

1.10 Interaction between protein translation initiation factors and binding proteins

Recently, two human cDNAs encoding novel eIF-4E binding protein (4E-BPs) which function as repressors of cap-dependent translation have been cloned. The mechanism of their interaction with eIF-4E is through negative regulation by phosphorylation when the cells are treated with growth factors (Mader, et al., 1995). EIF4G-1, which is involved in binding eIF-4E, contains a conserved motif. This conserved motif is also present in the regulatory proteins 4E-BP1 and 4E-BP2, which is required for binding to eIF-4E. 4E-BP1 is a heat and acid-stable protein (PHAS-1) which is phosphorylated by MAP kinase on serine 64 in response to insulin and growth factors that signal through the MAP kinase pathway (Lin, et al., 1994). This observation suggests that eIF4G-1 and 4E-BPs compete for binding to eIF-4E (Sonenberg, 1996). The region of interaction corresponds to a 49 aa region in human eIF4G-1 (aa 409-457)
that is identical to the murine sequence. This region is also present in the eIF4G-1 yeast homologues of 4G, TIF-4631 and TIF-4632. Deletion mutagenesis of the binding motif confirmed that it is essential for the interaction between 4E-BPs and eIF-4E, and for the interaction between eIF4G-1 and eIF-4E (Mader, et al., 1995). MAP kinase phosphorylates 4E-BP1, which decreases its affinity for eIF4E and thus relieves translational inhibition. (Haghighat, et al., 1995). EIF4E is hypophosphorylated during mitosis (Bonneau and Sonenberg, 1987a), following heat shock (Duncan et al., 1987) or infection with several viruses (Huang and Schneider, 1991), concomitant with a reduction in cap-dependent translation rates (Minich, et al., 1994).

While both 4E-BPs exhibit high sequence homology to PHAS-1 (Hu, et al., 1994) (4E-BP1: 93%; and 4E-BP2: 55% identity to PHAS-1), the two proteins exhibit a differential tissue distribution, and it is possible that they modulate the translation of specific mRNAs in a tissue-dependent manner (Haghighat, et al., 1995). In addition, 4E-BP2 contains a potential phosphorylation site for protein kinase A, which is absent in 4E-BP1, suggesting an additional level of regulation of translation by diverse extracellular signals.

Following poliovirus infection, which leads to the cleavage of eIF4G-1, crosslinking of eIF4E to the mRNA cap structure is reduced dramatically (Rozen and Sonenberg, 1987). Cleavage of eIF4G-1 alone is not sufficient for the complete inhibition of host protein synthesis after poliovirus infection. The subsequent dephosphorylation of 4E-BPs and increased affinity for eIF-4E also contribute to the complete shut down of host protein synthesis (Bonneau and Sonenberg, 1987b). Protein synthesis abrogation occurs in encephalomyocarditis (EMC) virus infection, even though there is no accompanying
cleavage of eIF4G-I, thereby supporting the view that dephosphorylation of 4E-BPs is a significant contributor to host protein synthesis shut off. (Bonneau and Sonenberg, 1987b).

Recent work has demonstrated that cap-dependent mRNA translation is independently regulated by the phosphorylation state of both eIF4E and 4EBP-1; moreover BP-1 is not involved in all pathways that inhibit cap-dependent translation. For instance heat shock blocks cap-dependent translation by inducing both eIF4E dephosphorylation and sequestration of dephosphorylated eIF4E by BP-1, while rapamycin inhibits BP-1 sequestration of eIF4E during heat shock without blocking the dephosphorylation of eIF-4E. These findings support a dual role for eIF4E dephosphorylation and sequestration by BP-1 in blocking protein synthesis during heat shock (Feigenblum and Schneider, 1996)

Experiments using adenovirus (Ad) infected cells show that early during infection, the virus induces phosphorylation of BP-1 and release of sequestered eIF4E, stimulating cap-dependent protein synthesis. The early Ad-induced phosphorylation of BP-1 persists into the late phase of infection, at a time when Ad also blocks eIF4E phosphorylation. Also, rapamycin blocks Ad-induced phosphorylation of BP-1 without influencing the dephosphorylation of eIF4E. Experiments with heat shocked and Ad-infected cells have shown that the regulation of eIF4E and BP-1 occurs by cycles of phosphorylation and dephosphorylation (Feigenblum and Schneider, 1996). The presence of at least two independent mechanisms of regulation of translation of cap-dependent mRNAs provides the cell with a fine-tuning mechanism for translational
selection of mRNA based on their dependence on eIF4F (Feigenblum and Schneider, 1996; Sonenberg, 1996).

1.11 Translational control and heat shock proteins

The essence of heat shock translational response is the ability of heat shock protein (hsp) mRNAs to be translated very efficiently when most of the non-heat shock mRNAs are severely repressed (Lindquist 1981; Duncan and Hershey 1989). It is thought that unique structural features in heat shock mRNAs enable them to bypass the initiation factor disruption due to heat shock. Some of the mechanism and molecular events underlying translational repression and restoration are briefly mentioned here.

a) Initiation is principally inhibited, but elongation may be affected as well.

The initiation phase is the primary repressed step following heat shock, as evidenced by a disaggregation of polyribosomes into inactive monosomes and ribosomal subunits.

b) A deficit in 43S preinitiation complex formation occurs in heat shocked cells (Panniers and Henshaw, 1984), suggesting that the binding of the ternary complex eIF2-GTP-Met-tRNA is impaired. Defective eIF2 function has been confirmed in this situation.

c) Initiation factors including eIF2 and eIF4F show reduced activity

A reconstituted system approach indicates that eIF2 and eIF3/eIF4F are significantly inhibited by heat shock (Duncan and Hershey 1984). Supplementation analyses in unfractionated lysates indicate that eIF4F can restore non-heat shock mRNA
translation in heat shock cell free systems from mammalian and drosophila cells (Panniers et al., 1985; Zapata et al., 1991).

d) Molecular alterations that cause translational repression

The ability of eIF2α to promote Met-tRNA binding to the 40S ribosomal subunit by phosphorylation at ser 51 residue of eIF2α subunit constitutes an important regulatory event under heat shock conditions. Heat shock increases eIF2α phosphorylation in a temperature-dependent manner (Duncan and Hershey, 1984). Phosphorylation of eIF4F increases its initiation activity (Morley et al., 1991). EIF4E phosphorylation decreases substantially during moderate to severe heat shock (>42°C) (Duncan and Hershey, 1989; Lamphear and Panniers, 1991), implicating eIF4F inactivation in heat shock induced translational repression. However, during milder heating (41-42°C), no detectable dephosphorylation occurs (Duncan and Hershey, 1989). Thus, this dephosphorylation of eIF4E occurs in some but not all heat shock circumstances, paralleling eIF2α phosphorylation.

At moderate to severe heat shock, the mammalian eIF4F complex dissociates, releasing “free” eIF4E (Duncan et al., 1987). Phosphorylation of eIF4E is a molecular event that regulates eIF4F heterotrimer association/dissociation (Lamphear and Panniers, 1991), and is controlled by the eIF4E-binding protein. Another initiation factor, eIF4B is loosely associated with eIF4F. It dissociates during moderate to severe heat shock and is substantially dephosphorylated in both mammalian and Drosophila cells (Duncan, et al., 1995). Both eIF2α and eIF4F (4E) are converted into their inactive forms during moderate to severe heat shock in mammalian cells. In addition to protein
synthesis inhibition, there is a preferential translation of heat shock mRNAs, due to their special structural features. The 5'UTR of HSP mRNAs contains sufficient primary sequence information for preferential translation. Some of the features are,

1. Relatively long (200-250 nucleotides) 5'UTRs,
2. Conserved sequence blocks in similar positions relative to the cap site, suggestive of an important regulatory function,
3. Very high adenosine content (~45-50%), resulting in a low potential for forming secondary structure.

Several lines of evidence strongly suggest that preferential translation is based on a capacity of hsp mRNAs to bypass an eIF4F lesion. There is also evidence of translation regulation by hsp70. During heat shock followed by recovery, hsp70 initially translates very efficiently and then its translation is rapidly repressed due to rapid hsp70 mRNA degradation (Petersen and Lindquist, 1989).

1.12 Viruses and translational control

The host cell shutoff by rhinoviruses, enteroviruses, and aphthoviruses is mediated, at least in part, by the virally induced cleavage of a protein synthesis initiation factor eIF4G-1. Although early evidence suggested that viral proteases play only an indirect role in eIF4G-1 proteolysis, recent results support direct cleavage of eIF4G-1 by virally encoded proteases (Lamphear, et al., 1995). In addition, in cells infected with adenovirus and influenza virus, the extent of eIF4E phosphorylation decreases (Huang and Schneider, 1991; Feigenblum and Schneider 1993). Such dephosphorylation is believed to contribute to host cell shutoff by placing cellular mRNAs at a disadvantage.
when they are competing against viral mRNAs. Phosphorylation of 4E-BP1 decreases concomitantly with the inhibition of host-protein synthesis in EMCV-infected cells, further suggesting that translation of the uncapped viral RNA is favored by diminished cap-binding activity.

The enzymes responsible for eIF4F cleavage are protease 2A in the enteroviruses (including poliovirus and coxsackievirus) and rhinoviruses (the common cold virus), and protease L (in foot and mouth disease virus). Cleavage of eIF4G-1 effectively separates it into two domains; an amino-terminal domain that interacts with eIF4E, and a carboxy-terminal domain that interacts with eIF4A and eIF3 (Lamphear, et al., 1995; Mader et al., 1995). Therefore, cap-dependent initiation is severely inhibited. Since the IRES-dependent initiation mechanism is unaffected or even facilitated (Ohlmann, et al., 1993), eIF4G-1 cleavage sets these picornavirus mRNAs at an advantage. Following poliovirus infection, cross-linking of eIF4A polypeptides to the cap structure is altered. The pattern of cap binding protein complexes recovered from infected cells is distinctly different from that of uninfected cells, suggesting that infection disrupts macromolecular complexes important for cap-dependent initiation (Etchinson and Smith, 1990; Lamphear, et al., 1993). No alteration in other eIF3 or eIF4A polypeptides is apparent consequent to infection. Protein complexes containing intact eIF4G-1 restore cap-dependent translation in extracts of infected cells. These studies suggest a domain model for eIF4G-1 wherein the cap recognition function is in the N-terminal one third of the molecule and the unwinding and ribosome binding functions are in the C-terminal two thirds. The region separating the N and C terminal domains may be a flexible hinge or loop which is more exposed to proteases. With such a domain model
for eIF4G-1, two models for the mRNA-binding step of initiation have been proposed:
1. the stepwise assembly and 2. the preformed complex. Recruitment of capped mRNAs requires the coupling of cap recognition, unwinding and scanning. The models differ primarily in which of these functions occur on the ribosomes.

1.13 New roles for protein translation initiation factors

1.13.1 eIF-4E as an oncogene

EIF4E subunit of the initiation complex is present in limiting amounts in the cell and is regulated by phosphorylation. Decreased phosphorylation of eIF4E following various treatments correlates with a decrease in cellular translation rate, while overexpression of eIF4E profoundly affects cellular growth properties. Overexpression of eIF4E in NIH 3T3 or Rat 2 fibroblasts caused their tumorigenic transformation, as determined by the formation of transformed foci on a monolayer of cells; anchorage independent growth and tumor formation in nude mice. Activation of eIF4E through phosphorylation occurs after stimulation by diverse extracellular agents including serum; platelet derived growth factor, insulin, TNF and TPA. In addition, viral tyrosine kinase components like v-src or v-fps also enhance phosphorylation of eIF4E.

Additional support for a role for eIF4E in control of cell growth comes from genetic studies in yeast. The S.cerevisiae cell cycle mutant, CDC-33 harbors a mutation in eIF4E, that is complemented by a mutation in the regulatory subunit of cAMP-dependent kinase, hinting that phosphorylation of eIF4E has a crucial role in regulating its activity (Lazaris, et al., 1990).
Microinjection of eIF4E mRNA into early embryos of *Xenopus laevis* leads to the induction of mesoderm in ectodermal explants. This induction occurs without a stimulation of overall protein synthesis and is blocked by the co-expression of a dominant negative mutant of the proto-oncogene ras or a truncated activin type II receptor. Other translation initiation factors have not been shown to play a direct role in development (Klein and Melton, 1994).

A mutated eIF4E in which ser 53 has been converted to alanine also leads to the elongation of ectodermal explants in *X. laevis*, although in a lower percent of cases. This mutant is inactive in cell transformation assays, but in some cell types, the ala 53 mutated eIF4E appears to be functional and highly phosphorylated, suggesting that alternative sites of phosphorylation are important for regulation in other cell types. The induction of mesoderm by eIF4E is carried out by a mechanism called homogenetic induction. Alternatively, increased levels of eIF4E may lead to preferential translation of a limited set of low abundance mRNAs that includes mesoderm-inducing factors. The transforming ability is attributed to translation of growth factor mRNAs with inhibitory secondary structure in the 5’ end, a feature commonly found in mRNAs encoding growth regulatory molecules (Lazaris, *et al.*, 1990; Benedetti and Rhoads, 1990). Finally, Polunovsky *et al.*, (1996) demonstrated that overexpression of eIF4E in NIH3T3 cells prevents apoptosis induced by serum depletion.
2. Materials and Methods

2.1 Background

As mentioned in the introduction, TTK is a novel kinase (Mills et al., 1992) that is regulated in a cell cycle dependent manner (Hogg et al., 1994). However, the biological role(s) of TTK remains unknown. We used the protein-protein yeast two-hybrid (Y2H) interaction system, in an effort to identify novel partners of this kinase.

2.2 Yeast two hybrid system

The Y2H system that identifies interaction between two proteins was devised by Stanley Fields (1989). In principle this system consists of a ‘bait’ fused in frame with a protein -DNA binding domain (lex A or GAL4) and a ‘prey’ built in-frame with an transcription activating domain of a transcription factor (VP16 vector carrying a murine cDNA library from mouse embryos 9.5/10.5 dpc). These hybrids were transformed into yeast using the lithium acetate protocol (Ausubel et al., 1989) (Fig 2). Positive clones were selected through the expression of the reporter gene lacZ and histidine (HIS) medium selection. The ‘bait’ used the TRP1 (Tryptophan) and the ‘prey’ LEU2 (Leucine) selectable markers respectively. True positive clones were determined by ‘curing’ the bait of the prey by extended growth in the absence of tryptophan selection, allowing the segregation and growth of daughter cells without the lexA fusion plasmid. The rescued prey was mated again with the same ‘bait’ or another ‘bait’ system to identify true positive clones.
The part of TTK chosen for the 'bait' was the region between codons 135-366. This region was selected as it was away from the kinase domain, which is lethal when expressed in yeast (not shown).

Two Y2H screenings were done. In the first screening clones eIF4G-1 (1.7) and eIF4G-3 (24) along with 7 other clones were obtained. In the second Y2H screening eIF4G-3 was isolated along with 4 other clones. Both eIF4G-1 and eIF4G-3 clones were considered important, as they encoded translation initiation factors. 

EIF4G-1 is also known as eIF4G (or gamma), EIF4G-3 is an isolog of eIF4G-1. A full description of these two factors was presented in the Introduction chapter. Since very little was known about eIF4G-3, it was decided to characterize this gene fully. EIF4G-1 was already cloned and characterized elsewhere (Yan et al., 1992).

2.3 cDNA screening for the full length eIF4G-3 clone

2.3.1 Description of the host strain for the Lambda ZAP II vector

The host strain for the Lambda ZAP II vector (containing the human umbilical endothelial venule-HUVEC cDNA library) is the recA E.coli host strain, XL1-Blue. The XL1-Blue produces blue plaques with non-recombinant phage and white plaques with recombinants.

