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Cloning of *S. pombe* EF-1α Using a Yeast Two-Hybrid Screen for Calmodulin-Binding Proteins

by

Christine Alaine Wiebe 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the the degree of Master of Science

Department of Biochemistry

Edmonton, Alberta

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University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled *Cloning of S. pombe EF-1α Using a Yeast Two-Hybrid Screen for Calmodulin-Binding Proteins* submitted by Christine Alaine Wiebe in partial fulfillment of the requirements for the degree of Master of Science.

Dr. Colin Rasmussen

Dr. Charles Holmes

Dr. David Begg

Dr. Michael Schultz

Date: December 5, 1997

To my mother Darlene,
with much love

Abstract

Cell growth and division are regulated by the intracellular calcium receptor calmodulin (CaM). More than 30 different enzymes are regulated by CaM in a Ca^{2+}-dependent manner. Since CaM must physically interact with the proteins it regulates we used two-hybrid selection to isolated CaM-interacting proteins. A screen of a *Schizosaccharomyces pombe* cDNA expression library yielded several clones, one containing a partial cDNA with homology (78% similarity over 100 amino acids) to human protein translation elongation factor 1α (EF-1α). We have since isolated and sequenced a full length cDNA for *S. pombe* EF-1α. An interaction between CaM and EF-1α was confirmed both *in vitro* and *in vivo*. Northern blot analysis demonstrated the presence of a single mRNA species of 1.6 kb which persisted at relatively constant levels throughout various stages of the cell cycle and also during stationary phase. Interestingly, overexpression of EF-1α in *S. pombe* resulted in a marked reduction in the rate of cell proliferation.

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aa-tRNA	aminoacyl transfer ribonucleic acid
ade	adenine
Asp	aspartate
ATP	adenosine triphosphate
BD	binding domain
BSA	bovine serum albumin
β-gal	β-galactosidase
Ca^{2+}	calcium ion
CaM	calmodulin
CaMKII	calmodulin dependent protein kinase II
cAMP	3',5'-cyclic adenosine monophosphate
cdc	cell division cycle
cDNA	complimentary deoxyribonucleic acid
cGMP	3',5'- cyclic guanosine monophosphate
CHO	Chinese hamster ovary
Ci	Curie
°C	degree(s) celsius
DAG	diacylglycerol
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate

DTT	dithiothreitol
EF-1α	elongation factor 1α
EF-1H	elongation factor 1 holoenzyme
EGTA	ethylene glycol-bis[β-aminoethyl ether]-N,N,N',N'-tetracetic acid
G_0	quiescence
G_1	gap 1 phase
G_2	gap 2 phase
Glu	glutamate
GDP	guanosine diphohsphate
GST	glutathione-*S*-transferase
GTP	guanosine triphosphate
h	hour(s)
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesufonic acid
his	histidine
HIV	human immunodeficiency virus
HSP	heat shock protein
IP_3	inositol 1,4,5-triphosphate
IPTG	isopropyl-1-β-*D*-thiogalactopyranoside
J	Joule
kb	kilobase
kDa	kiloDalton
leu	leucine
L	litre
M	molar
M (-phase)	mitosis
MAP	microtubule associated protein

MARCKS	myristoylated alanine-rich C kinase substrate
Mb	megabases
min	minute
µg	microgram
µl	microlitre
MLCK	myosin light chain kinase
mM	millimolar
mRNA	messenger ribonucleic acid
MT	microtubule
NaCl	sodium chloride
NAD	nicotinamide adenine dinucleotide
ng	nanogram
nm	nanometre
nmt	no message in thiamine
NO	nitric oxide
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PI4K	phosphatidylinositol 4-kinase
PI4P	phosphatidylinositol 4-phosphate
PI45BP	phosphatidylinositol 4,5-bisphosphate
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
S	Svedberg
S (-phase)	DNA synthesis phase
SDS	sodium dodecyl sulfate

sec	second(s)
Ser	serine
Sp	*S. pombe*
SSC	sodium chloride, sodium citrate
TE	Tris, ethylenediaminetetraacetate
Thr	threonine
trp	tryptophan
ura	uracil
UTR	untranslated region
UV	ultraviolet
vol	volume

1. INTRODUCTION

In order to function appropriately, a cell must be able to sense and respond to its surrounding environment. External signals or stimuli such as nutrients, growth factors, hormones, neurotransmitters, and cell contact are received and detected at the cell's surface. Through an array of signaling pathways, external signals are transduced into an intracellular signal that ultimately elicits a cellular response. This process of signal transduction requires the coordinate regulation of a series of intracellular communication networks in order to produce the required cellular responses to external stimuli. Such responses include proliferation, differentiation, secretion, altered cell motility and morphology, and changes in metabolism, and gene expression.

Receptor proteins on the cell surface are responsible for the detection of the initial signaling event. Surface receptors recognize and bind a variety of agents and substances including other proteins, ions, and other small molecules. This receptor binding results in the transfer of a signal across the plasma membrane into the cytosol. In the signal transduction process, various intracellular signaling molecules, termed second messengers, play a pivotal role. Second messengers relay signals from activated cell surface receptors, either directly or indirectly, triggering a rapid alteration in activity of one or more intracellular enzymes which ultimately affect specific cellular processes. In this way, a stimulus need not enter the cell, since its biological effects are mediated by a second messenger molecule. Among the most important and well studied second messengers are 3',5'-cyclic AMP (cAMP), 3',5'-cyclic GMP (cGMP), diacylglycerol (DAG), inositol 1,4,5-triphosphate (IP_3), and calcium ion (Ca^{2+}).