The F’ episome in the XL1-blue strain contains the DM15 mutation of the lacZ gene required for alpha complementation of the amino terminus of the lacZ gene present within the Lambda ZAP vector. The expression of these partial lacZ genes both from the vector and the host strain is necessary for the generation of a functional β-
galactosidase protein from the Lambda ZAP II vector. The F' episome also contains the
genesis for the expression of the bacterial pili which are required for filamentous phage
infection.

The conversion of a recombinant Lambda ZAP clones to the pBluescript phagemid
requires superinfection with a filamentous helper phage. The F' episome encodes the
lac repressor which is important for blocking transcription from the lacZ promoter in
the absence of the inducer, IPTG (isopropyl β-thiogalactoside). This repressor is
important for controlling expression of fusion proteins that may be toxic to E.coli. The
presence of the Tn10 tetracycline gene on the episome allows it to be selectively
maintained in the presence of the antibiotic.

2.3.2 In vivo excision of the pBluescript SK (-) plasmid from the
Lambda ZAP II vector

The lambda ZAP II system has been designed to allow in vivo excision and
recircularization of any cloned insert contained within the lambda vector to form a
phagemid containing the cloned insert. The in vivo excision is dependent on the
proteins derived from a fl bacteriophage. For 'positive strand synthesis' the fl
bacteriophage is dependent on the recognition of the origin of replication (Ori). The Ori
is divided into two parts, 1. An initiation site and 2. A termination site for DNA
synthesis, both of which have been cloned separately into the Lambda ZAP II vector.
When XL1-Blue cells bearing a Lambda ZAP II phage are infected with the fl
bacteriophage, the 'helper' proteins from the fl bacteriophage recognize the initiator
DNA that is within the lambda vector. These proteins nick the DNA at the site of
initiation and replicate a single new DNA strand up to the 3' termination signal. The resultant single stranded DNA molecule is circularized by another fI gene product and includes all sequences of the phagemid, pBluescript SK (-) and the cDNA insert.

The necessary elements required for the packaging of the newly created phagemid is present within the fI terminator origin DNA sequence. They permit the circularized DNA to be ‘packaged’ and secreted from the E.coli. By heat treatment of the E.coli that kills the E.coli but retains the heat resistant phagemid, the circularized DNA to be packaged is saved. For production of double stranded DNA, the ‘packaged’ pBluescript DNA is mixed with fresh E.coli cells and spread on LB/ampicillin plates to produce colonies.

2.3.3 In vivo excision

A part of the Lambda ZAP II cloning kit (Stratagene, USA) is the ExAssist/SOLR system. The ExAssist/SOLR system is designed to allow efficient excision of the pBluescript SK (-) phagemid from the Lambda ZAP II vector. The ExAssist helper phage contains an amber mutation that prevents replication of the phage genome in a non-suppressing E.coli strain such as SOLR cells. This allows only the excised phagemid to replicate in the host, removing the possibility of coinfection from the ExAssist helper phage. This system cannot be used for single strand rescue due to its inability to replicate in the SOLR strain.
2.3.4 *Titration of phage stock*

To determine the titre of the phage HUVEC-cDNA library, I infected *E. coli* strain XL1-Blue with several dilutions of the library. Specifically, 1μl of the library stock was diluted to concentrations of $10^3$, $10^4$, $10^5$ and $10^6$ in SM buffer. One μl of each dilution was used to infect 200μl XL1-Blue cells in log phase (grown over 3hrs in LB media to a density of $0.5_{od}$, spun down at 5000G and resuspended in 10mM Mg$_2$SO$_4$. The cells were incubated at 37°C for 15 minutes and then top agarose preheated to 42°C was added. The cell suspension was quickly and gently mixed and then poured directly onto N2Y agar plates prewarmed to 37°C. The plates were then incubated at 37°C for 16-20 hrs and the number of plaques counted. I calculated the library titre as follows:

\[
\text{Titre} = \frac{\text{plaque number/dilution}}{\mu l \times 1000 \mu l/ml} = \text{Library plaques/ml.}
\]

The titration was adjusted to observe 50,000 plaques/plate.

2.3.5 *Primary Screening*

About 20 plates were screened with a final dilution of $10^6$ phages/ml giving me a total of $5 \times 10^4$ phages/plate.

Nylon filters were placed on the plaques formed, with the center of the filter touching the plate first and allowed slowly to spread on the plate. The filters were allowed to sit on the plate for 2-5 minutes and marked distinctly in 3 different spots with India ink. The filters were then removed and air-dried for a few minutes on 3MM filter paper. The filters were placed in a series of three solutions for 1-2 minutes each. The solutions were a) 1.5M NaCl/0.5M NaOH b) 0.5M Tris-HCl (pH8.0)/1.5M NaCl
and c) 0.2M Tris-HCl (pH 7.5)/2xSSC. The filters were then air dried for 30 minutes and cross-linked in an UV-cross linker at 1200x100 pJoules/cm² for 2 minutes.

The cross-linked filters were transferred to a prewarmed prehybridization solution of 1M NaCl and 1% SDS at 60°C. Prehybridization was carried out for 1-2 hrs at 60°C in circular Pyrex glass containers. 100ml of hybridization solution (1% SDS, 1M NaCl and 5% dextran sulphate) was then added to the filters in the hybridization chamber along with the entire probe prepared in 400μl and hybridization was performed overnight at 60°C. 2x10⁶ cpd/ml of high specific activity probe were added to the hybridization solution.

2.3.6 Probe Preparation

The clone #24 obtained previously from Y2H screening was grown on LB/ampicillin plates. Single colonies were picked up and grown in a 2ml culture for ‘mini-preparation’ DNA by alkaline lysis. The mini-prep DNA was digested with Not I restriction enzyme (New England Biolabs, USA), run on a mini-gel and the insert eluted from the gel using the Geneclean II kit (BIO 101 Inc, USA). The purified insert from clone #24 was used as the probe in the cDNA library screening.

5μl of #24 mini-prep DNA was taken and made up to a volume of 34μl using TE buffer. Labelling was done using ³²P-dCTP and the oligolabelling kit (Pharmacia Biotech, USA). This kit allows labeling of 25-50ng of DNA to 1-2x10⁹ dpm/μg. After making up the volume to 34μl the DNA was denatured by heating for 2-3 minutes in a water bath at 95-100°C and cooled on ice for 2 minutes. This sample was then centrifuged briefly and made up to 49μl with 10μl of reagent mix, 5μl of α-³²P
labeled nucleotides, 1μl of Klenow fragment and distilled water. The mixture was centrifuged briefly and incubated at 37°C for 30-60 minutes.

The probe was purified using the Nick columns (Pharmacia Biotech, USA). These are prepacked disposable columns containing Sephadex G-50 DNA grade for rapid separations of nick-translated DNA from unincorporated 32P labeled nucleotides. The probe was eluted using TE buffer. Two μl of eluate were then added to a scintillation vial containing 2ml of cytoscent fluid to determine the amount of 32P incorporated using the scintillation counter. The eluted probe was boiled for 5 minutes before being added to the hybridization solution and the mixture of probe and hybridization was poured into a hybridization cylinder containing the filters.

The filters were washed 7-8 times in 0.2xSSC/0.1% SDS at 55°C. Each filter was wrapped individually in saran wrap and exposed to a x-ray film at -70°C overnight.

The plates were aligned in the correct orientation on the x-ray film with the help of the markings made in the plate with India ink and pointers made on the filters (scintillation spots). Together these were placed on a light box to mark the phage clones that hybridized to the probe. These positive phage clones were removed from the plate using the blunt end of a Pasteur pipette. The phage plugs were placed into 1ml of SM buffer and 40μl of chloroform, vortexed and kept at 4°C overnight to allow the elution of the phage.
2.3.7 Secondary screening

Top agar and NZY plates were prepared again as in primary screening. The eluted phages from the primary screenings were harvested from the supernatant and were titrated into three different dilutions of 1:10, 1:100 and 1:000 in SM buffer. Only 1-2 dilutions were selected for filter lifts. The selected dilutions were mixed with a constant volume of 200µl of XL1-Blue bacterial cells. This was incubated at 37°C for 20 minutes.

After 20 minutes the mixture was added to 2.5ml of top agar, poured over NZY plates and allowed to incubate overnight at 37°C.

Filter lifts were made for 9 plates and the filters were marked appropriately. Plaque colony forming units were counted for each plate. These filters were processed in the same manner as in primary screening, air dried and UV-cross linked. The wet filters were prehybridized at 60°C as before and hybridized overnight at 60°C with 1-5x10⁶ counts/ml. The filters were washed first with 2xSSC at room temperature and 8-10 times with 0.2xSSC/0.1%SDS at 55°C. Filters were dried, saran wrapped individually and exposed to a x-ray film overnight at 70°C.

No tertiary screenings were carried out, as I successfully isolated individual clones at this stage. I prepared positive phagmids from using the ExAssist SOLR system as follows.
2.4 The ExAssist/SOLR in vivo excision system

The ExAssist/SOLR system is designed to allow efficient excision of the pBluescript phagemid from the Lambda ZAP II vector. The host strains for this system are SOLR and XL1-Blue strains.

The SOLR and XL1-Blue strain cells were grown in LB media with 0.2% maltose and 10mM MgSO₄. They were plated then on LB/Kanamycin (50μg/ml) and LB/Tetracycline (12.5μg/ml). Plating cultures were started from a single colony and grown overnight with vigorous shaking at 30°C in appropriate media. The cells were spun at 1000-2000g for 10 minutes and gently resuspended in 0.5 volumes of 10mM MgSO₄.

The plaques of interest from secondary screening were picked up in a tube containing 500μl of SM buffer and 20μl of chloroform. The tube was vortexed to release the phage particles into the SM buffer. The following were added to a 50ml conical flask.

a) 200μl of OD₆₀₀=1.0 XL1-Blue cells
b) 100μl of phage stock (containing > 1x10⁵ phage particles) and
c) 1μl of ExAssist helper phage (> 1x10⁶ pfu/ml).

The mixture was incubated at 37°C for 15-30 minutes. 3ml of 2xYT media were added and the resultant mixture was incubated for 2-3 hrs at 37°C with shaking. The tube was heated at 70°C for 20 minutes and spun for 15 minutes at 2500g. The supernatant containing the plasmid packaged as a filamentous phage particle was transferred to a sterile tube. This phage stock was titrated against the SOLR cells as
follows, 1:200μl; 2:200μl; 1.5:250μl and 3:500μl. These different titrations were incubated at 37°C for 15 minutes, and were then plated on LB/Ampicillin plates (50μg/ml) in concentrations of 100, 125, 150, 200, 250 and 500μl, and incubated overnight at 37°C.

The rescued phagmids were grown in mini-prep cultures of 2ml each overnight at 37°C in LB/Ampicillin media. The insert was excised from the phagemid by using the restriction enzymes EcoRI and Xho1 (GIBCO, BRL). This double restriction enzyme digestion released an insert of 3.5 kb in length designated B3.5.

2.5 Restriction Enzyme Mapping

To determine the restriction sites present within the clone, mini-prep DNA from this clone was subjected to an array of restriction enzyme digestions including SacI, BstXI, SacII, XbaI, SpeI, BamHI, Smal, PstI, Hinc II, KpnI, DraII, A2al, EcoRV, HindIII, ClaI, SalI and NotI. The restriction digestions were analyzed on a 1% agarose electrophoretic gel. A control plasmid was also run without any restriction enzyme digestion. Double restriction enzyme digestions were also carried out including, EcoRI + PstI; BamHI + PstI; ClaI + BamHI and ClaI + PstI. All restriction enzymes used were from GIBCO, BRL (Fig. 5)

2.6 Subcloning

To sequence the entire B3.5 clone I constructed subclones using restriction enzyme digestion and ligation. I carried out digestions with BamHI and PstI followed by self-ligation yielding clones with a 2.2Kb and a 3.2 Kb respectively.
I also double digested B3.5 with BamHI + PstI and subcloned the 1.4 and 0.8kb inserts into pBSII.

The ligation reactions were setup as follows after the DNA was purified from the gel using the Geneclean II kit (BIO 101 Inc., USA)

a) Self-ligations (BamHI and PstI, GIBCO-BRL)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>BamHI/PstI digested DNA</td>
<td>10μl</td>
</tr>
<tr>
<td>10mM ATP</td>
<td>1μl</td>
</tr>
<tr>
<td>Ligase Buffer</td>
<td>1μl</td>
</tr>
<tr>
<td>T4 ligase</td>
<td>1μl</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>7μl</td>
</tr>
</tbody>
</table>

b) Ligation in Bluescript

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>BamHI + PstI digested DNA</td>
<td>8μl</td>
</tr>
<tr>
<td>BamHI + PstI digested pBS</td>
<td>8μl</td>
</tr>
<tr>
<td>10mM ATP</td>
<td>1μl</td>
</tr>
<tr>
<td>Ligase Buffer</td>
<td>1μl</td>
</tr>
<tr>
<td>T4 ligase</td>
<td>1μl</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>1μl</td>
</tr>
</tbody>
</table>

The ligation kit used was from GIBCO, BRL. Appropriate controls were carried out without the T4 ligase enzyme. A positive control with a supercoiled pBS DNA was
also added to the experiments to determine the transformation efficiency.

Transformations were done in DH5α cells.

These subcloned fragments were sequenced manually first using the Universal and M13 reverse primers and later using the ABI-Perkin Elmer automated DNA sequencing machine with a series of primers whose sequences are given in Table 2.

2.7 Sequencing - manual and automated

2.7.1 Principle

Dideoxy sequencing depends upon base specific termination of enzyme catalyzed primer-extension reactions. Reactions are performed containing primer template and the four deoxynucleotides (dATP, dCTP, dGTP and dTTP). Each reaction is terminated with the particular dideoxynucleotide present in the reaction. Thus, the occurrence of the corresponding deoxynucleotide in each reaction represents the terminated chain fragments. When these reaction mixtures are separated electrophoretically on a sequencing gel or through a capillary, the sequence in which nucleotides are added to the primer can be deduced from the sequence in which successively larger fragments occur in the four lanes. The positions of these fragments are detected by the incorporation of radioactive label either before or during the primer-extension reactions.

The steps involved in using T7 DNA polymerase to sequence DNA using a radioactive label are as follows:

The template DNA to be sequenced is first isolated and purified using polyethylene glycol (PEG). The primer anneals to the template adjacent to the target sequence.
Labeling reactions are carried out where enzyme catalyzed extensions of the primer is initiated in the presence of a limiting concentrations of all four deoxynucleotides. Any one of the four deoxynucleotides could be radiolabeled. Termination reactions take place at where the labeled and extended primers from the labeling reaction are terminated in four separate reactions. Each of the reaction contains a specific dideoxynucleotide in addition to non-limiting concentrations of all four deoxynucleotides.

Sequencing gels were prepared from 6% acrylamide (50ml), TEMED (40μl) and 10% ammonium persulphate (400μl), mixed gently and loaded between the plates using a syringe taking care not to introduce any bubbles. The gels were allowed to polymerize at room temperature. Electrophoresis was run at a voltage of 750 volts. The running buffer used was 1xTBE. Electrophoretically separated DNA base pair fragments were detected by autoradiography.

Manual sequencing of the eIF4G-3 clone was done using the 77 Sequencing kit from Pharmacia Biotech, USA, which utilizes the dideoxy sequencing reactions using T7 DNA polymerase. The mini-prep DNA used for sequencing purpose was purified using PEG.