Calcium as a second messenger

Calcium (Ca^{2+}) plays a critical role in many cell functions as an intracellular second messenger (reviewed in Berridge, 1995; Niki et al., 1996). Calcium has been demonstrated to be a key second messenger in a wide variety of biological phenomena including muscle contraction, secretory events, cell cycle, differentiation and transcription. Increased cytosolic Ca^{2+} levels can occur via multiple mechanisms. Calcium can enter the cell from the external environment through voltage regulated ion channels. Alternatively, upon hormonal stimulation of cell surface receptors, and subsequent IP_3 signaling, Ca^{2+} may be released from internal stores, such as the endoplasmic or sarcoplasmic reticulum lumen, into the cytosol (Lodish et al., 1995). The mobilized Ca^{2+} binds to intracellular Ca^{2+} receptors, or Ca^{2+}-binding proteins in order to transmit the Ca^{2+} signal. These Ca^{2+}/Ca^{2+}-binding protein complexes in turn interact with other cellular targets, thereby transducing the Ca^{2+} signal into specific biological functions.

Calmodulin

The primary mediator of Ca^{2+}-dependent signaling processes in eukaryotes is the ubiquitous intracellular Ca^{2+} receptor, calmodulin (CaM). Through CaM, Ca^{2+} signals can be relayed to many target proteins. Calmodulin itself does not possess any enzymatic activity, but upon Ca^{2+} binding undergoes a conformational change which enables it to bind to and stimulate the activity of a variety of proteins.

Structure of Calmodulin

CaM is a small, acidic protein comprised of 148 amino acid residues. It is a highly conserved protein with all but one vertebrate CaM being identical to each other at the amino acid level (Munjaal et al., 1981). In addition, throughout the evolution of eukaryotes, CaM has retained a high degree of structural conservation such that from yeast to human there is greater than 60% identity (Davis et al., 1986). Crystal and solution structures demonstrate that CaM is a dumb-bell shaped molecule, composed of two homologous globular domains separated by a short flexible random coiled helix (reviewed in Torok and Whitaker, 1994). Within each globular domain are two high affinity Ca^{2+}-binding domains each comprised of a linear sequence of 12 amino acid residues forming a helix-turn-helix, or EF-hand motif (Kretsinger and Nockolds, 1973). Other proteins which utilize this motif to bind Ca^{2+} include troponin C and parvalbumin. CaM binds Ca^{2+} in a co-operative fashion, therefore a small change in the level of cytosolic Ca^{2+} leads to a large change in the level of active CaM (Vogel, 1994). The binding of Ca^{2+} encourages a range of conformations that facilitate interaction(s) with different targets. For example, hydrophobic pockets in each globular domain, which are thought to be involved in binding to target proteins, become exposed after Ca^{2+} binding (La Porte et al., 1980). Recently, Tan and colleagues have demonstrated that Ca^{2+} binding induces the opening of the interfaces between helical segments in both domains of CaM (Tan et al., 1996). A key feature of the versatility of CaM's molecular recognition mechanism is the flexible middle linking region. As CaM binds its target protein, the linking region changes in length and flexion, allowing the two globular domains to come together to enfold or wrap around the target peptide (reviewed in Torok and Whitaker, 1994).

Calmodulin Binding Domains

Most CaM-binding proteins generally possess a single CaM binding region, though two CaM binding domains have been suggested for phosphorylase kinase (Dasgupta et al., 1989), phosphofructokinase (Buschmeier et al., 1987), adenylate cyclase from *Bordetella pertussis* (Ladant, 1988) and a Na^+/H^+ exchanger isoform 1 (NHE 1) (Bertrand et al., 1994).

There is a common trend for a CaM binding domain to exist near the carboxyl terminus of a protein often nearby or even overlapping with other regulatory domains of proteins. These include domains responsible for interaction with other proteins (e.g. actin binding site of caldesmon), phosphorylation sites, and in the case of several protein kinases, pseudosubstrate domains (e.g. protein kinases and phosphatases), (reviewed in Rhoads and Friedberg, 1997).

The CaM binding regions of target proteins do not exhibit a strong sequence homology. A common binding site at the level of primary amino acid sequence is not observed. Using deletion mutagenesis or chemical methods CaM binding domains of many proteins have been narrowed down to a minimal fragment of approximately 20 amino acid residues. Studies utilizing native and synthetic peptides have provided for the prediction of common structural features essential for CaM binding. Most Ca^{2+}/CaM binding domains possess the following features.

1. *A large number of positively charged residues (arginine, lysine, histidine) that are interspersed with hydrophobic residues.*

2. *An almost complete lack of negatively charged residues (aspartate or glutamate).*

3. *The ability to fold into a basic and amphipathic helix when bound to CaM.*

Two related motifs for Ca^{2+} dependent CaM binding have been characterized. These are known as the 1-8-14 and 1-5-10 motifs and are numbered as such based on the position of conserved hydrophobic residues within these motifs (Rhoads and Friedberg, 1997). The motifs and examples for the Ca^{2+}-dependent CaM binding proteins are shown in Table 1. Although considerable sequence diversity is observed among the different binding regions, it is generally a protein's ability to form a "surface-seeking" basic, amphipathic α-helix which makes it a good target for CaM binding.