Equal amounts of 13% PEG in 1.6M NaCl₂ was added to the amount of DNA present (50μl DNA + 50μl PEG). This mixture was cooled overnight on ice in the cold room. The sample was spun for 15 minutes at 13,200 rpms in the cold room and washed with 70% cold ethanol and inverted a couple of times and spun again briefly for
about a minute. The sample was vacuum dried for 30 minutes and resuspended in 50μl of TE buffer (pH8.0).

The sequence of eIF4G-3 (B3.5) generated by manual sequencing was confirmed and refined using the automated sequencing machine, ABI PRISM 310 Genetic Analyzer, Perkin Elmer, USA.

2.7.2 Automated sequencing

The ABI PRISM 310 Genetic analyzer is a laser induced fluorescence capillary electrophoresis system. Rhodamine labeled DNA samples are loaded on the autosampler. Upto 98 samples can be loaded in the autosampler. The machine automatically introduces the samples into a polymer filled capillary for electrophoresis.

The rhodamine dye labeled DNA fragments electrophorese through the polymer and DNA fragments separate according to size. When the labeled samples migrate through the capillary and into the window, they are illuminated. The laser beam excites the fluorescent dyes attached to the fragments and makes them emit light at a specific wavelength for each dye. Based on the wavelength the light is collected and separated by a spectrograph. A charge-coupled device (CCD) camera collects this in turn. All four types of fluorescent emissions are detected simultaneously. The data collection software collects the light intensities using software selectable filters (spectral or wavelength) and stores them as electrical signals for eventual processing. At the end of the run, the computer automatically analyzes the collected data and prints electropherograms on a colour printer.
2.7.3 Sample preparation for automated sequencing

The reagents supplied with the sample preparation kit were mixed in a 1.5ml eppendorf tube as follows.

8.0μl of terminator ready reaction mix were mixed with 1.5-2.5μl of double stranded DNA of 0.2μg/μl and primer of 3.2 pmole concentrations made up to a volume of 20μl with ddH₂O. The reaction mix was then amplified using PCR for 25cycles following a rapid thermal ramp to 96°C for 10 seconds, moving to a rapid thermal ramp to 50°C with a hold for 5 seconds. After this, the thermal ramp moves up to 60°C with a hold time of 4 minutes. The cycle was repeated for 25 times and held at 4°C at the end of 25 cycles.

The PCR sample was ethanol precipitated and dried in a vacuum centrifuge at room temperature. The sample was resuspended in 25μl of template suppression reagent (Perkin Elmer, USA) and heated at 95°C for two minutes to denature. The sample was vortexed, spun and placed on ice before it was ready to be loaded onto the machine.

2.8 Obtaining the 5’end of the cDNA clone

During the complete sequencing of the B3.5clone, two other groups independently published the sequence of the murine eIF4G-3 homologue. Comparison of the murine clones with our sequence confirmed my suspicion that B3.5 lacked the 5’ coding sequences. To isolate the entire coding region of the human cDNA, I designed primers to the 5’end of the mouse cDNA clone containing the proper ‘start’codon with a complete open reading frame leading to a full length translation of protein. The primers
(Table 3) were used against a teratocarcinoma cDNA library (Tera-2) constructed by one of our lab member, Dr. Liu Ling.

Library DNA PCR was performed using combinations of sense (5' end of murine 'start' codon sequence) and antisense (from my B3.5 clone in the 3'→5' direction) primers. The PCR product (500 bp) obtained this way (using primers #1 and 4) was cloned into the PCR-script Amp SK (+) cloning vector (Stratagene, USA). The PCR product was digested with NcoI and PstI, and the B3.5 (eIF4G-3) clone was digested with PstI and SmaI. The restriction enzyme digested products were run on a gel, purified from the gel using the Geneclean II kit (BIO 101 Inc., USA), and cloned into the expression vector EE(TM1) between NcoI and SmaI in a tri-molecular ligation reaction (Fig. 6)

2.8.1 Restriction digestion of eIF4G-3 with SalI and BamHI (truncation of eIF4G3)

EIF4G-3 was isolated as one of the true positive clones from the Y2H screenings. To show that the association of eIF4G-3 with TTK is a true association, I decided to do co-immunoprecipitation experiments. EIF4G-3 shared the same molecular mass of 97 kDa with TTK. To differentiate the eIF4G-3 protein from TTK a carboxy terminal truncated version of eIF4G-3 protein was derived by digesting it with SalI and BamHI or with BamHI alone as described below.

The eIF4G-3 PEG purified DNA was digested with SalI and BamHI first. It was blunted with 1µl of Klenow along with 1µl of dNTPs at 37°C for five minutes. The
Klenow was removed by denaturation for 15 minutes at 65°C. Subsequently the DNA was run on a gel, purified and ligated in 14°C water bath.

2.8.2 Restriction digestion of eIF4G-3 with Bam HI alone (truncation of eIF4G3)

The eIF4G-3 PEG purified DNA was digested with BamHI to yield a carboxy terminal truncated version of eIF4G-3 protein. I denatured the restriction enzyme by heating to 65°C for 15 minutes. T4 DNA polymerase and dNTPs were incubated for 5 minutes at 37°C followed by denaturation of the enzyme for 15 minutes at 65°C. The digested products were run on a DNA-gel and geneclean purified and used in ligation. The ligated products were transformed into DH5α cells. Mini-preparation DNA from positive clones was screened with CiaI in the case of BamHI alone ligation product, as restriction digestion with BamHI alone followed by ligation was expected to create a new CiaI site. The truncated eIF4G-3 construct lost a 1.1 kb fragment as evidenced by screening with HindIII. On a protein gel the corresponding polypeptide had a MW of 85-89 kDa as predicted.

2.9 mRNA expression and northern blot analysis

2.9.1 Tissues and cell lines

Breast cancer tissues (50N, 51N), LY2, GPE (NIH 3T3 derived), Jurkat, GM607 (OCI), colo320, OCI2Y8C3P, HUVEC cells, Daudi, Karpas, normal liver, normal
kidney, normal pancreas, normal testis, normal thymus and testicular tumours were tested for eIF4G-3 mRNA expression.

The probe 2.2 kb obtained by BamHI restriction digestion was used in northern blotting against Germ cell tumour (GCT) RNA blots. The cell lines present in Gel 1 are,

577, 833, 1156, 1618, 1685, 2102, 2965, PA-1, Tera-1, Tera-2, 2806, FHS, T98G, KG1a, L1937, L2-1, Jurkat, NS/PBL, PHA/PBL and TPA/R3 (Fig 9).

2.9.2 RNA isolation

The supernatant was gently removed from the cells containing flasks. 5ml of solution D (guanidium isothiocynate+ sodium citrate+sarcosyl+ddH₂O) containing 2-mercaptoethanol were transferred to these flasks which contain the cells from which RNA was isolated. Following this, 0.5 ml 2M sodium acetate, 5.0 ml phenol and 1.0 ml chloroform mixture were mixed well into the flasks and placed on ice for 15 minutes. The cells were spun at 6000 rpm for 20 minutes. Centrifugation produced two phases containing the RNA in the upper aqueous phase. This phase was isolated using a pasteur pipette and 5.0 ml isopropanol was added; the mixture was then placed in a -20°C freezer for 1hr. The RNA was isolated by a second spin at 6000 rpm for 20 minutes. The isopropanol was removed after the second spin, 300µl of solution D were added, and the RNA was resuspended. An additional 300µl of isopropanol were added, the mixture was incubated for 1 hour in a -20°C freezer, and then centrifuged again at very high speed. The supernatant was removed and 300µl of cold 70% ethanol were added to remove the salts from the RNA. The isolated RNA in ethanol was spun again
in the cold room for 10 minutes. The 70% ethanol was removed as a supernatant and the RNA sample was isolated as a pellet in a speed vacuum for 10-15 minutes. The pellet was resuspended in 50μl of DEPC water and stored in a -70°C freezer.

2.9.3 RNA gel preparation and running

The gel tray and tank were washed in soap and ddH₂O and soaked in 0.5 M NaOH and 0.5% SDS mixture for 1hr, and then washed again in ddH₂O and 95% ethanol.

The RNA gel was prepared by melting 1g agarose in 80 ml DEPC water, 10 ml of 10xMOPS (3-[N-Morpholino] propanesulfonic acid) were added with 21ml of formaldehyde in the fume hood. The gel running buffer was 1xMOPS diluted from 10x MOPS in ddH₂O. 5-10μg of RNA were used with 16μl of loading buffer. The RNA samples were heated in loading buffer at 68°C for 5 minutes and the gel was run at 100 V/30 mA for 2hrs. After completion of the run, the gel was left in ddH₂O for half an hour alongside a ruler and photographed. A nitrocellulose membrane was pre-wet with water and then with 20xSSC, and RNA was transferred to the membrane with 20xSSC over a period of 2 days at 100V in cold room.

2.9.4 Prehybridization and Hybridization of Northern blot

Solution A and B were used for hybridization.

Solution A:

4.9ml formamide, 2.5ml 20xSSPE, 2.0ml 10%SDS, 1.0ml 50xDenhardts

Solution B:

0.6ml formamide, 0.1ml salmon sperm
Solution A was kept at 42°C and solution B was boiled for 2-3 minutes and cooled on ice for 2-3 minutes. The blots were washed twice in 2xSSC and UV-cross linked.

Solution A was added to the transfer membranes, which were prehybridized at 42°C for 2 hours. Hybridization was done exactly like the prehybridization but the labeled probe was added to solution B before boiling. Hybridization was carried out overnight at 42°C. The hybridization solution and the probe were removed and the blots rinsed twice with 2xSSC at room temperature. The blots were washed in the following series of solutions for 10-15 minutes each at 55°C:

- 0.4xSSC/0.1% SDS
- 0.2xSSC/0.1% SDS
- 0.1xSSC/0.1% SDS

The signal on the membrane was checked using a Geiger counter and the number of washes adjusted on this basis. After washing the membranes, the blots were wrapped in saran wrap and exposed to a x-ray film at -70°C.

### 2.10 Cloning of eIF4G-1 and eIF4G-3 in the vaccinia virus expression system (EE,TM1)

The transient mammalian expression vector designated EE,TM1 was obtained from the lab of Dr. Jim Woodgett, Ontario Cancer Institute (OCI). Vaccinia virus was introduced in 1982, as a vector for transient expression of genes in mammalian cells. This expression system differs from others in that transcription occurs in the cytoplasm of the cell rather than in the nucleus. This vector permits cloning of fragments up to > 20 kb and allows a high level of protein synthesis with appropriate post-translational modifications as dictated by the primary structure of the expressed proteins and the cell type used. I used COS cells for my transfection experiments.
The EE\textsubscript{p}TM1 vector contains the T7 promoter, which drives the expression of the protein. A polyglutamic acid tag (EE) at the NH\textsubscript{2} terminal end of the protein allows detection of the product with an anti-EE monoclonal antibody (Santa Cruz Biotechnology, USA).

We obtained the eIF\textsubscript{4}G-1 cDNA clone from Dr.Rhoads, Louisiana State University. The eIF\textsubscript{4}G-1 was present in pSK-HFC1, which was made from λgt11-HFC1 (Yan \textit{et al.}, 1992).\textit{EIF}4G-I was excised using EcoRI and Eco RV restriction enzymes and reinserted into pSK (-) downstream of the T7 promoter. From this modified vector, I isolated eIF\textsubscript{4}G-1 by using EcoR1 restriction enzyme (NewEngland Biolabs, USA) and cloned the insert comprising the T7 promoter and the eIF\textsubscript{4}G-1 cDNA into EE\textsubscript{p}TM1. All the subsequent work connected with the eIF\textsubscript{4}G-1 clone was carried out by Dr.Wei Wang in our laboratory. For cloning of eIF\textsubscript{4}G-3 see section 2.8.

2.11 \textit{In vitro} transcription and translation

To express and verify the correctness of the various constructs, and to translate mRNA \textit{in vitro}, I constructed a vector containing a T7 promoter. The desired protein is synthesized from the T7 polymerase expressed mRNA as a \textsuperscript{35}S-labeled or unlabeled polypeptide. A major advantage of this method is that altering the DNA template can generate any desired mutant protein in an easy manner.
2.11.1  **TNT-coupled reticulocyte lysate system (Promega, USA)**

*In vitro* transcription and translation were performed done using the TNT coupled reticulocyte lysate system from Promega, USA. The reaction components were mixed in a 1.5 ml microcentrifuge tube. After addition of all the components, the lysate was mixed gently by pipetting the reaction mix up and down.

The reaction mixture contained TNT rabbit reticulocyte lysate (25μl), TNT reaction buffer (2μl), TNT RNA (T7) polymerase (1μl), amino acid mix minus methionine, 1mM (1μl), ^35^S-Methionine (1000 Ci/mmol) at 10mCi/ml (4μl), RNAsin ribonuclease inhibitor, 40U/μl (1μl), DNA template (1μg) and nuclease free water to a final volume of 50μl. The reaction mixture was incubated at 30°C for 60-120 minutes. The reaction mixture was run on a gel, fixed, dried and autoradiographed.

2.12  **Protein expression in COS cells (transfection)**

The COS cells were incubated at 37°C in the CO₂ incubator until the cells were 75-80% confluent. This usually required 18-24 hours after splitting the cells 1:3.

2.12.1  **Infection of cultured cells with vaccinia virus**

Frozen vaccinia virus was thawed at 37°C and 200μl were used per 100 mm dish. An equal amount of trypsin was added to the virus solution to digest the virus proteins, the mixture was vortexed and incubated at 37°C for 30 minutes, and the digested viral solution was added to 4 ml of Opti-mem (10% Fetal Bovine Serum).
The cultured COS cells were rinsed with either 1xPBS or Hanks solution (balanced salt solution). The virus-Opti-mem mixture was then added to the culture dish and incubated for 30 minutes at 37°C.

2.12.2 Transfection of COS cells with TTK, TTK\textsuperscript{(mutant)}, eIF4G-3 and truncated eIF4G-3

TTK\textsuperscript{(mutant)} was derived by a point mutation in the kinase domain that altered a conserved Arg residue at the ATP binding site and hence inactivated the kinase completely. This allowed us to study the kinase TTK in the absence of its protein phosphorylation activity.

\[
\begin{array}{c|cccc}
\text{Translation} & Q & R & I & S \\
\text{Wild type TTK} & \text{CAG AGG ATA TCC} \\
\text{TTK\textsuperscript{(mutant)}} & \text{CAG CTG ATA TCC} \\
\text{Translation} & Q & L & I & S
\end{array}
\]

The ‘CAG CTG’ sequence introduced a new restriction site for PvuII, which was used to screen the mutants.

2.12.3 Transfection

Lipofectamine reagent is a 3:1 (w/w) liposome formation of the polycationic lipid 2,3-dioleyloxy-N[2(sperminecarboxamido)ethyl]-N, N-dimethyl-1-propanaminium trifluoroacetate (DOSPA) and the neutral lipid dioleoyl phosphatidylethanolamine (DOPE) in membrane filtered water. It is used for the transfection of DNA into
cultured eukaryotic cells. The positively charged and neutral lipids form liposomes that can complex with nucleic acids. The lipid-nucleic acid complex, when applied to cultured cells, facilitates the uptake of the nucleic acid into the cells.

Solution ‘A’ was made for each transfection with 5-10μg of DNA diluted into 100μl of Opti-mem. Lipofectamine reagent (GIBCO, BRL) 10μl diluted into 100μl of Optimem formed solution ‘B’.

Solutions ‘A’ and ‘B’ were mixed gently and incubated at room temperature for 45 minutes to allow DNA-liposome complex formation. While the DNA-liposome complex was forming, the cultured cells were rinsed with serum free opti-mem and incubated in serum free opti-mem for 45 minutes at 37°C in the CO₂ incubator. 3.5 ml of Opti-mem were added gently to the DNA-liposome complex to a final volume of 4 ml for each dish. This mixture was gently overlayed onto the cultured cells. The transfected dishes were incubated for 5 hours at 37°C in the CO₂ incubator. After the 5 hours incubation, 4 ml of D-MEM containing twice the normal concentration of serum (20% FBS) was added to the transfected cells without removing the transfection mixture.

After 18 hours the medium was replaced with fresh complete D-MEM (10% FBS). Transfected cells were harvested to be analyzed for expression of the respective constructs 24-72 hours after the start of transfection. The transfected cells were washed with 1xPBS or Hanks buffered solution. Cells were scraped and transferred to a centrifuge tube and spun for 5-10 minutes at 1000 rpm. The medium was removed and
the cells were resuspended in 400μl of NP-40/digitonin lysis buffer. The lysed cell extract was used for western blotting, immunoprecipitation and \textit{in vitro} kinase assays.

\subsection*{2.12.4 Western blotting and detection of proteins}

Western blotting is a rapid and sensitive assay for the detection and characterization of proteins. It allows identifying particular proteins by utilizing the specificity inherent in antigen-antibody recognition.

The antigenic mixture of interest was solubilized, usually with sodium dodecylsulphate (SDS), urea and the reducing agent 2-mercaptoethanol. Following solubilization, the proteins were size separated by SDS-PAGE and the antigens were then transferred electrophoretically to nitrocellulose. The membrane was blocked with 15 ml of 5\% skim milk powder in (0.1\% Tween 20) at room temperature for 2 hours, or overnight in the cold room to prevent non-specific binding of antibody, and was then probed with the antibody of interest.

The primary antibodies used were a) TTK (C-19), Santa Cruz Biotechnology Inc., USA, an affinity purified rabbit polyclonal antibody raised against a peptide corresponding to amino acids 823-841 at the carboxy terminus of human TTK. It was used in a 1:3000 dilution. The secondary antibody for this was goat anti-rabbit polyclonal antibody used in a 1:5000 dilution.

b) Anti-eIF4G-3: This antibody was obtained from Dr. Nahum Sonenberg, McGill University, Canada. It is a polyclonal rabbit antibody used in a 1:4000 dilution. The secondary antibody for this was goat anti-rabbit polyclonal antibody used in a 1:5000 dilution.
c) Anti-EE: This antibody, which recognizes the epitope tag of polyglutamic acid used in the vaccinia virus system, was obtained from Santa Cruz Biotechnology Inc., USA. This is a murine monoclonal antibody and was used in a 1:30 dilution. The secondary antibody was rat anti-mouse immunoperoxidase conjugated secondary antibody used in a 1:5000 dilution.

The probed blot was washed thrice with 1xTBST for 10 minutes each. The blot was then incubated with the secondary antibody at room temperature for 1 hour, washed again thrice with 1xTBST again for 10 minutes each. The proteins blotted on the nitrocellulose, and recognized by primary and secondary antibodies were visualized using the ‘Enhanced Chemiluminescence’ reagents (Amersham International, UK). The Chemiluminescence was based on the reaction in which the enzyme horseradish peroxidase (HRP) catalyzed light emission by the oxidation of the chemical luminol.

Membranes were exposed to a mixture of reagents ‘A’ and ‘B’ for several minutes, and were then wrapped in saran wrap, and exposed to a x-ray film for 10, 5, 2.5 and 1 minute duration.

2.13 Co-immunoprecipitation of proteins

Immunoprecipitation consists of multiple ordered steps. It involves binding of a specific antigen to an antibody, precipitation of the antigen-antibody complex, washing, purifying the antigen-antibody complex and finally, analysis of the proteins contained within the complex, usually by electrophoretic methods.

In this experiment TTK, TTK\textsuperscript{(mutant)} eIF4G-3 and truncated-eIF4G-3 were transfected together, to see if co-expression of the proteins would be achieved, and if so, whether
these proteins were co-immunoprecipitated together in COS and T98G human cell lines.

2.13.1 Procedure

Protein-A sepharose beads were swelled by adding 5 mg of beads (per 100-200μg of total lysate protein) in 500μl of immunoprecipitation buffer (IP) at room temperature for 1 hour. The beads were washed thrice with IP buffer at high speed for 30 minutes each, blocked for an hour in cold room with 1% bovine serum albumin (BSA) made with IP buffer and allowed to mix by rotating slowly in cold room. This mixture was washed again thrice with IP buffer and spun down at high speed for 30 minutes. 5μl of TTK antibody was added along with 200μl of IP buffer to each tube containing the swollen beads in the cold room with slow rotation. Simultaneously, the same number of tubes was set up for pre-clearing the lysate. The beads were swollen, washed, and 200μl lysate were added to each tube. The lysate was allowed to mix with the beads in slow rotation for 2 hours in cold room.

The supernatant was removed from the primary antibody containing tube after spinning it down at high speed for a minute. The pre-cleared supernatant was added to the antibody containing tubes after spinning them down also for one minute. The antigen-antibody complex was then allowed to form.

After two hours, the complex was washed thrice with 0.2% digitonin buffer and the pellet was stored in -20°C. To 200μl of the lysate half the amount of sample buffer with 2-mercaptoethanol was added, boiled for 5 minutes, spun at high speed for a minute and loaded on SDS-PAGE gel.
The proteins were subsequently transferred to a nitrocellulose membrane and detected with appropriate antibodies to assess co-expression and co-association.

2.14 In vitro Kinase Assay

The in vitro kinase assay facilitates the observation of phosphorylation of proteins. This also helps in establishing whether a kinase in study could phosphorylate a particular substrate. The change in molecular mass of a protein after phosphorylation may sometimes be detected by this assay.

The protein-A antibody-antigen complex was washed thrice with 0.2% digitonin buffer containing a protease inhibitor cocktail (leupeptin, aprotinin, trypsin inhibitors with equal amounts of PMSF in a final concentration of 2mM each) 3μl/ml.

The complex was washed again with ice-cold kinase assay buffer (hepes+NaCl₂+NP40+MgCl₂+MnCl₂) thrice, the residual medium was removed completely and 45μl of kinase buffer was added to each pellet followed by 1μCi of ³²P-γ ATP. This mixture was incubated for 15 minutes at 37°C or 30 minutes at room temperature.

15μl of sample buffer (4x) of was added to each tube, the mixture boiled for 5 minutes and the samples were microfuged for 10 minutes at high speed. The supernatant was analyzed on a SDS-PAGE gel, the gel was dried and exposed to a x-ray film at -70°C freezer.
3.0 Results

3.1 Background

The biological role(s) of TTK has remained unclear to this time, as the position of TTK in signal transduction cascades is unknown. To address this question, we used the yeast two hybrid system (Y2H) system in an effort to identify interacting proteins. We reasoned that identifying protein partners of TTK would allow us to position this kinase within known cellular signaling pathways. Using this approach, we successfully isolated two protein translation initiation factors and went on to show that they do indeed interact with TTK in mammalian cells.

3.2 Yeast two hybridization (Y2H) for TTK-interacting proteins (figure 2)

The Y2H identifies interaction between two proteins and was devised by Stanley Fields (1989). In principle, this system consists of a ‘bait’ fused in frame to a protein-DNA binding domain (in our case: a TTK-lexA chimera) and a ‘prey’ protein that is built in-frame with a transcription-activating domain (in our case: a murine cDNA library from mouse embryos 9.5/10.5 dpc fused to VP16). These hybrids were transformed into yeast using the lithium acetate protocol (Ausubel et al., 1989). If the bait and prey interact within the yeast cells, the resultant complex will then direct transcription of one or more reporter genes. In the lexA system that we employed, the reporter gene lacZ and histidine (HIS) separately detected the interaction of the proteins and positive clones. The reporter genes lacZ and histidine (HIS) respectively allowed
detection of positive clones by color change or by growth of the parent yeast strain on His (-) medium.

We chose to construct the bait from codons 125-366 of TTK. This region was selected as it was away from the kinase domain, which is lethal to yeast; because it bears no resemblance to other proteins (and thus might contain hitherto unrecognized interaction sequences); and because small baits work better than large ones in the LexA Y2H system (personal communication, S.Hollenberg). The 'bait' used the *Trp1* (Tryptophan) and the 'prey' *Leu2* (Leucine) selectable markers respectively. The TTK bait construct was transformed into L40 yeast using the lithium acetate protocol (Ausubel *et al.*, 1989) and selected on trp (-) medium; a single plasmid-containing clone was grown up and subjected to a second transfection and those expressing the 'prey' cDNA library were similarly selected on trp (-)/leu (-) medium to determine the transfection efficiency. We simultaneously selected interacting clones by growth on trp (-)/leu (-)/his (-) medium and identified over 100 individual isolates. True positive clones were determined by 'curing' the bait of the prey by extended growth in the absence of tryptophan selection, allowing the segregation and growth of daughter cells without the LexA fusion plasmid. Those mato clones that reverted to a negative phenotype were mated back to MatA yeast containing the original bait to identify true positive, β-galactosidase expressing clones.

Two Y2H screenings were done, the second of which I carried out in collaboration with Toni Amandeola in Dr.Hogg's laboratory. In the first screening clones *eIF4G-1* (p220-clome 1.7) and *eIF4G-3* (p97-clome 24) along with 7 other clones were obtained.
In the second Y2H screening, eIF4G-3 was isolated along with 4 other clones. Examination of the sequences of eIF4G-1 and eIF4G-3 revealed that they were isologs (a full description of these two factors was presented in the introduction chapter). Because of the close relationship between eIF4G-1 and eIF4G-3, it seemed likely that clones 1.7 and clone 24 represented peptide fragments of true positive TTK-interacting proteins. Since at this time, eIF4G-3 had not been described in the literature, I decided to characterize this cDNA fully. EIF4G-1 had already been cloned and characterized in the laboratory of Dr. Rhoads (Yan et al., 1992).

3.3 Isolation of a human cDNA clone of eIF4G-3 (figures 4A & 4B)

A putative eIF4G-3 clone (#24) obtained from the second Y2H screening was used to screen a HUVEC human endothelial cDNA library (Stratagene, USA). The HUVEC cDNA library has the following characteristics: The library comprises ≥ 2x10^6 pfu (plaque forming units) made with a single amplification from a unidirectional primed cDNA synthesis of HUVEC mRNA using unidirectional oligo (dT). Excision of phagemid from Lambda ZAP II library is greatly simplified by the use of the ExAssist™ excision system. I performed a primary and secondary screening of the cDNA library (described in detail in the Materials and Methods chapter). In the primary screening 20 plates were screened with a final dilution of 10^6 phages/ml giving me a total of 5x10^4 phages/plate (1x10^6 phage total). Ten phage isolates from the primary screening were each titrated into three different dilutions of 1:10, 1:100 and 1:000 in SM buffer and grown on secondary plates. Filter lifts were made for 9 of these plates and plaque colony forming units (pfu) were counted for each plate (as described
in section 3.5 of Materials and Methods). No tertiary screenings were carried out, as I successfully isolated individual clones at the secondary stage.

A partial cDNA of the eIF4G-3 was isolated from this HUVEC cDNA library using the ExAssist/SOLR system. The rescued phagmids were grown in mini-prep cultures of 2 ml each overnight at 37°C in LB/Ampicillin media and insert excised from the phagemid using the restriction enzymes EcoRI and XhoI (GIBCO/BRL). This double restriction enzyme digestion released an insert of 3.5 kb in length designated B3.5.

3.4 Sequencing of the full length eIF4G-3 cDNA (figures. 4-8)

I elected to sequence this clone in its entirety so that its structure could be determined and to confirm that eIF4G-3 shared homology with other eukaryotic translation initiation factors (particularly eIF4G-1). In addition, further experiments involving protein expression and function required that the complete nucleotide sequence of the full-length cDNA be known.

I adopted the following sequencing strategy. First, I derived a restriction map of the clone by digesting it with different restriction enzymes, singly and in combination (Figs. 5 & 7). This allowed me to identify the position of each major restriction site in the clone and enabled sub-cloning of the 3.5 kb cDNA fragment. I carried out restriction digestions with BamHI and PstI followed by self-ligation of the digested clones using ligase, which yielded clones with 2.2 kb and 3.2 kb inserts respectively. I also double digested B3.5 with BamHI + PstI and subcloned the resultant product of 1.4 and 0.8 kb inserts into the pBS II vector (Fig 4). Second, I sequenced the original B3.5 clone and the derived sub-clones using the M13 Universal and Reverse primers.
Finally, I designed primers specific to eIF4G-3 sequences where necessary. A complete list of primers and their sequences are given in the Materials and Methods section (Table 2). I performed sequencing manually using a gel and $^{35}$S-labeling (T7 sequencing kit-Pharmacia Biotech) and later extended and verified eIF4G-3 sequences using the ABI 310 genetic analyzer (Perkin Elmer, USA). In this fashion, the entire sequence of B3.5 (eIF4G-3) was obtained (Fig. 8) However, when we assembled the B3.5 cDNA and translated it in all three different open reading frames (ORF) we noted that no ‘start site’ was present, suggesting that this clone lacked some 5’ sequences.

At the same time that we finished sequencing the B3.5 clone, the homologous mouse (Mus musculus) sequence was published in GenBank (accession number U63323). We employed this published sequence to obtain the human 5’sequence as follows. I designed a sense primer to the mouse 5’UTR GTG sequence (sense primer #3; see Materials and Methods) and an antisense-primer (anti-sense reverse complement #1) to the human sequence. I then used these primers to amplify 5’ eIF4G-3 sequences from a Tera-2 (teratocarcinoma) carcinoma cell-line cDNA library (constructed in Dr.Hogg's laboratory by Dr. Ling Liu). I obtained a PCR amplified fragment of 400bp, containing a 5’ Nco I site (engineered into the sense primer) and an internal 3’ Pst I site (corresponding to a Pst I site in clone B3.5). To construct the eIF4G-3 cDNA comprising the entire protein coding sequence, I digested the PCR product with Nco I and Pst I; the B3.5 clone with Pst I and Sma I; and the vaccinia virus expression vector EEpTM1 with Nco I and Sma I. All three linearized DNA fragments were subjected to tri-molecular ligation to yield the construct of EEpTM1-p97 (eIF4G-3) (Fig. 6). I confirmed the orientation and sequence of this construct (EEpTM1-p97) by automated
sequencing on the ABI 310. This construct was of considerable importance to us, as it encoded the full-length protein comprising 893 aa of calculated mass 97 kDa. We observed that there is more than 95% homology between the human and mouse eIF4G-3 sequences (Fig 10). Analysis of the full-length sequence of eIF4G-3 (Fig. 8) demonstrated 28% identity and 36% similarity to that of the carboxyterminal two thirds of eIF4G-1. The complete human eIF4G-3 sequences were published at this time (Imataka et al., 1997).

3.5 Northern Blotting (figure 9)

I next sought to determine whether the expression of eIF4G-3 was specific to any tissues or cell lines, or whether the transcript was expressed ubiquitously. To this end, I probed a series of northern blots using a 2.2 kb BamHI fragment of the B3.5 clone. I observed high levels of eIF4G-3 mRNA expression in all cell lines screened. Similar results were obtained using tissue samples from a series of breast cancers; unfortunately, we were unable to obtain samples of normal tissues for comparison. The ubiquitous expression of eIF4G-3 mRNA fits well with its presumptive role in the general control of protein translation.

3.6 eIF4G-1 and eIF4G-3 expression and interaction with TTK in COS cells (figures 11-15)

After ascertaining the mRNA profile of eIF4G-3, we wanted to express and study its interaction with TTK. We utilized the vaccinia virus transient mammalian
expression system (EEpTMI) (Fig. 11), which is driven by the T7 promoter and produces abundant amounts of protein in COS cells infected with vaccinia virus.