Table 1. Ca^{2+}-dependent CaM binding motifs[a]

Type	Motif[b]	Proteins
1-8-14 Motif		
Type A	(FILVW)xxx(FAILVW)xx(FAILVW)xxxxx(FILVW) Charge: 3+ -> 6+	Calcineurin, NO synthase, skeletal muscle MLCK, and most other Ca^{2+}-dependent binding proteins
Type B[c]	(FILVW)xxxxxx(FAILVW)xxxxx(FILVW) Charge: 2+ -> 4+	α-Fodrin, Caldesmon, smooth muscle MLCK, and Titin
1-5-10 Motif	xxx(FILVW)xxx(FILV)xxxx(FILVW) Charge: 3+ -> 6+	CaM kinases I and II, MARCKS, HSP90 heat shock protein, and synapsins

[a] This table is adapted from Rhoads and Friedberg (1997)

[b] The standard amino acid letter code is used. The letter x stands for any amino acid. Net electrostatic charge for the 14-residue or 13- residue (1-5-10 motif) motif was based on assigning -1 for residues E and D and +1 for K and R.

[c] The type B motif possesses a lower net electrostatic charge or lacks a hydrophobic residue in position 5.

Calmodulin Binding Proteins

From a functional perspective Ca^{2+}/CaM acts both directly, by interacting with target enzymes (e.g. phosphodiesterase and adenylate cyclase), and structural proteins (e.g. tubulin and microtubule binding proteins, MAPs), and indirectly, through specific protein kinases (CaM activates at least 5 protein kinases and a single protein phosphatase, calcineurin), that in turn regulate the activities of their substrates.

To date CaM has been reported to interact with over 30 proteins including kinases, at least one phosphatase, kinesins, myosins, ATPases, metabolic enzymes, and cytoskeletal enzymes. Table 2 contains a summary of CaM-binding proteins. Although based on a review by Hans Vogel (1994), it has been supplemented by other sources cited in the Bibliography.

Table 2. CaM-regulated enzymes and proteins grouped by their functions

Function	Enzyme or protein
Cell messengers	Plasma membrane Ca^{2+}-ATPase Phospholamban (regulates sarcoplasmic reticulum-Ca^{2+}-ATPase) Cyclic nucleotide phosphodiesterase Adenylate cyclase Nitric oxide synthases Calcium and other ion channels Inositol 1,4,5-triphosphate kinase
Protein phosphorylation and dephosphorylation	CaM kinases Phosphorylase kinase Calcineurin (protein phosphatase IIb) Growth factor receptor kinases
Gene expression and cell proliferation (nuclear CaM)	CaM kinases and calcineurin Basic helix-loop-helix transcription factors CaM-binding structural proteins in the nucleus CaM-dependent endonuclease (DNA repair) RNA helicase
Muscle contraction	MLCK (smooth and skeletal muscle) Caldesmon Calponin
Others	NAD kinase Phosphofructokinase Heat shock proteins Synapsin B50 (neuromodulin) MAP-2; tau Fodrin Translation elongation factor 1α Type I inositol 1,4,5-triphosphate receptor HIV transmembrane glycoprotein

Functions of Calmodulin

The vast array of its targets attests to the importance and complexity of CaM's role in the cell. CaM is involved both directly and indirectly through CaM-activated enzymes in the regulation of a wide variety of cellular processes such as: glycogen and cyclic nucleotide metabolism, smooth muscle contraction, neurotransmitter release, T-cell activation, cytoskeletal organization, and the cell cycle (reviewed in Lu and Means, 1993; Rasmussen and Means, 1989a). Hence it is not surprising that CaM is an essential protein, influencing cell growth, proliferation, and differentiation. Gene disruption experiments performed in the ascomycete yeasts *Saccharomyces cerevisiae* (Davis et al., 1986) and *Schizosaccharomyces pombe* (Takeda and Yamamoto, 1987), and the filamentous fungus *Aspergillus nidulans* (Rasmussen et al., 1990) demonstrate that loss of CaM function is lethal. Evidence has also been provided demonstrating the essential function of CaM in mammalian cells (Rasmussen and Means, 1987, Rasmussen and Means, 1989b). Thus the indispensable role of CaM for cellular growth is conserved in a variety of eukaryotic cells.

The role of Ca^{2+}/CaM in cell cycle events is well documented (reviewed in Lu and Means, 1993; Means, 1994; Rasmussen et al., 1992; Rasmussen and Means, 1989a). In mammalian cells CaM is essential for cell-cycle progression at multiple points including the G_1/S, G_2/M and metaphase/anaphase transitions (Rasmussen and Means, 1987, Rasmussen and Means, 1989b). Decreasing CaM levels by expression of antisense mRNA in proliferating mouse C127 cells caused a cell cycle arrest in G_1, G_2 and metaphase of mitosis (Rasmussen and Means, 1989b). When CaM was overexpressed, proliferating cells showed an accelerated progression through G_1/S and G_2/M transitions and a shortened duration of G_1 phase (Rasmussen and Means, 1987). In *Aspergillus nidulans*, cell cycle studies using a strain conditional for CaM

expression indicate that CaM is required at the G_1/S and G_2/M transitions (Rasmussen et al., 1990), and more recent studies have specifically shown that a depletion of CaM in *A. nidulans* results in a failure to progress from a *nim*T23 cell cycle block at G_2/M (Lu et al., 1993). In *S. cerevisiae*, a temperature-sensitive CaM mutant strain lost viability during mitosis and arrested as large budded cells in which the spindle failed to separate the DNA (Davis, 1992). Using a genetic approach in order to generate temperature-sensitive mutations in *S. cerevisiae* that abolish single functions of CaM while leaving others intact, CaM has been implicated in a number of critical cellular processes including spindle-pole-body duplication and spindle formation, nuclear division, cell surface growth, actin organization and cytokinesis (Ohya and Botstein, 1994). Of these functions, two essential interactions have been identified. Calmodulin must interact with a 110 kDa protein component of the spindle pole body, the yeast equivalent of the microtubule organizing center, for an essential mitotic function (Geiser et al., 1993, Stirling et al., 1994) and must interact with Myo2p, an unconventional type V myosin, for polarized yeast growth (Brockerhoff et al., 1994).