We made use of the following constructs of vaccinia virus to express the protein in COS cells (Figs. 12 & 13): EEpTMI-eIF4G-1; EEpTMI-TTK; EEpTMI-TTKm; EEpTMI-eIF4G-3; EEpTMI-eIF4G-3t. Dr. Wei Wang in Dr. Hogg's laboratory constructed a kinase-dead mutant of TTK (TTKm) and a TTK construct bearing a hemagglutinin epitope tag at the carboxy terminus (TTK-HA). These constructs also carried a polyglutamine tag at the amino-terminus, which helped us in the identification of the proteins by an anti-polyglutamic antibody. An eIF4G-1 cDNA, a kind gift of Dr. Rhoads from the Louisiana State University, USA was cloned into the EcoRI site of EEpTMI, while the eIF4G-3 and TTK cDNAs were cloned into the NcoI and SmaI and NcoI and EcoRI sites, respectively, of the same vector (Fig. 5). The vector diagrams with the corresponding inserts were all drawn using the Vector NTI 5.0 software (Figs. 4-6).

3.6.1 Truncation of the eIF4G-3 protein

The calculated theoretical molecular weight of the eIF4G-3 protein is 97 kDa (893 aa), and this agrees with experimental data (Imataka et al., 1997). We found it difficult to differentiate between TTK and eIF4G-3 on a protein gel as they migrate close to one another. To solve this problem we subcloned a truncated eIF4G-3 cDNA into the EEpTMI expression vector. Specifically, we designed a carboxy-terminal-truncated version of eIF4G-3 that lacked 82 aa. As the original clone isolated from the Y2H screening (clone 24-eIF4G-3) represented amino terminal sequences of eIF4G-3 (Fig.
4) this carboxy-terminal truncated form was expected to retain the ability to interact with TTK. We digested the eIF4G-3 clone with the BamHI restriction enzyme (for complete protocol see Materials and Methods) blunted it with T4 polymerase, purified the digested fragment, ligated it into EE9TM1 at the Bam HI site and transformed the resultant construct into DH5α cells. Expression of this construct in vaccinia infected cells together with TTK allowed us to easily differentiate between both proteins on a SDS-PAGE protein gel, as the truncated version of eIF4G-3 migrated at a molecular mass of 89 kDa (see Figs. 14 & 15). As predicted, we were able to show through our experiments that truncated eIF4G-3' did associate with TTK (see Fig. 16).

3.6.2 Sequence alignment study (figure 10)

As shown in Figs. 10 & 24, both Y2H clones do localize to the same approximate region in the homology alignment map. To our surprise, these clones did not precisely overlap; the carboxyterminal end of clone 24 (eIF4G-3) is separated from the aminoterminal end of clone 1.7 (eIF4G-1) by 6 amino acids. Of interest, this particular region is highly homologous between eIF4G-1 and eIF4G-3, suggesting that it truly represent a TTK interaction domain. This data is further discussed in the Discussion chapter. In Fig. 10 it can be seen that the newly described poly A binding protein-interacting protein (PAIP-1) also contains a similar domain that shares a high sequence similarity with eIF4G-1 and eIF4G-3. The corresponding PAIP-1 domain shows 25% identity and 39% similarity with the central portion of eIF4G-1 (Craig et al., 1998).
3.6.3 In vitro transcription and translation (figure 14)

Before we began the interaction studies, we wanted to test the ability of the constructs to express their respective proteins in vitro and to confirm that the truncated version of eIF4G-3 (eIF4G-3') protein differed in its molecular mass from TTK. To this end, we used an in vitro transcription-translation kit (Promega, USA).

The genes inserted in EE7TM1 were each under the transcriptional control of the T7 RNA polymerase promoter, which allowed us to use T7 polymerase in the TNT lysate system. 0.2-2.0µg of plasmid DNA were added directly to TNT lysate, incubated in a 50µl reaction volume for 1-2 hours at 30°C and the synthesized proteins analyzed by SDS-PAGE and autoradiography. As seen in Fig. 14, both full length and truncated eIF4G-3 can be distinctly seen migrating at different molecular masses both when expressed separately or together. Polyclonal anti-eIF4G-3 antibody was used to screen this western blot at a dilution of 1:5000; note that it identifies both forms of eIF4G-3. Moreover, native eIF4G-3 is present in the translation reaction, although expression of the corresponding full-length cDNA increased the total amount of eIF4G-3 protein. All the EE-tagged constructs expressed well in an in vitro system confirming that the constructs are functional and were suitable for expression studies in mammalian cells.
3.6.4 Co-transfection and co-expression studies in COS cells (figures 16 & 17)

Satisfied by the expression of the constructs in an *in vitro* system and the results obtained therein, we went on to express the constructs *in vivo* using COS cells. We used the lipofectamine protocol (GIBO BRL, USA) to transfect these constructs into the mammalian cells.

We hypothesized that TTK interacts with both eIF4G-1 and eIF4G-3. To prove this hypothesis, we co-expressed the constructs in COS cells, immunoprecipitated the complex with α-TTK antisera, separated the proteins on a SDS-PAGE gel, and then probed the blot for the initiation factors with specific antibodies. We transfected the constructs alone and in the following combinations: TTK+eIF4G-1; TTK* + eIF4G-1; TTK + eIF4G-3; TTK* + eIF4G-3; TTK + eIF4G-3*; and TTK* + eIF4G-3*. We first demonstrated that we could co-express TTK and the eIF4G-1 constructs by a direct western analysis of extracts from singly or co-transfected cells. Specifically, the proteins from the lysates of the COS-transfected cells were harvested using 0.2% digitonin buffer, run on a SDS-PAGE gel and transferred to nylon membrane. The protein products were analyzed using an anti-EE monoclonal antibody at a 1:30 dilution; as seen in the fig. 15, both co-expressed TTK and eIF4G-3 (which carry the EE-epitope tag) are detected by the anti-EE MAb. Having successfully demonstrated expression of each protein in COS cells; we then went on to examine whether TTK associates with one or both of eIF4G-1 and eIF4G-3.
3.6.5 Association of TTK and eIF4G-1 (figures 16 & 17)

Dr. Wei Wang of our laboratory demonstrated that TTK and eIF4G-1 associate by means of co-immunoprecipitation experiments. He performed co-transfections of TTK and eIF4G-1 in COS cells and harvested the proteins from the lysates using 0.2% digitonin. He immunoprecipitated the proteins using the α-TTK antisera. The immunoprecipitated proteins were washed several times in digitonin buffer and run on a SDS-PAGE gel. The western blot was first probed by anti-TTK antibody at a 1:2000 dilution to confirm the presence of TTK in the immunoprecipitated complex. The same blot was probed again with anti-eIF4G-1 to show that the complex associated with the α-TTK-antisera contained eIF4G-1, co-migrating at the 185 kDa. Dr. Wang also carried out the converse experiment by immunoprecipitating the lysate from double transfected cells (TTK and eIF4G-1) with a series of anti-peptide antisera against eIF4G-1 (a gift from Dr. Rhoads, Louisiana State University); data not shown here. Finally, to show that TTK phosphorylates eIF4G-1, the blot was washed and re-probed with an anti-phosphotyrosine antibody at a 1:5000 dilution, which detected a strong band co-migrating with eIF4G-1 at the 185 kDa range. Finally, while eIF4G-1 also associated with TTKα or TTK-HA in immunoprecipitation experiments, there was no phosphorylation of eIF4G-1 by either TTKα or TTK-HA (note that this later construct lacks kinase activity). Subsequently, in vitro kinase assays confirmed that kinase-active TTK phosphorylates the same 185 kDa protein (see section 3.5.4.3 below). Thus, co-immunoprecipitation experiments demonstrated that TTK does interact with and phosphorylates eIF4G-1 in COS.
3.6.6 Association of TTK and eIF4G-3 in COS cells (figure 16)

Similarly, to demonstrate an association between TTK and eIF4G-3, Dr. Wang and I performed further co-immunoprecipitation experiments using the TTK and eIF4G-3 constructs. We performed double transfections in COS cells using TTK and eIF4G-3, TTK\textsuperscript{m} and eIF4G-3\textsuperscript{t} in the combinations described in section 3.5.4. TTK and TTK\textsuperscript{m} were both detected by anti-EE and anti-TTK antibodies (Santa Cruz Biotechnology, USA), while anti-EE antibody and anti-eIF4G-3 antibodies (the latter a gift from Dr. Sonenberg, McGill University) detected eIF4G-3 and eIF4G-3\textsuperscript{t}. Lysates from transfected cells were harvested using 0.2% digitonin and the protein complex immunoprecipitated with anti-TTK antibody. The co-immunoprecipitated complex was run on a SDS-PAGE gel and the blot probed with anti-EE and \(\alpha\)-eIF4G-3 antibodies. As shown in Fig. 16, both wild type and truncated eIF4G-3 interact with TTK; we presumed that this interaction takes place through the amino-terminus region of eIF4G-3 as shown by sequence alignment (section 3.5.2 and Figs. 4 & 10). In support of this assumption, the carboxyterminal-truncated eIF4G-3 was also shown to interact with both TTK and mutant version of TTK. Whether eIF4G-3 is phosphorylated by TTK needs further clarification, and experiments are underway in Dr. Hogg's laboratory to determine this.

To prove that this association between TTK and eIF4G-1 and eIF4G-3 is not an artifact due to the presence of overabundant proteins expressed through the vaccinia expression system in the cell, we sought to prove an association between these proteins in untransfected cells (Fig. 16). We used the T98G glioblastoma cell line to verify the
association between TTK and eIF4G-1 and eIF4G-3. T98G cells were grown to 85% confluence harvested using 0.2% digitonin buffer and the lysate immunoprecipitated with anti-TTK antibody. The immunoprecipitated complex was separated on a SDS-PAGE gel, transferred to nylon membrane, and probed with eIF4G-1 specific antibodies. The western blot did indeed show the presence of eIF4G-1 and eIF4G-3 in association with TTK as detected by the respective antibodies (Fig 16).

This experiment and its outcome reinforced and supported our hypothesis that association of TTK and eIF4G-1 and eIF4G-3 does occur in untransfected mammalian cells in a native complex.

3.6.7 In Vitro Kinase assay (figure 18)

The next question we wanted to address was whether this association between TTK and eIF4G-1 and eIF4G-3 resulted in the phosphorylation of the latter proteins. To this end, we performed a series of in vitro kinase assays. Dr. Wei Wang carried out the majority of these experiments.

We performed co-immunoprecipitation experiments as described above (sections 3.5.4.1 and 3.4.5.2), but divided the lysates into two portions and one part of the lysate was used in an in vitro kinase assay. The immunoprecipitated complex was first washed with 0.2% digitonin followed by washes with kinase assay buffer. After removing the residual medium, radiolabelled \(^{32}\)P-\(\gamma\)-ATP was added to the immunoprecipitate pellet, incubated at room temperature for 30 minutes and autoradiographed. As seen in Fig. 18, TTK phosphorylates itself as well as a band co-migrating with eIF4G-1 at the 185 kDa. No phosphorylated bands were detected when
these co-transfection experiments were carried out with kinase dead mutant TTK

Recall that western blots probed with a α-ptyr antibody detected a strong band that co-
migrated with eIF4G-1 in the wild type TTK lane (Fig. 17; section 3.5.4.1).

We are conducting similar in vitro experiments on lysates containing TTK, and
eIF4G-3 or eIF4G-3'. We conclude that TTK both autophosphorylates, and in addition,
phosphorylate one of its protein partners, namely eIF4G-1. Mutant TTK, while
associating with eIF4G-1, does not phosphorylate it. Finally, we suggest that while
TTK phosphorylates eIF4G-1 on tyr residues (as detected by α-ptyr antibody), this
kinase likely autophosphorylates on ser and thr residues, since the α-ptyr antibody does
not recognize a band that co-migrate with TTK.
4.0 Discussion

This thesis project evolved from previous characterization of the novel kinase TTK (Threonine and Tyrosine Kinase) that was cloned and characterized in the laboratory of Drs. Hogg and Mills (1992). These investigators originally cloned TTK from an NK cell line expression library by screening for novel kinases using an \( \alpha \)-ptyr antibody. They observed that TTK was, highly conserved across species as diverse as man, mouse and yeast. TTK mRNA was expressed ubiquitously in proliferating cells and tissues and in malignant tumors. Moreover, TTK is expressed in all proliferating cell lines tested, and in fact is highly cell cycle regulated, demonstrating maximal activity in M-phase and very little activity in mid-late G1. Taken together, these findings suggested that TTK may play an important and highly conserved role in cell proliferation.

4.1 TTK and the cell cycle

TTK is a mixed function kinase that phosphorylates serine, threonine and tyrosine residues, as do some cell cycle dependent kinases (cdks). In support of this finding, TTK does not contain the HRDL sequence which is invariably present in members of the Tyr kinase family but rather contains the DLK motif which is found in members of the Ser/Thr kinase family and in kinases which phosphorylate Ser, Thr, and Tyr residues. TTK also contains the DLKPAN motif, which is found in most kinases, which phosphorylate ser, thr and tyr residues (Mills, et al., 1992; Hanks, et al., 1988; Stern, et
TTK itself may be regulated by phosphorylation by other ser/thr kinases, as it contains consensus phosphorylation motifs for a number of different protein kinases (Mills, et al., 1992). Because of the tight association between TTK expression and cell proliferation, Hogg et al (1994) proposed that this kinase might be regulated in the cell cycle. In support of this hypothesis, they found very low levels of TTK mRNA and protein in starved T98G cells. When these cells were induced to enter the cell cycle, levels of TTK mRNA, protein and kinase activity increased at the G1/S phase and peaked in the G2/M phase of the cell cycle. That TTK may actually play a role in cell cycle control was supported by the observation that deletion of a homologous yeast kinase called RPK1 (regulatory cell proliferation kinase) led to early mitosis and subsequent mitotic catastrophe in S. cerevisiae, while overexpression of the RPK1 gene gave rise to polyploidy. RPK1 thus may function in a checkpoint control which couples DNA replication to mitosis and is involved in a pathway that coordinates cell proliferation and differentiation (Poch, et al., 1994). However, the role of human TTK in specific signal transduction pathways has remained unknown to this point.

4.2 eIF4G-1 and eIF4G-3 interact with TTK in the Y2H system

To shed light on the cellular roles of TTK, we sought to determine what other molecules interacted with this kinase. We reasoned that this knowledge would in turn provide further insight into some of the functions that TTK subserves. To this end, we employed the yeast two hybrid system with TTK as bait to screen a murine embryonic cDNA library. We isolated a number of clones, of which two represented members of the eIF4G-I family involved in protein translation initiation, namely eIF4G-I and
eIF4G-3. TTK binds to and phosphorylates both full-length proteins, suggesting that it plays a role in controlling protein translation in a cell cycle regulated fashion.

We focused on the eIF4G-1 and eIF4G-3 clones for the following reasons. First, when the sequences of these clones were matched against the database, they were both identified as isologous translation initiation factors (Figs. 10 & 24). Second, eIF4G-3 was isolated in two separate Y2H screens, albeit from the same cDNA library. Third, the eIF4G-1 and eIF4G-3 clones isolated from the Y2H screen does not overlap precisely, but are separated by a gap of six amino acids. Thus, while TTK may not bind to the same sequences on eIF4G-1 and eIF4G-3, the region encompassed by clones 1.7 and 24 may represent a true TTK interaction domain. Finally, while eIF4G-1 had already been cloned and characterized elsewhere, eIF4G-3 had not at the time we initiated this project, so we undertook to characterize the corresponding cDNA as well as the nature of its interaction with TTK.