Calmodulin has been shown to interact with many cytoskeletal components including MAP-2, tau, tubulin and intermediate filament proteins (reviewed in Chant, 1994; Davis, 1992; Ohya and Anraku, 1992; Rasmussen and Means, 1989a; Vogel, 1994). Some of these proteins, such as MAP-2 and tau have been shown to be substrates for phosphorylation by CaM kinase II (Yamamoto et al., 1983). Thus CaM may function directly or indirectly in the regulation of various cytoskeletal changes involved in controlling functions such as cell growth, shape and movement.

Consistent with its role in mitosis, CaM is found highly concentrated on the mitotic spindle apparatus, specifically the kinetochore microtubules, in dividing cells (Welsh et al., 1978; Welsh et al., 1979; Sweet et al., 1988; Vantgard et al., 1995).

Microinjection of CaM into PtK_1 cells transiently protects kinetochore microtubules from the destabilizing effects of nocodazole (Sweet et al., 1988). Spindle function and microtubule dynamics during mitosis are regulated at least in part by CaM kinase II. During mitosis, CaM kinase II was found to be a dynamic component of the mitotic apparatus, present particularly at the microtubule-organizing centers (Ohta et al., 1990). Also demonstrated has been the Ca^{2+}/CaM dependent phosphorylation of a 62 kDa protein which induces microtubule depolymerization of the sea urchin mitotic apparatus (Wordeman and Cande, 1987). Studies observing the functional consequences of expressing specific mutant variations of a consensus CaM in *S. cerevisiae* demonstrated accumulated prominent arrays and large bundles of polymerized microtubules (Harris et al., 1994). Since the lack of functional CaM resulted in a marked lengthening and bundling of microtubules emanating from spindle pole bodies, it was suggested that CaM is necessary for the depolymerization of microtubules and/or their detachment from the spindle body at the completion of mitosis (Harris et al., 1994). As defects in CaM function lead to aberrations in microtubule and spindle pole body architecture, CaM may also play an important role in the nucleation of microtubule assembly by the spindle pole body. Since the poleward movement of chromosomes at anaphase requires depolymerization of spindle microtubules, the role of CaM in mitosis may be the regulation of microtubule stability. To date however, the exact molecular mechanism of CaM in regulating the function of the mitotic spindle is still unclear. In a study looking at the consequences of constitutive elevation of CaM in a mouse cell line it was observed that the cells demonstrated a flattened cell morphology and differences in appearance of microfilament, intermediate filament and microtubule networks were noted (Rasmussen and Means, 1992). An increase in CaM levels was also associated with a

decrease of β-tubulin mRNA stability consistent with an increase in unpolymerized tubulin.

Calmodulin is also implicated in the regulation of microfilaments. During interphase CaM is found associated with actin based stress filaments in 3T3 cells (Welsh et al., 1978). CaM has been found to bind several actin binding proteins including myosin I (Howe and Mooseker, 1983), caldesmon (Sobue et al., 1981), and spectrin (Tanaka et al., 1991). Investigations of the Ca^{2+} dependent regulation of the erythroid membrane cytoskeleton suggested that, in red blood cells, the major target for CaM is the cytoskeleton-associated protein 4.1. Thus, CaM is thought to regulate cell shape through an interaction with complexes containing spectrin, protein 4.1, and actin (Tanaka et al., 1991). In proliferating liver cells, CaM may regulate a nuclear contractile system that includes actin (Bachs et al., 1990). In *S. cerevisiae* the relationship between CaM and actin in polarized cell growth has been examined (Brockerhoff and Davis, 1992; Brockerhoff et al., 1994). Results demonstrated that polarized distributions of actin and CaM are interdependent (Brockerhoff and Davis, 1992). CaM depends on actin for proper localization and disruption of CaM function disorganizes the actin network in most cells. Also required for polarized growth is Myo2p (Brockerhoff et al., 1994), an unconventional myosin and CaM binding protein that is suggested to function as a molecular motor protein in the transportation of secretory vesicles along actin cables to the site of bud development (Brockerhoff et al., 1994; Johnston et al., 1991).

Identification of Calmodulin Binding Proteins

Much of our current understanding of calmodulin-mediated control of cellular functions has arisen from the identification of CaM-binding proteins. For the purpose of analysis a CaM-binding protein must associate with CaM in a high affinity Ca^{2+}-dependent manner (Billingsley et al., 1985). In the past, several techniques have been useful in the identification and purification of CaM-binding proteins. Some of these have included CaM affinity columns, photoaffinity X-linking protocols, and ^{125}I - labeled and biotin - labeled calmodulin gel overlay techniques. These techniques are all well-accepted, but have their limitations. Generally they are labour-intensive, involving several steps to obtain purified protein. When performing gel overlays, proteins are resolved under denaturing conditions and subsequently immobilized onto nitrocellulose membranes. The formation of a basic amphipathic helix may be compromised, reducing the ability of CaM to recognize and interact with any potential targets, with the ultimate result being the inability to detect some legitimate CaM binding proteins.