To this end, we used the putative eIF4G-3 clone (#24) obtained from the second Y2H screening to probe a HUVEC cDNA library and obtained a 3.5kb clone. Manual and automated sequencing of this isolate revealed that we lacked the 5' end of the full length open reading frame (ORF). At this time, the full-length mouse homolog of eIF4G-3 appeared in the genome database (GDB). To clone the remaining eIF4G-3 human sequence, we designed primers to the 5' end of the mouse clone and used these primers to screen a teratocarcinoma cDNA library. We isolated a 400bp clone that was highly homologous to the murine sequences. By ligating this isolate to the 3.5 clone we obtained a full-length eIF4G-3 cDNA containing a full length ORF. This clone was
subsequently expressed in the Vaccine virus expression system to produce the predicted 97 kDa protein.

4.3 Translation initiation factors eIF4G-1 and eIF4G-3

As described in the introduction, multiprotein complexes are formed at the 5’ end of the mRNA during translation initiation. EIF4G-1 serves as a scaffold for eIF4E and eIF4A to form the eIF4F complex in, which eIF4E binds to the cap structure to position the complex near the 5’ terminus of the mRNA. EIF4A (an RNA-dependent ATPase and ATP-dependent RNA helicase) unwinds the secondary structure of the 5’ untranslated region (5’UTR) of the mRNA to facilitate ribosome binding. Finally, eIF3 (a large protein complex consisting of at least eight polypeptides) binds to eIF4G-1 and serves to juxtapose the 40S ribosome next to the 5’ end of the mRNA (Pain, 1996; Merrick and Hershey, 1996).

Several viruses, including members of the picornaviruses family inhibit cap dependent translation of cellular proteins by cleaving the N-terminal third of eIF4G-1. This proteolysis separates the binding site for eIF4E from those for eIF4A and eIF3. Translation of viral RNA is not affected, since the C-terminal fragment of eIF4G-1 can support cap independent translation (Ohlmann et al., 1995; Ohlmann et al., 1996). Interestingly, eIF4G-3 has homology to the C-terminal fragment of the picornavirus cleaved eIF4G-1.
4.3.1 Cloning of eIF4G-3

I used the full-length human cDNA clone of eIF4G-3 in northern blotting experiments and observed mRNA expression in all cells and tissues tested, suggesting that the protein is required in all cells. To confirm that the human eIF4G-3 cDNA did indeed encode a protein of the expected molecular mass, I inserted it into the EE_{p}TM1 expression vector. Transfection of this construct into vaccinia virus infected cells resulted in the expression of the predicted 97 kDa protein bearing a polyglutamic (poly-E) tag at the amino terminus. Moreover, a truncated version of the eIF4G-3-EE construct yielded a protein of 87 kDa (this latter reagent was later used in co-immunoprecipitation experiments). In the murine and human eIF4G-3 cDNA, the initiator codon is GUG rather than ATG codon. Imataka et al (1997) mutated the murine initiation codon to AUG and noted a four-fold increase in eIF4G-3 protein levels, while mutation of the GUG to GGG abrogated eIF4G-3 translation. Alignment of human eIF4G-3 and eIF4G-1 (p220) amino acid sequence reveals that eIF4G-3 exhibits overall 28% identity and 36% similarity to the C-terminal two thirds of eIF4G-1. The N-terminal third of eIF4G-1, to which eIF4E binds (Lamphear et al, 1995; Mader et al., 1995) bears no similarity to eIF4G-3, and thus eIF4G-3 lacks an eIF4E binding site (Mader et al., 1995). As predicted, immunoprecipitation experiments revealed that eIF4A and eIF3 co-precipitated with eIF4G-3 and eIF4G-1, while eIF4E co-precipitated with eIF4G-1 only (Imataka et al., 1996).
4.3.2 Association of TTK with eIF4G-1 and eIF4G-3 in mammalian cells

We first precipitated TTK together with eIF4G-1 and eIF4G-3 and detected these two translation initiation factors using α-EE antibodies or specific antisera. Association of TTK with its targets could be demonstrated both in transfection experiments (in vaccinia infected COS cells) and as a native complex in the T98G glioblastoma cell line.

In our co-expression and co-immunoprecipitation experiments with TTK, eIF4G-1 and eIF4G-3 we observed bands migrating at the 97 kDa range with anti-TTK and anti-eIF4G-3 Abs in all TTK and eIF4G-3 transfectants. Using the anti-eIF4G-1 Ab we observed a band at 220 kDa only in those lanes containing cotransfected TTK+eIF4G-1 or TTKm+eIF4G-1. A negative control using an irrelevant antibody (anti-p16) did not immunoprecipitate TTK or eIF4G-1 separately or together. This experiment helped us to conclude that TTK and eIF4G-1 exist as a complex in the transfected cells. An in vitro kinase assay of the co-immunoprecipitate revealed that eIF4G-1 is phosphorylated by TTK. When the blot was probed with anti-phosphotyrosine Ab, a strong band was detected at 220 kDa that co-migrated with eIF4G-1. We conclude that TTK does interact with eIF4G-1 and likely phosphorylates it on tyrosine residues, although proof of this hypothesis awaits phosphoamino acid analyses (see Figs.16-18). As TTK is capable of phosphorylating tyr, ser or thr residues, it is conceivable that this kinase autophosphorylates on ser and thr sites, but targets tyr residues in other proteins. Bu et al., 1993 have shown that phosphorylation of eIF4G-1 recruits the eIF4F complex to the m7G cap structure, but not eIF4E alone, perhaps by enhancing the affinity of eIF4G-1
for eIF4E. It is also possible that several kinases phosphorylate eIF4G-1, so that alternative signaling pathways may dictate the eIF4G-1 residues to be phosphorylated and thereby modulate its biological function.

TTK and eIF4G-3 both migrate as 97 kDa bands on a protein gel. To differentiate between the two molecules we truncated eIF4G-3 at the COOH terminal to yield an 86 kDa protein (which as predicted, still interacted with TTK). We carried out a series of co-immunoprecipitation experiments to establish that TTK did indeed associate with eIF4G-3. Following immunoprecipitation with α-TTK antibody, we performed western blotting experiments using α- eIF4G-3 antisera. Immunoprecipitation of TTK followed by western blotting with α-eIF4G-3 antisera demonstrated a 97 kDa band in the immunoprecipitate containing full-length eIF4G-3. In contrast, western blotting identified an 86 kDa band when the truncated form of eIF4G-3 was transfected (Figs. 16). These studies established that there is an association between TTK and eIF4G-3, as well as between TTK and eIF4G-1. We have not yet established whether TTK phosphorylates eIF4G-3. If this is so, then phosphorylation/dephosphorylation of eIF4G-3 might alter its affinity for eIF4A and eIF3.

4.4 Phosphorylation in translational control

The interactions between the mRNA cap, eIF4E and eIF4G-1 and the 4E-BPs have a profound impact on the rate of translation. To control and modulate the binding of eIF4E to 4E-BPs, the cell employs at least two major signal transduction cascades to phosphorylate these proteins independently.
Translation is concurrently regulated by the phosphorylation states of eIF4E and 4E-BP1 following exposure to heat shock or infection by adenovirus (Ad) (Feigenblum and Schneider, 1996) by simultaneously inducing dephosphorylation of eIF4E and 4E-BP1. These data suggest that cells might coordinately regulate translation of capped mRNAs by impairing both the activity and the availability of eIF4E. Likewise, adenovirus induced phosphorylation of 4E-BP1 is mediated by the rapamycin sensitive pathway that involves the p70\textsuperscript{65k} Rsk kinase signalling cascade, which may be triggered by growth factors or hormonal signals (Beretta et al., 1996; Graves et al., 1995). An inhibitor of MAP kinase kinase (MEK), PD058059 inhibited insulin-stimulated MAP kinase activity in 3T3-L1 cells but had no effect on the phosphorylation of 4E-BP1 (Lin et al., 1995). The insulin growth factor, IGF-1 increased the phosphorylation of 4E-BP1 in aortic smooth muscle cells without an increase in MAP kinase activity (Graves et al., 1995). These results indicate that the major 4E-BP1 phosphorylation pathway is rapamycin sensitive and does not include the MAP kinase pathway as initially supposed (Haystead et al., 1994). Exposure of cells to growth factors or other mitogens triggers hyperphosphorylation of 4E-BP1, which then dissociates from eIF4E. Conversely, treatment of cells with rapamycin or wortmannin blocks such phosphorylation, allows the 4E-BP to associate tightly with eIF-4E, and leads to a G0/G1 growth arrest (von Manteuffel et al., 1997; Beretta et al., 1996; Gingras et al.; 1998). At least in yeast, this cell cycle arrest is directly due to a severe and general inhibition of protein synthesis (Barbet et al., 1996).

In contrast, phosphorylation of eIF4E is thought to result from signalling, at least in part, through the MAPK pathway (Wang et al., 1998). The kinase that directly
phosphorylates eIF4E is probably Mnk-1 (activated by MAPK) (Morley and Mckendrick, 1997; Sonenberg and Gingras, 1998). Phosphorylation of eIF4E decreases its binding to 4E-BPs and increases the rate of protein synthesis.

4.5 The translational signaling cascade (FRAP/RAFT1 pathway)

As described above, the kinase(s) that phosphorylates 4E-BP1/2 is now thought to be a member of the FRAP/RAFT1/mTOR pathway. This pathway has been conserved from yeast to humans and is largely dedicated to the detection of nutrient supply (yeast and humans) (Burnett et al., 1998; Wang et al., 1998) and growth factor signals that invoke protein translation initiation (human) (Burnett et al., 1998; Wang et al., 1998). Central to the pathway is the FRAP kinase, which corresponds to TOR1p and TOR2p in yeast (Beretta et al., 1996; Gingras et al., 1998; Pedersen et al., 1997; West et al., 1998). FRAP is a member of the phosphoinositide 3-kinase (PI3K) family, which also includes ATM, ATR and PI3K itself. Signals are transduced from upstream of FRAP by PI3K via the AKT/PKB kinase. PI3Kinase activity is inhibited by wortmannin, while FRAP is similarly affected by rapamycin. Inhibition of either kinase induces a profound block in protein synthesis (Gingras et al., 1998; Pedersen et al., 1997; Wagle et al., 1998) (Fig. 19). The anti-proliferative effects of rapamycin are further associated with prevention of mitogen induced downregulation of the cyclin-dependent kinase inhibitor p27kip1: Murine BC3H1 cells, selected for resistance to growth inhibition by rapamycin, exhibited an intact p70s6k pathway but had very low p27 levels that were no longer responsive to mitogens or rapamycin. Fibroblasts and T lymphocytes from mice with a targeted disruption of the p27kip1 gene had impaired growth inhibitory responses to
rapamycin. These results suggest that the ability to regulate p27kip1 levels is important for rapamycin to exert its anti-proliferative effects (Luo et al., 1996).

FRAP activation results in phosphorylation of the p70 ribosomal protein S6 kinase (p706k) and the 4E-BP family members. It is unlikely that FRAP phosphorylates 4E-BP directly; this action appears to be carried out by one or more protein kinases directed against S/TP motifs (Wagle et al., 1998; Pedersen et al., 1997; Gingras et al., 1998). Likewise, another uncharacterised kinase probably phosphorylates p706k, which then phosphorylates its target (the S6 ribosomal protein). Finally, p706k itself may also phosphorylate 4E-BP (Gingras et al., 1998; Pedersen et al., 1997).

EF4G-1 and eIF4G-3 have complex 5' secondary structures and a 5'polypyrmidine tract (TOP), and are hence candidates for regulation by S6 ribosomal protein phosphorylation. The role of secondary elements, 5'TOP elements, phosphorylation of both S6 and 4E-BP1 are currently under investigation (Brown and Schreiber, 1996).

It is clear that phosphorylation of eIF4G-1 occurs at multiple sites in stimulated cells (Morley et al., 1991; Morley and Traugh, 1990; Donaldson et al., 1991). In yeast, inhibition of TOR1/TOR2 with a rapamycin promotes the rapid degradation of eIF4G-1 (Berset et al., 1998); similar studies have not yet been carried out in mammalian cells. Therefore, the finding that eIF4G-1 and eIF4G-3 associate with TTK is of considerable interest.

4.6 Phosphorylation of eIF4G-1 and eIF4G-3

To date no specific kinase has been shown to target eIF4G-1 or eIF4G-3. Many hormones, mitogens and growth factors have been shown to induce phosphorylation of
eIF4G-1, suggesting that multiple signaling pathways impinge on this translation initiation factor proteins. In fact, such phosphorylation may conceivably act inversely on the two eIFG family members: eIF4G-1 may become less able to direct translation following phosphorylation, while eIF4G-3 may act as a more efficient repressor. We are currently conducting experiments to address this hypothesis. We speculate that TTK phosphorylates both eIF4G-1 and eIF4G-3 in vivo, and in doing so, alters the efficiency of protein translation (Figs. 16-18).

We have not yet identified the residue (s) in either initiation factor that are targeted by TTK; however, TTK appears to phosphorylate eIF4G-1 on tyrosine (Fig. 17), on the basis of western blotting experiments using an α-phosphotyrosine antibody as probe. To address this issue, further experiments with deleted/mutated eIF4G-1 and eIF4G-3 constructs are planned.

Phosphorylation of eIF4G-1 and eIF4G-3 could alter their binding affinities to target proteins and thus modulate their biological functions. While eIF4G-1 upregulates translation efficiency, eIF4G-3 downregulates it by a factor of two (Imataka et al., 1996). These experiments and others (Craig et al., 1998; Gradi et al., 1998) suggest that eIF4G-3 is a general suppressor of translation that acts by forming a protein complex with eIF4A and eIF3, but not eIF4E (Imataka et al., 1996). This complex may allow translation only at IRES sequences, but not from 5' capped mRNA. Another mechanism by which eIF4G-3 may inhibit translation is based on the results by Tarun and Sachs (1996), who showed that in yeast the poly A binding protein (Pabpl) enhances translation by interacting with eIF4G-1. If this mechanism is also true in the mammalian system, eIF4G-3 could compete with eIF4G-1 for interaction with the poly
A binding protein. If the fundamental role of eIF4G-3 is to repress translation, when does it act and by what mechanisms? Does phosphorylation of eIF4G-3 by TTK control its activity?

4.7 Subcellular localization of TTK

TTK carries a ‘KKRGKK’ motif on the carboxy terminal (Fig.3). Proteins that possess a di-lysine motif, in which the lysines are at positions –3 and –4 from the C-terminus (KKXX), are recycled to the endoplasmic reticulum (ER) from the golgi complex (Pelham, 1994). In contrast, the KDEL signal targets proteins to the ER lumina. Both in animal cells and in yeast, proteins bearing the KKXX motif are found in the ER but often acquire golgi-specific carbohydrate modifications. Yeast and mammalian ER retention motifs interact specifically in cell lysates with the coatomer, a polypeptide complex implicated in membrane traffic. The molecular mechanism for ER retrieval of di-lysine tagged proteins is unknown (Cosson and Letourneur, 1994; Letourneur et al., 1994). Unpublished data (this lab) using immunohistochemistry and confocal microscopy has revealed that native TTK is localized to the cytoplasm. Despite the difference between the consensus ER recycling sequence (KKXX) and the carboxy terminus of TTK (KKRGKK), it is possible that TTK is localized to the endoplasmic reticulum (ER), in keeping with the putative role of TTK in protein translation. To address this possibility we have constructed a green fluorescent protein (GFP)-TTK fusion construct for use in transfection studies. This construct will be used in tandem with immuno-histochemistry of specific proteins to determine the precise subcellular location of TTK.
4.8 Two model systems for translational control

We propose two models of translational control by TTK based on its association with eIF4G-1 and eIF4G-3 (Figs. 20-23). First, eIF4G-3 may inhibit translation initiation by interacting with the polyA binding protein (Pab1p) at the C-terminal end thereby sequestering eIF4A and eIF3. EIF4A is important for both cap-dependent and cap-independent translation, as dominant negative mutants of eIF4A repress both cap-dependent and cap-independent translation (Pause et al., 1994). TTK phosphorylation of eIF4G-3 might increase its binding affinity towards eIF4A, eIF3 and other rate-limiting translation initiation factors. (Figs. 20 & 21).