While many enzymes activated by CaM have been identified by these biochemical methods, little is known regarding which CaM-dependent interactions are physiologically relevant *in vivo*. Our laboratory decided to utilize the yeast two-hybrid screening strategy to isolate CaM-interacting proteins. The two-hybrid system developed by Stanley Fields and coworkers (Chien et al., 1991) is a powerful technique for detecting protein-protein interactions *in vivo* utilizing the well developed molecular genetics of the yeast *Saccharomyces cerevisiae*. There are several advantages to this screening method. Interaction occurs *in vivo* under conditions that may be similar to those that occur naturally. In addition it may be possible to detect

weak or transient interactions. Finally, this method allows for the immediate availability of the cloned cDNA encoding the interacting protein.

Schizosaccharomyces pombe as a Model Organism

The fission yeast *S. pombe* is used as a model system in our laboratory. It proves an attractive model organism for examining eukaryotic cellular function due to the availability of genetic and molecular analyses. *S. pombe* is a simple unicellular organism with a genome size of 14 megabases (Mb), contained on 3 chromosomes.

With respect to CaM, *S.pombe* CaM is more related to that of higher organisms, sharing 74% identity at the amino acid level to vertebrate CaM (Takeda and Yamamoto, 1987), whereas *S. cerevisiae* CaM is only 60% identical to vertebrate CaM (Davis et al., 1986). *Schizosaccharomyces pombe* is not closely related to the budding yeast, *S. cerevisiae*, and in fact comparison of gene sequences suggests *S. pombe* to be more similar to mammalian cells than is *S. cerevisiae*. This is especially true for such features as cell cycle regulation, chromosome structure, and RNA splicing (Moreno et al., 1991). Interestingly, *S. cerevisiae* CaM possesses only 3 functional Ca^{2+} binding sites and cannot substitute for the loss of CaM function in higher organsims including *S. pombe* (Moser et al., 1995) further underscoring this point. However a *S. pombe* CaM cDNA can functionally compliment *S. cerevisiae* lacking a functional CaM gene. Despite this, the essential functions of CaM in *S. pombe* have yet to be identified.

Using the yeast two-hybrid strategy to select for CaM-binding proteins, a screen of a *Schizosaccharomyces pombe* cDNA expression library yielded several clones, one containing a partial cDNA with homology (78% similarity over 100 amino acids) to a portion of human protein translation elongation factor 1α (EF-1α).

Recently a trypanosome homolog of EF-1α was shown to bind CaM *in vitro* (Kaur and Ruben, 1994) supporting the utility of the two-hybrid system to select CaM-binding proteins.

After obtaining the initial result from the two-hybrid screen, the goals of this project were to carry out the following specific aims.

1. Screen a *S. pombe* cDNA library, utilizing the partial cDNA isolated from the two-hybrid screen as a hybridization probe, to isolate and subsequently sequence a full-length cDNA encoding EF-1α.

2. Demonstrate an interaction between the CaM and EF-1α proteins both *in vitro* and *in vivo*.

3. Characterize the expression levels of EF-1α mRNA in both proliferating and non-proliferating cells, and during various stages of the cell cycle.

4. Overexpress EF-1α in *S. pombe* and determine if overexpression results in a measurable phenotypic change.

This thesis describes the results of experiments undertaken to address the above-mentioned aims.

2. MATERIALS AND METHODS

Strains

The following *S. cerevisiae* strains were employed in the yeast two hybrid system: HF7c (*MATa, ura3-52, his3-200, lys2-801, ade2-101, trp1-901, leu2-3, 112, gal4-542, gal80-538, LYS2::GAL1-HIS3, URA3::(GAL4 17mers)$_3$-CYC1-lacZ*), and SFY526 (*MATa, ura3-52, his3-200, lys2-801, ade2-101, trp1-901, leu2-3, 112, can^r, gal4-542, gal80-538, URA3::GAL1-lacZ*) (Clontech Laboratories Inc). *S. cerevisiae* strains were cultured as describd in the MatchMaker Two-Hybrid System Manual (Clontech Laboratories Inc.). The *S. pombe* strains used in this study were Q358 (*h-, ade6-210, ura4D18, leu1-32*) and *cdc25* (*h-, cdc25-22, leu1-32*) and were cultured as described (Moreno et al., 1991). *Escherichia coli* strains XL1 Blue and DH5α were cultured as described (Sambrook et al., 1989).

Plasmid Isolation and Cloning Conditions

Small scale preparation of plasmid DNA was performed by the alkaline lysis method (Sambrook et al., 1989) utilizing the Wizard miniprep purification kit (Promega). For large scale preparations, alkaline lysis followed by polyethylene glycol / NaCl precipitation was used (Sambrook et al., 1989). Following isolation, DNA was resuspended in TE buffer and quantified by spectrophotometric analysis. Molecular cloning methods were performed as outlined (Sambrook et al., 1989). Restriction enzymes and DNA modifying enzymes were purchased from Promega, and used according to manufacturer's specifications.

Synthesis of Radiolabeled Probes for Hybridization

DNA probes utilized for library screening and Northern blotting procedures were prepared as follows. Plasmid DNA was digested with the appropriate restriction enzyme and fragments separated by electrophoresis using low melting point agarose. Each specific fragment of interest was excised from the gel, incubated at 70°C for 10 min and purified using a Wizard DNA Clean Up kit (Promega) according to manufacturer's instructions. For each labeling reaction approximately 200 ng of DNA was labeled with [^{32}P]-dCTP (3000 Ci/mM, Amersham) by the random primer method (Sambrook et al., 1989) using the Prime-A-Gene labeling kit (Promega). G-50 sephadex chromatography was used to separate labeled DNA from unicorporated nucleotides. Incorporation of ^{32}P was quantified by liquid scintillation counting.

Oligonucleotide Synthesis

Deoxyoligonucleotides were synthesized on an Applied Biosystems 392 DNA Synthesizer in the DNA Core Facility in the Dept. of Biochemistry, University of Alberta.

DNA Sequencing

Manual sequencing of DNA was carried out by the chain termination method (Sanger et al., 1977) using a Sequenase version 2.0 kit (U.S. Biochem Corp.) and ^{35}S-dATP (3000Ci/mM, Amersham). Following electrophoresis the gel was dried under vacuum at 80°C for 2 h and then exposed to Kodak X-OMAR AR film at room temperature overnight. Computer analysis of DNA sequences was performed using

MacDNASIS software (Hitachi Software Engineering America, Ltd., Brisbane, CA), and searches of the GenBank database were performed using Blast Client software.

Yeast Two Hybrid Screen

Lithium acetate transformation of *S. cerevisiae* strain HF7c was performed as described in the Matchmaker Two-Hybrid System Manual (Clontech Laboratories Inc.). The HF7c cells were first transformed with the bait vector pGBT9-SpCaM, which contains the *S. pombe* calmodulin cDNA cloned into pGBT9. Cells were then plated on synthetic complete media lacking tryptophan and incubated at 30°C for 5-7 days. A trp^+ colony was selected (which has been named HF7c[pGBT9-SpCaM]) and used for further manipulation. An *S. pombe* MatchMaker plasmid-based cDNA library was then transformed into HF7c [pGBT9-SpCaM] and the cells then plated onto synthetic complete media lacking leucine, tryptophan, and histidine, and incubated at 30°C for 7-10 days. His^+ colonies were analyzed for β-galactosidase activity using the filter assay as described in the Matchmaker Two-Hybrid System Manual (Clontech Laboratories Inc.). Library-derived $His^+/β\text{-}gal^+$ clones were rescued and transformed into *Escherichia coli* for plasmid preparation and sequencing. A partial cDNA clone isolated in this screen was named CMTH-A6.

To verify interactions between known proteins the yeast strain SFY526 was co-transformed with pGBT9-SpCaM or pGBT10-SpCaM and pGAD-EF1α and plated onto synthetic complete media lacking tryptophan and leucine. Protein-protein interactions were then verified using the β-galactosidase filter assay according to the manufacturer's instructions. The filters were incubated for 40 min to overnight at 30°C. Blue colouration after incubation was indicative of β-galactosidase activity.

S. pombe cDNA Library Screening

An *S. pombe* cDNA library constructed in λZAP (Stratagene) was generously supplied by Alison Pidoux and Zac Cande, University of California, Berkeley. Using CMTH-A6 as a probe, the library was screened using standard procedures (Sambrook et al., 1989). Bluescript SK phagemid (pSK) containing positive inserts were excised from the lambda phage *in vivo* by coinfection with R408 helper phage. From this screen a complete cDNA was obtained which has been used for all subsequent subcloning and expression studies.

Synchronization of S. pombe

Synchronization of *cdc25-22* cells was performed as previously described (Plochocka-Zulinska et al., 1995). Briefly, *cdc25-22* cells were grown at 25°C (permissive temperature) to a cell density of 5 x 10^6 cells/ml. To synchronize the cells, the culture was shifted to 35°C (restrictive temperature) for 4 h, which results in a G2 arrest. To release the cells, an equal volume of chilled media was added and the culture incubated at 25°C, during which time cells were harvested from the culture at 20 min intervals. For each time point 1 ml samples were fixed with 3.7% formaldehyde to examine septation index as indicators of cell cycle synchrony. For RNA isolation, 5 x10^8 cells were frozen in liquid nitrogen and stored at -70°C until needed.

RNA Isolation and Northern Blotting

Total RNA was prepared using Trizol reagent (Life Technologies) following the manufacturer's instructions. Upon isolation, total RNA was resuspended in

DEPC-treated water and quantifid by spectrophotometric analysis. Northern blot analysis was performed as previously described (Plochocka-Zulinska et al., 1995; Sambrook et al., 1989).

For dot-blot analysis, 5 µg of RNA in DEPC treated water were resuspended in 200 µl SSCF (3 vol 20 x SSC, 2 vol 37% formaldehyde) and denatured at 65°C for 15 min. The sample was chilled on ice and an equal volume of 15 x SSC was added. For Northern blot analysis, samples were prepared the same way, and then resolved in formaldehyde-containing agarose gels (Rasmussen et al., 1994). Blotting onto Magna Nylon membrane was performed using either a dot blot apparatus (Tyler Research) or in the case of Northern blots a vacuum blotter (Tyler Research). Membranes were then air-dried and the RNA crosslinked using UV light (1200 J/cm^2) in a Stratalinker 2400 (Stratagene).

Purification of EF-1α as a GST Fusion Protein

The details of the contruction of the expression plasmid pGEX-EF1α used for the production of recombinant protein in *E. coli* is described in the Results section. To produce GST-EF-1α fusion proteins, *E. coli* containing the pGEX-EF1α plasmid were initially grown at 37°C overnight, and then fresh medium inoculated with the overnight culture at a 1:100 dilution. The fresh culture was then incubated at 37°C for 2 h. IPTG was added to a final concentration of 1 mM, and the culture incubated an additional 90 min at 37°C. The GST-EF-1α was purified from *E. coli* by affinity chromatography on glutathione-Sepharose according to the GST Gene Fusion System manual (Pharmacia Biotech).