Second, eIF4G-3 may interact indirectly with PABP through an ‘intermediate’ protein (protein x—see figs. 22 & 23). Craig et al., (1998) have recently described a novel protein designated PAIP-1 (poly-A binding protein-interacting protein) that increases translation efficiency by interacting via its C-terminus (aa 415-480) with PABP (poly-A binding protein-Pab1p). We speculate that TTK may be phosphorylate either PAIP-1, eIF4G-3 or both, alter the interaction between these proteins, and hence repress translation (see figs.22&23). In support of this hypothesis, we note that there is significant homology between PAIP-1 and eIF4G-3 at the same region of eIF4G-1 and eIF4G-3 implicated in TTK interaction (Fig. 10).

It is very likely that TTK plays additional roles in cell cycle control other than translational control. The homologous yeast RPK1/MPS1 kinase is thought to enforce a checkpoint that monitors the mitotic pole spindle (equivalent to the centrosome in
human cells). Whether TTK serves a similar function in mammalian cells is of interest, but was not addressed in my thesis work.
5.0 Future studies

To determine whether TTK does indeed play a role in the cell cycle regulation of protein translation, our laboratory will have to address the following questions.

5.1 Does interaction of TTK with eIF4G-1 family members vary during the cell cycle?

We have shown that TTK associates with eIF4G-1 and eIF4G-3 in proliferating cells. We plan to carry out a series of experiments to determine whether this interaction with, and phosphorylation by TTK, varies in degree during the cell cycle by using synchronized cell lines (Hogg et al., 1994). These experiments are the first step in establishing a link between the cell cycle regulation of TTK and its effect on protein translation.

We hypothesize that the interaction between TTK and eIF4G-1/3 will increase concurrently with increasing TTK kinase activity. However, the activity of TTK as measured by autophosphorylation may not necessarily correlate with its binding to the translation initiation factors. If this is the case, we may have to reassess our model of TTK as a negative regulator of translation.

5.2 Does TTK alter the efficiency of protein translation?

The next step in confirming that TTK plays a role in translation initiation is to directly examine the effect of this kinase on translation in the cellular milieu. To this
end, we will transfect cells with kinase active and kinase dead TTK constructs, alone or in combination with eIF4G-1 and eIF4G-3 and analyze protein translation efficiency. While the technical details of these experiments are beyond the scope of this discussion, we will make use of a CMV-cap-IRES vector (a kind gift of Nahum Sonenberg). Transcription of this construct from the CMV promoter expresses a mRNA bearing in order: a 5’-cap, a chloramphenicol acetyltransferase (CAT) reporter gene; an IRES (internal ribosome entry site); and a luciferase reporter gene (Roberts et al., 1998). A ribosome that initiates from the cap site will translate the CAT cassette, while one that scans from the IRES will encounter and translate the luciferase gene. The overall translational efficiency may be judged by comparing the mRNA levels (which we will measure by the RNAse protection assay) to those of the expressed proteins; the efficiency of cap-dependent versus cap-independent translation may be judged by examining the ratio of CAT to luciferase activity (Roberts et al., 1998). This system can detect a 2-3 fold variation in protein translation efficiency in mammalian cells.

We have also constructed pcDNA expression vectors that express eIF4G-1 and eIF4G-3. Expression of eIF4G-1 increases protein translation, while expression of eIF4G-3 suppresses it. However, the effect of co-expression of TTK and members of the eIF4G-1 family is unknown. To address this question, we will use the eIF4G-1 constructs in additional cap-IRES readout experiments in which we transfect combinations of: active or kinase-dead TTK; and eIF4G-1 or eIF4G-3. We hypothesize that TTK, eIF4G-1 will act in opposition (negatively and positively, respectively), while co-transfection of TTK, and eIF4G-3 should depress translation synergistically.
5.3 Mapping the interaction domains of TTK and eIF4G-1 family members

The Y2H screen fortuitously suggested the region of the TTK binding domain of eIF4G-1 and eIF4G-3, as these two isolates encompassed, but did not overlap homologous sequences (see Figs. 10 & 24). However, we do not know whether there is one or more than one TTK binding domain in these three translation initiation factors. In addition, as shown in Fig. 10, PAIP-1 shares considerable homology with the eIF4G-1 family members around the presumptive TTK binding site. It is therefore possible that PAIP-1 and TTK interact, even though the former protein was not originally isolated in the Y2H screen. This question is of considerable importance, as phosphorylation of PAIP-1 by TTK could conceivably alter the interaction of the polyadenylated tail of the mRNA with the cap-binding complex (see section 4.8).

To this end, we will perform additional binding assays using the Y2H system. We have chosen this approach because of its ability to screen large numbers of individual constructs in a speedy, sensitive and quantitative fashion. We will use PCR to construct and mutagenize various deletion mutants of eIF4G-1, eIF4G-3, PAIP-1 and TTK and then mate the bait (TTK) to the prey (other factors) constructs in yeast. The interactions between TTK and its putative partners will be measured by a liquid β-galactosidase assay that is accurate and reproducible in our hands. These experiments should allow us to map the interaction domains (at least in eIF4G-1, eIF4G-3 and TTK).
5.4 Is TTK in the FRAP signaling pathway?

Schmandt et al (1994) demonstrated that TTK expression is blocked by either cyclosporin A or FK520, which inhibits IL-2 production; this blockade could be bypassed by the addition of exogenous IL-2. Furthermore, TTK expression was prevented by incubation of the cells (T cells) with rapamycin, which blocks IL-2 signaling. This experimental evidence suggests that TTK may be present in the FRAP signaling pathway. However, it is also possible that rapamycin merely induces a G0/G1 cell cycle arrest; in this case, low levels of TTK would merely reflect a loss of proliferative activity.

To address the role of TTK in the FRAP pathway, we will determine the time course of TTK inhibition by rapamycin. If the blockade of TTK activity occurs early after rapamycin treatment, it is likely that both kinases lie in the same or related signalling pathways. Conversely, TTK activity may decrease only when cells undergoing rapamycin treatment exit the cell cycle. This observation would suggest that there is little direct communication between FRAP and TTK. We suspect that this latter hypothesis is correct. Specifically, we propose that growth signals that propel the cell through G1/S increase protein translation through FRAP/RAFT-1/mTOR pathway. Conversely, entry into mitosis forces a cessation of translation, which is mediated at least in part through TTK.
6.0 Conclusion

I have cloned and expressed full length and functional cDNA of eIF4G-3 and TTK in a transient expression construct and expressed them in mammalian cells. I have also shown, in collaboration with Dr. Wei Wang that TTK associates with the translational initiation factors eIF4G-1 and eIF4G-3 as proved by co-immunoprecipitation and in vitro kinase experiments. The consequence of this interaction between TTK and eIF4G-1 and with that of eIF4G-3 is yet to be determined. This biological interaction should shed considerable light on the signaling pathways involved in translational control.
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rapamycin-sensitive point immediately upstream of p70^{65k}. Mol Cell Biol 17, pp.5426-36.


3.0 Table Legends

Table 1: The key players in human, rat, and mouse protein translation initiation are, rat and mouse.

eIF = eukaryotic initiation factor

Table 2: The oligonucleotides used in the sequencing of the cDNA clone B3.5 are shown

S = sense primer; AS = anti sense primer; rev-com = reverse complement primer.

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<td>AS-EEpTM1: 1599-171</td>
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Table 3: 5’ RACE specific oligonucleotides used to obtain the 5’ end of the cDNA clone B3.5 are

S = sense primer; S1 to the 5’end of mouse: 29-50 nt; S2: 198-218 nt

Anti-sense from human eIF4G-3: 603-622 nt
Table 1. The key players in protein translation initiation (adapted from Merrick and Hershey, 1996)

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<td>94</td>
<td></td>
<td>stabilizes ternary complex in presence of RNA</td>
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<td>elf3</td>
<td></td>
<td>650</td>
<td></td>
<td>dissociates ribosomes; promotes Met-tRNAi and mRNA</td>
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<td>p35</td>
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<td>p115</td>
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<td>105.3</td>
<td>binds RNA</td>
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<td>major phosphorylated subunit</td>
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<td>elf4A</td>
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<td>46</td>
<td>44.4</td>
<td>ATPase, helicase, binds RNA</td>
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<td>80</td>
<td>69.8</td>
<td>binds RNA, promotes helicase activity</td>
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<td>25</td>
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<td>m'G cap-binding unit</td>
<td>M15353</td>
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<td>46</td>
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<td>ATPase, helicase</td>
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<td>elf4G-1</td>
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<td>220</td>
<td>153.4</td>
<td>binds elf4A, 4E and elf3</td>
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<td>human</td>
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<td>elf5</td>
<td></td>
<td>58</td>
<td>48.9</td>
<td>promotes GTPase with elf2 and ejection of elfs</td>
<td>L11651</td>
<td>rat</td>
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<td>elf6</td>
<td></td>
<td>25</td>
<td></td>
<td>binds to 60S ribosomes, promotes dissociation</td>
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<td>p97</td>
<td></td>
<td>97</td>
<td></td>
<td>translational suppressor; positive regulator of apoptosis</td>
<td>U63323</td>
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<tr>
<td>No.</td>
<td>Name</td>
<td>Sequence</td>
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<td>3’TGACCGGCAGCAAAAATG 5’</td>
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<td>3.</td>
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<td>AS-cloning primer 3</td>
<td>5’CCATTCTCAAGAAAAGCCTTGCGAGG 3’</td>
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<td>6.</td>
<td>New clone S1</td>
<td>5’GCAGCAGTTACTCTCTAGTC 3’</td>
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<td>8.</td>
<td>B3.5-S8</td>
<td>5’CATCCTCTGCTCCTTCCAAG 3’</td>
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<td>9.</td>
<td>B3.5-S9</td>
<td>5’GCAAGCTTGACTTTCACCAG 3’</td>
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<td>10.</td>
<td>New clone-AS1</td>
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<td>11.</td>
<td>New clone-S1</td>
<td>5’CGTCGTCAATTGGAGACTCAG 3’</td>
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<td>12.</td>
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<td>13.</td>
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<td>14.</td>
<td>EEpTM1-S1</td>
<td>5’ACGTGTTTTCCTTTGAATTAAAC 3’</td>
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<td>4G2/AS-3</td>
<td>5’GTGGCATATCCTCCTTCCTGTGG 3’</td>
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<td>16.</td>
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<td>5’GTGCCACTGTTCCTATTCATTAC 3’</td>
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<td>17.</td>
<td>4G2/S7</td>
<td>5’CGTTGGAATAGAATAAGTGC 3’</td>
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<td>18.</td>
<td>4G2/S6</td>
<td>5’GTAGGAAGCAGGAAGTTTC 3’</td>
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<td>19.</td>
<td>AS-rev.com</td>
<td>5’CTGCTCTAAGTGTCTTTGG 3’</td>
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<td>21.</td>
<td>Sense124216-2</td>
<td>5’TCAACCAGTAAAGTGTC 3’</td>
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Table 3. List of 5' RACE specific oligonucleotides and their sequences

<table>
<thead>
<tr>
<th>Number</th>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>S1 to the 5' end of mouse</td>
<td>5'GCAGCAGTTACTCCTCTGAGTC 3'</td>
</tr>
<tr>
<td>2.</td>
<td>S2</td>
<td>5'GGCTTTTCATTCTACCACCACCC 3'</td>
</tr>
<tr>
<td>3.</td>
<td>S3</td>
<td>5'ACATCCATGGAGTGCGATTCGAG 3'</td>
</tr>
<tr>
<td>4.</td>
<td>Anti-sense from human elf4C-3 clone</td>
<td>5'CTACACCCACATGAGGAGG 3'</td>
</tr>
</tbody>
</table>
4.0 Figure Legends

4.1 Fig.1 Overview of protein translation initiation (adapted from Thach, 1992)

Schematic representation of protein translation initiation, the first step in the process of protein synthesis in eukaryotes. The present understanding is that the different components of the eIF4F complex are recycled and assemble to form the functional eIF4F comprising eIF4G-1, eIF4E, eIF4A and eIF4B. eIF4E is the cap recognition protein; eIF4G-1 the adapter molecule; and eIF4A the ATP dependent RNA helicase at the carboxy terminus respectively; eIF4B co-operates with eIF4A to enhance the rate of mRNA helix unwinding.

4.2 Fig. 2 Yeast two-hybrid system (Y2H)

The Y2H is an assay system for protein-protein interaction devised by Stanley Fields (1989). The presence of either the DNA binding domain or the activating domain alone does not result in transcription of the reporter gene, as the interaction of both domains is necessary for transcription. In our experiments, TTK-lexA DNA binding domain fusion protein is the ‘bait’ and a murine cDNA library from mouse embryos 9.5 to 10.5 days post-coitum fused to VP16 is the ‘prey’. In the TTK-lexA system, we used lacZ and histidine genes as a reporter and selectable marker, respectively.
4.3 Fig. 3 TTK protein structure

Schematic representation of TTK protein structure. It comprises 858 aa encoding a 97 kDa protein. The diagram shows the ‘bait’ region (125-366) used in the Y2H screen, potential phosphorylation sites, the kinase domain and a carboxy ‘KKRGKK’ endoplasmic localization signal.

S= serine; T=threonine; P=proline

4.4 Figs. 4A & 4B Yeast two-hybrid screen approach and B3.5 restriction map and sub-cloning sites

Clone B3.5 (eIF4G-3) was isolated from a human umbilical vein endothelial cell (HUVEC) cDNA library. The cDNA is located between the EcoRI and XhoI sites in the polylinker of the parent vector (blue script). Single restriction enzyme digestions with BamHI and PstI followed by self-ligation of the digested clones using ligase yielded clones with a 2.2 kb and a 3.2 kb inserts respectively. I also double digested B3.5 with BamHI + PstI and subcloned the resultant product of 1.4 and 0.8 kb inserts into pBS vector.

Fig. 4A showing the Y2H approach and the two prey clones differing from each other by a short 6 aa stretch.

Fig.4B also shows the overlap of clone 24 (isolated from Y2H ) between nucleotides 339-803 with the B3.5 clone and used as a probe in cDNA screening
4.5 Fig. 5 Restriction mapping of the cDNA clone B3.5

To determine the different restriction sites in the clone B3.5, I digested the cDNA clone with different restriction enzymes as shown here. The different restriction enzymes are: Hind III, SacII, Xba I, Spe I, Kpn I, Sma I, BstX I, Eco RV, Pst I, Cla I, control. The first lane shows the molecular weight markers.

4.6 Fig. 6 Obtaining the 5' end of eIF4G-3

When the B3.5 clone was sequenced and compared to the mouse sequence, we realized that the cDNA clone B3.5 lacked the 5'end sequences. The correct start codon was also found missing the cDNA clone B3.5. I designed sense primers to the start site of the mouse cDNA eIF4G-3 sequences and an anti-sense primer to B3.5 and used these primers together in a PCR reaction using a teratocarcinoma cDNA library. Missing 5' end sequences, thus obtained, were then ligated to B3.5. The figure shows the tri molecular ligation between the 5' PCR product, the B3.5 clone and the expression vector EEpTM1. The resultant full-length clone is constructed between Nco I and Sma I in the expression vector.

4.7 Fig. 7 cDNA clone B3.5 (3820 bp) showing the restriction enzyme sites

Figure showing some of the restriction sites of the cDNA clone B3.5. The Hind III restriction enzyme was used to screen the full-length construct of B3.5.
4.8 Fig. 8 cDNA sequence of *eIF4G-3*

The original B3.5 clone was only 3.5 kb in length. I performed a 5' RACE to obtain the missing 5' sequences and ligated the RACE product to the original clone to yield us a full-length cDNA of 3.8 kb. *eIF4G-3* exhibits overall 28% identity and 36% similarity to the C-terminal two thirds of *eIF4G-1*. The N-terminal third of *eIF4G-1*, to which *eIF4E* binds bears no similarity to *eIF4G-3*. Thus, it is predicted that *eIF4G-3* would bind to *eIF4A* and *eIF3*, but not to *eIF4E*.

4.9 Fig. 9 Northern blotting

Northern blots showing steady state levels of *eIF4G-3* mRNA in all cell lines and tissues, using a 2.2 kb BamHI fragment of the B3.5 clone as a probe. Total RNA was isolated using the solution D (guanidium iso-thiocynate) protocol and 10μg of each sample were subsequently analyzed. High levels of *eIF4G-3* mRNA expression is seen in all germ cell lines and breast cancer tissues screened.

PA-1=human ovarian carcinoma; Tera-1&2=embryonic carcinoma; L02rl.1=bone marrow stromal cells transformed with large T-antigen; Jurkat=T-cell line; T98G=glioblastoma; PHA=activated T-cell; FHS=human embryonic fibroblasts; TPA=stimulated T-cells.

Germ cell tumors (GCT) cell lines: 577, 833, 1156, 1618, 1685, 2102, 2806 and 2965
4.10 Fig 10. Sequence alignment of TTK clones 1.7, 24, eIF4G-1 and eIF4G-3

Multiple sequence alignments were performed using the Align 1.0 software program, (InformMax, Gaithersburg, MD). Identical amino acids are shown in blue, while green indicates similar but not identical residues. The consensus sequence between the different proteins is shown at the bottom. EIF4G-3 exhibits overall 28% identity and 36% similarity to the C-terminal two thirds of eIF4G-1. The N-terminal third of eIF4G-1, to which eIF4E binds, bears no similarity to eIF4G-3 (Imataka et al., 1997). PAIP-1 is the poly-A binding protein interacting protein, which also shows sequence homology to eIF4G-1 and eIF4G-3 (Craig et al., 1998).

4.11 Fig. 11. Vaccinia virus expression vector

Schematic representation of vaccinia virus transient expression system comprises 5396 bp (5.3 kb). It carries a polyglutamine tag at the amino terminus of the protein and is driven by a T7 polymerase promoter.

4.12 Fig.12 EE_pTMI-TTK expression vector

Schematic representation of TTK cDNA inserted into the vaccinia virus vector. The open reading frame of TTK encompasses 2685 bp from nucleotides 865 to 3550 (Mills et al., 1992). The coding region (2.6 kb) is subcloned in the vector (5.3 kb) between the NcoI and EcoRI restriction sites, yielding a protein of 97 kDa.
4.13 Fig.13 $EE_pTM1$-eIF4G-1 expression vector

$EIf4G-1$ was subcloned into the EcoRI restriction site in the vaccinia virus expression system. $EIf4G-1$ clone was (a kind gift of Dr. Rhoads from the Louisiana State University, USA), was derived from the original pSK-HFC1 vector.

4.14 Fig.14 Western blot of in vitro transcription and translation

Western blot showing the expression of proteins in an in vitro transcription-translation system. We designed a carboxy terminal truncated version of eIF4G-3 and cloned it into $EE_pTM1$. Single and double transfections were done in vitro using the TNT rabbit reticulocyte transcription-translation system (Promega, USA). 0.2-2.0µg of plasmid DNA were added directly to TNT lysate. The mixture was incubated in a 50µl reaction volume for 1-2 hours at 30°C, and the synthesized proteins were then separated by SDS-PAGE and transferred to nylon membrane. The western blot was probed by an anti-eIF4G-3 polyclonal Ab (1:5000).

4.15 Fig.15 Western blot of co-transfections

Western blot showing the expression of proteins by the vaccinia virus expression vector-$EE_pTM1$. TTK, eIF4G-1 and eIF4G-3 constructs in vaccinia virus expression vector ($EE_pTM1$), each carrying the polyglutamine (EE) tag at their amino terminus, were transfected into vaccinia virus treated COS cells using the lipofectamine method. Single and double transfections were carried out. Cell lysates were prepared using
0.2% digitonin and the proteins were separated on an SDS-PAGE protein gel. The proteins were transferred to a nylon membrane and probed with an anti-EE MAb in 1:30 dilution. Here, the first five lanes show single transfections of the respective constructs. Lanes 7-10 carry co-transfectants of TTK and eIF4G-3. Since, TTK and eIF4G-3 share the same molecular mass of 97 kDa they appear as a single band as seen in lanes 6 & 8. Regular and truncated eIF4G-3 are both seen to be expressed along with kinase dead mutant of TTK in lanes 8 & 9. The migration of the truncated eIF4G-3 is clearly seen in lanes 4, 7 and 9 at a molecular mass (89 kDa) of less than the regular eIF4G-3.

In the first six lanes, the polyclonal antibody detected both regular and truncated eIF4G-3. Note that the polyclonal antibody recognized both the native and truncated eIF4G-3 (lanes 1, 3 & 5). In addition, the antibody detected native eIF4G-3 in the TTK alone transfection. Negative control lacks input DNA.

**4.16 Fig. 16 TTK associates with both eIF4G-1 and eIF4G-3**

Co-immunoprecipitation/western blots showing association of TTK with eIF4G-1 and eIF4G-3 in transfected and untransfected cells.

Vaccinia-infected COS cells were transfected with TTK, eIF4G-1 and eIF4G-3 constructs; cell lysates were prepared using 0.2% digitonin, and were immunoprecipitated with anti-TTK antibody (lanes 1-4) or with α-MDM2 (lanes 5-6). The immunoprecipitates were separated on an SDS-PAGE protein gel and transferred to
a nylon membrane. The membrane was cut vertically; the left 2 lanes were probed with anti-eIF4G-1, while lanes 3-6 probed with anti-eIF4G-3. In the lane co-transfected with TTK and eIF4G-I, the eIF4G-1 protein was detected; while lanes 3 & 4 (transfected with either TTK and native eIF4G-3 or truncated eIF4G-3 forms) demonstrated the presence of the two respective eIF4G-3 forms (Note that a small amount of native eIF4G-3 is seen in the lanes containing the truncated protein). Lanes 5-6, precipitated with the irrelevant α-MDM2 antibody demonstrated that the association between TTK and eIF4G-3 is not an artifact of immunoprecipitation.

The second western blot shows the association of TTK and eIF4G-1 in native T98G glioblastoma cells. The cell lysate was immunoprecipitated with an anti-TTK antibody, separated and transferred as above, and probed by an anti-eIF4G-1 antiserum. A band of 185 kDa MW is detected by the antibody. Immunoprecipitation with irrelevant antibodies (or no antibody) revealed no eIF4G-1. α-MDM2 and α-Rb (retinoblastoma) were the irrelevant antibodies used in this experiment.

4.17 Fig. 17 TTK phosphorylates eIF4G-1 on tyrosine

Three western blots demonstrating the association and phosphorylation of eIF4G-1 by TTK following co-immunoprecipitation.

Cell lysates were immunoprecipitated with anti-p16 (irrelevant antibody-control) or anti-TTK. The immunoprecipitates were separated on a SDS-PAGE protein gel, transferred to a nylon membrane and probed with an anti-phosphotyrosine antibody. The irrelevant antibody control lane lacks specific bands, while the anti-TTK
immunoprecipitate reveals complex shows a very strong band co-migrating with eIF4G-1. In contrast, the kinase-dead mutant TTK did not phosphorylate this 185 kDa band.

The same western blot was then stripped and probed with anti-TTK antibody, TTK is present in all the lanes, including the mutant TTK (TTK$_{mut}$) lane (note that despite the lower amount of TTK$_{mut}$ protein, it is still possible to conclude that the α-ptyr antibody detects no associated proteins). The western blot was then immediately reprobed with anti-eIF4G-1 antibody. This demonstrates again that active TTK associates with eIF4G-1. The mutant TTK also associates with eIF4G-1 (note faint band at 185 kDa).

4.18 Fig. 18 TTK phosphorylates eIF4G-1 in an in vitro kinase assay

Cell lysates were prepared by 0.2% digitonin; immunoprecipitated with anti-TTK antibody and washed in kinase assay buffer; radiolabelled $^{32}$P-$\gamma$-ATP was added to the immunoprecipitate pellet for 30 minutes, the complex separated on a protein SDS-PAGE gel and autoradiographed. Note that TTK autophosphorylates strongly, but that TTK, eIF4G-1 or TTK$_{mut}$ alone did not demonstrate any phosphorylating band at the $>185$ kDa range (lanes 2, 3, 4 & 5). Co-transfected TTK and eIF4G-1 demonstrate a band co-migrating with eIF4G-1 at $>185$ kDa, strongly suggesting that eIF4G-1 is indeed phosphorylated by active TTK. Finally, note that TTK-HA (lane 2) and TTK$_{mut}$ (lane 4) do not autophosphorylate.
The second autoradiograph represents a different experiment, and demonstrates again that active TTK phosphorylates eIF4G-1. The 250 kDa band may represent a dimer of TTK, or may represent another, as yet unidentified protein. The negative control lane shows empty vector alone.

4.19 Fig. 19 A model illustrating the signaling cascades leading to an increase in protein synthesis (adapted from Gingras et al., 1998)

Both wortmannin and rapamycin are inhibitors of protein translation. Wortmannin inhibits protein translation upstream in the translation signaling cascade by inhibiting the PI (3)K kinase. Rapamycin similarly inhibits the FRAP/mTOR kinase. In this model, I suggest that TTK may transduce a mitotic signal to eIF4G-1 that block protein translation.

4.20 Figs. 20 & 21 A Model of direct translation repression by eIF4G-3

Figs. 20 & 21 Schematic diagram of direct translation repression by eIF4G-3 (eIF4G-3) showing the eIF4F complex assembled at the 5’cap end of the mRNA. EIF4E binds the cap structure along with the other components of eIF4F namely, eIF4G-1, eIF3 and eIF4A. PABP also interacts with eIF4G-1, thus circularizing the mRNA. In contrast, eIF4G-3 interacts through its carboxy terminal with eIF3 and eIF4A.
EIF4G-3 inhibits both cap-dependent and independent protein translation initiation process (Imataka et al., 1997). I propose that eIF4G-3 is phosphorylated by TTK; phosphorylated eIF4G-3 directly represses translation initiation by sequestering eIF3 and eIF4A or PABP.

In Fig. 21 The model shows unphosphorylated eIF4G-3 sequestering eIF3 and eIF4A or interacting directly with PABP to stall protein translation initiation.

4.21 Figs. 22 & 23 A Model of indirect translation repression by eIF4G-3 through an ‘intermediate’ protein

Figs. 22 & 23. Schematic diagram of indirect protein translation repression by eIF4G-3 showing the eIF4F complex assembled at the 5'cap end of the mRNA. EIF4E binds to the cap structure along with the other components of eIF4F namely, eIF4G-1, eIF3 and eIF4A. PABP also interacts with eIF4G-1, thus completing the circularization of the mRNA. EIF4G-3 interacts through its carboxy terminal with eIF3 and eIF4A. Here, eIF4G-3 is also shown interacting with PAIP-1, which is necessary for mRNA expression and protection from degradation.

Craig et al., 1998 have shown recently that a protein PAIP-1 (polyA binding protein interacting protein) interacts with PABP and eIF4A, selecting only intact mRNAs (containing both cap and poly (A) tail) as templates for translation.

I am proposing a model in which eIF4G-3 interferes with this assembly process by sequestering PAIP-1 thus preventing the circularization of the mRNA (Wells et al.,
This model suggests that TTK might phosphorylate any or all of eIF4G-3, PABP and PAIP-1 effecting translation repression.

Fig. 23. The model shows unphosphorylated eIF4G-3 sequestering PAIP-1, preventing its interaction with PABP and thus repressing translation initiation complex circularization.
Fig. 1 Overview of protein translation initiation

- eIF4F
- + m7Gpppppp
- mRNA
- AUG codon

Formation of 5' cap structure

Binding of Poly-A tail to cap structure through PABP and PaIP-1
Fig. 2 Yeast two hybrid system

No Transcription Activating Domain

No Transcription Binding domain

Transcription=Yes
Activating+Binding domains
Fig. 3 TTK protein structure

Bait for Y2H screen

Complete TTK coding sequence
858 aa
Fig. 4A Yeast two hybrid screen - approach

Clone bait fragment into prey vector

Screen murine embryonic library X 2

Screen #1: eIF4G-1 + eIF4G-3 (one clone each, plus other cDNAs)
Screen #2: eIF4G-3 (one clone, plus other cDNAs)

= "prey" clones from Y2H screens

6 aa gap between prey clones
Fig. 4B B3.5 restriction map and sub-cloning sites

Clone 24 from Y2H overlapping region with B 3.5 (339-803 nt)

3.5 kb
Fig. 5 Restriction mapping of clone B 3.5
Fig. 6 Obtaining the 5' end of eIF4G-3

The new eIF4G-3 cDNA clone constructed in Vaccinia virus expression vector
Fig. 7 Human eIF4G-3 (3820 bp) showing the restriction sites

(not drawn to scale)
Fig. 8 cDNA sequence of *eIF4G-3*

```
Kpn I

1  CAGCAAGAGC AGGGCCCTCT ATGGGAGGTTG CAGGGTCTAC CAGGGCAGGC
   GGCAGGCTG AGGGCCCTCTCT CAGGGTCTAC CAGGGCAGGC

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BamHI

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Fig. 9 Northern blots showing eIF4G-3 mRNA expression
Fig 10. Sequence alignment of TTK clones 1.7, 24, eIF4G-1 and eIF4G-3
Fig. 11 Vaccinia virus expression factor

EEpTM1
5396 bp

Hind III (2)

T7 promoter

Hind III (1076)

Kpn I (1297)

EE epitope tag

BamHI (1493)
Fig. 12 EEpTM1-TTK expression vector
Fig. 13 EEpTM1-eIF4G-1 expression vector
Fig. 14 Western blot of in vitro-transcription & translation

detected with anti-eIF4G-3 polyclonal Ab (1:5000)
Fig. 15 Western blot of co-transfections

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97 kd

detected with anti-EE MAb (1:30)
Fig. 16 TTK associates with both eIF4G-1 and eIF4G-3

Untransfected T98G cells: samples immunoprecipitated with antibody as noted
Fig. 17 TTK phosphorylates eIF4G-1 on tyrosine
Fig. 18 TTK phosphorylates eIF4G in an *in vitro* kinase assay

All samples immunoprecipitated with α-TTK
Fig. 19 A model illustrating the signaling cascades leading to an increase in protein synthesis
Fig. 20 A model of direct translation repression by eIF4G-3
Fig. 21 A model of direct translation repression by unphosphorylated eIF4G-3 (eIF4G-3)
Fig. 22 A model of indirect translation repression by eIF4G-3 (eIF4G-3) through an ‘intermediate’ protein
Fig. 23 A model of indirect translation repression by unphosphorylated eIF4G-3 through an ‘intermediate’ protein