Casein Kinase II Assays

The casein kinase II assays were performed modifying a previously published protocol (Hockman and Schultz, 1996). Purified casein kinase II was kindly provided by Ataollah Ghavidel, Dept. of Biochemistry, University of Alberta. Briefly, assay buffer conditions were as follows: 20 mM Hepes pH 7.8, 10 mM $MgCl_2$, 1 mM DTT. For each assay approximately 80 ng of casein kinase II holoenzyme and 1 µl [γ-^{32}P] ATP (3000Ci/mM, Amersham) was used. Reactions were carried out at 30°C for 40 min and then terminated by the addition of SDS sample buffer followed by incubation at 95°C for 5 min. Samples were resolved by SDS-PAGE, and following electrophoresis the gel was dried under vacuum at 80°C for 2 h and exposed to Kodak X-OMAR AR film at -70°C for 20 min.

Calmodulin Kinase II Assays

Calmodulin kinase II assays were performed as previously described (Rasmussen and Garen, 1993) with the inclusion of purified GST-EF-1α or GST. Briefly, assay buffer conditions were as follows: 20 mM Hepes, pH 7.5, 0.2 mM $CaCl_2$, 2mM $MgCl_2$, 1 mM DTT, 50 µM ATP. For each kinase reaction 2 µL (approximately 1µg) of affinity purified GST-CaMKII and 1 µl [γ ^{32}P]ATP was used. The amounts of CaM, GST-EF-1α, and GST used in each reaction are described in the figure legends. Reactions were carried out at 30°C for 15 min. Reactions were terminated by the addition of SDS-PAGE sample buffer, the sample incubated at 95°C for 5 min and then resolved by SDS-PAGE. Following electrophoresis the gel was dried under vacuum at 80°C for 2 h and exposed to Kodak X-OMAR AR film at -70°C for 30 min. For densitometric analysis, the dried gel was exposed to a FUJI Imaging

Type BAS-IIIS phosphoimager plate for 10 min, and the data analyzed using the FUJIX BAS1000 system and MacBAS software.

Overexpression of EF-1α in S. pombe

The expression vector pREP1, in which gene expression is under control of the *nmt* promotor (Maundrell, 1990; Maundrell, 1993) was used for overexpression of EF-1α in *S. pombe*. The details of the construction of the pREP1-EF1α expression plasmid are described in the Results section. *S. pombe* strain Q358 was transformed with pREP1-EF1α using the lithium acetate procedure as described (Alfa et al., 1992) and transformants plated onto the appropriate medium containing thiamine (100 µl of 100mM stock solution applied topically) in order to repress expression of EF-1α. A leu^+ colony was selected from the transformation and used in subsequent manipulations. This strain was named YEG43. For studies on the effect of EF1α overexpression on cell growth in *S. pombe*, the strain YEG43 was grown in liquid culture either in the presence or absence of 2µM thiamine. Overexpression of EF-1α mRNA was verified by Northern blotting as described above.

YEG43 cells were incubated in the presence of 2 µM thiamine overnight at 30°C. Cells were then diluted to a density of 10^5 cells/ml and incubated +/- 2 µM thiamine at 30°C for 4 - 5 days. At various time intervals, aliquots were removed for microscopic analysis and for determination of culture density.

3. RESULTS

Two Hybrid Selection of Calmodulin Binding Proteins

The two-hybrid system developed by Stanley Field and coworkers (Chien et al., 1991) is a powerful technique for detecting protein-protein interactions utilizing the well-developed molecular genetics of the yeast *Saccharomyces cerevisiae.* The method is based on properties of the yeast GAL4 protein which is comprised of separable domains responsible for DNA binding and transcriptional activation (Keegan et al., 1986; Ma and Ptashne, 1987). Plasmids are constructed to encode two different hybrid proteins. The first hybrid protein consists of the GAL4 DNA binding domain fused to a known protein, commonly termed the "bait". The second hybrid consists of the GAL4 activation domain fused to protein sequences encoded by a library of DNA fragments. An interaction between the known protein and a protein encoded by one of the library plasmids leads to transcriptional activation of a reporter gene containing a binding site for GAL4. In our screen both the *HIS3* and *lacZ* genes were reporter genes in the yeast strain utilized. Thus a protein-protein interaction is identified *in vivo* through reconstitution of the activity of the transcriptional activator. Advantages of this screening method are multifold. Interactions are detected *in vivo*, under conditions that may be similar to those that occur naturally. In addition, it may be possible to detect weak or transient interactions. Finally, this method allows for the immediate availability of the cloned gene encoding the interacting protein.

In this study, the yeast two-hybrid system was used in order to select for cDNA clones encoding proteins that interact with and are potentially regulated by CaM. *S. pombe* CaM, expressed as a fusion protein with the GAL4 DNA binding domain (BD) was used as the "bait" in the two-hybrid screen. An *S. pombe* cDNA

library, in which cDNAs were expressed as GAL4 activation domain fusion proteins, was screened for the expression of fusion proteins which along with the GAL4BD-SpCAM hybrid would reconstitute GAL4 activity thus resulting in the appearance of a his^+ phenotype. From this screen multiple his^+ colonies were obtained. Each colony was streaked out 3 times to obtain pure clones, and *lacZ* expression confirmed by assaying β-galactosidase activity. It was also determined whether or not *lacZ* expression was dependent on the presence of the GAL4BD-SpCaM fusion protein. Specificity of protein-protein interactions was confirmed by measuring the ability of the two-hybrid fusion proteins to support his^+ protrophy and *lacZ* expression when expressed with the "corresponding" GAL4 binding domain lacking a fusion. This tested for false positives, such as cDNAs that had transcriptional activating activity, independent of the GAL4BD-SpCaM bait protein. From this test, several positive clones were obtained and each subjected to DNA sequence analysis. One positive clone, CMTH-A6, contained an insert 458 nucleotides in length which encoded a predicted amino acid sequence possessing 78% similarity to a portion of human protein translation factor EF-1α.

Cloning and Sequencing of S. pombe EF-1α cDNA

In order to obtain a full length cDNA encoding EF-1α, the insert of CMTH-A6 was used as a hybridization probe in a screen of an *S. pombe* cDNA library. Out of 10^4 plaques screened, 12 positive clones were obtained and plaque-purified. Bluescript SK phagemids containing inserts were synthesized from the λ phage *in vivo* by co-infection with R408 helper phage. These phagemid clones were then used for DNA sequence analysis. From this screen, a full length cDNA was obtained, mapped by digestion with restriction endonucleases, and various subfragments

subcloned for sequencing. The sequence of the full length EF-1α cDNA is shown in Figure 1 and has been deposited into the Genbank Database, Accession No. U42189.

The cDNA is 1383 bp in length, encoding a predicted protein of 461 amino acids with a predicted molecular mass of 49.7 kDa and a pI of 9.2. The cDNA sequence is highly conserved from yeast to human with *S. pombe* and human EF-1α possessing greater than 80% sequence identity (Figure 2).

In vivo Protein-Protein Interactions

To verify the interaction between CaM and EF-1α *in vivo* we tested the ability of CaM to interact with the full length EF-1α isolated from the library screen using the yeast two hybrid system. The "bait" vectors used were either pGBT9-SpCaM or pGBT10-SpCaM. Each of these vectors was constructed by the in-frame ligation of a cDNA encoding *S. pombe* CaM into either pGBT9 or pGBT10 respectively. Two constructs were produced in order to change the relative orientation of the CaM portion of the hybrid protein relative to the GAL4 binding domain portion. The length of the linkers between the GAL4BD and CaM differs by 2 amino acids in the pGBT9-SpCaM vs. pGBT10-SpCaM vectors. The "target" vector pGAD-EF1α was constructed by subcloning the full length EF-1α cDNA excised from pGEM-EF1α into the vector pGAD424. To do this, EF-1α was excised as an EcoRI fragment, the overhanging ends filled in by treatment with the Klenow fragment of DNA polymerase I and dNTPs, followed by ligation into SmaI-digested pGAD424. Clones containing the EF-1α cDNA in the sense, named pGAD-EF1α(+), and antisense, named pGAD-EF1α(-), orientations were selected. The yeast reporter host strain SFY526 was used for testing for an interaction between CaM and EF-1α. SFY526 cells were co-transformed with either pGAD-EF1α(+) or pGAD-EF1α(-) and either pGBT9-SpCaM

or pGBT10-SpCaM as described in the Materials and Methods. In parallel, SFY526 cells were transformed with the plasmid pCL1, which encodes the wild type GAL4 protein, and then used as a positive control for detection of β-galactosidase activity. The results from the β-galactosidase filter assay show that CaM and EF-1α do interact *in vivo* (Figure 3). Interestingly, a significantly greater level of β-galactosidase activity was detected when CaM was expressed as a GAL4BD fusion in pGBT10 as compared to pGBT9. This suggests that the spatial orientation of the two binding partners in a two hybrid assay (in this case CaM and EF-1α) affects the ability to stably interact. The results shown here represent the first evidence of a direct interaction between CaM and EF-1α *in vivo*.

Expression of EF-1α mRNA

Cells were harvested from exponentially growing cultures (10^7 cells/ml), cells just prior to stationary phase (10^8 cells/ml), and cells which had been allowed to grow to saturation (defined as the point when no further increase in cell number could be observed). Total RNA was prepared from the three different samples and equal amounts of RNA were resolved on agarose-formaldehyde gels. Prior to transfer to Magna nylon membranes agarose-formaldehyde gels were stained with ethidium bromide and photographed in order to visualize 25S and 18S rRNA and confirm equal loading. After transfer and cross-linking, the membrane was stained with methylene blue to visualize rRNA to confirm equal transfer of samples. Hybridization to ^{32}P-labeled EF-1α cDNA was performed according to standard procedures. The results show that the EF-1α cDNA hybridizes to a single mRNA species of approximately 1.6 kb (Figure 4). In addition, the levels of EF-1α mRNA remain unchanged even after the cells have entered stationary phase.

Cell Cycle Regulation of EF-1α Expression

Because CaM dependent events have been shown to be required for cell cycle progression (Rasmussen et al., 1992), and EF-1α's microtubule severing activity has been observed in sea urchin mitotic extracts (Ohta et al., 1990), EF-1α mRNA expression was examined during the cell cycle. *S. pombe cdc25* cells were synchronized as described in Materials and Methods and measurement of septation index used as an indicator of synchrony (Figure 5a). At 20 min intervals, cells were harvested and total RNA prepared. Equal amounts of total RNA were analyzed both by Northern blot and dot-blot analysis. The results demonstrate that EF-1α mRNA expression remained relatively constant during the course of a synchronous cell cycle (Figure 5b). In contrast, when the same dot-blot was used in a hybridization experiment with a recently isolated CaM kinase I cDNA from *S. pombe* (Wiebe and Rasmussen, manuscript in preparation), a clear pattern of cell cycle dependent variation in mRNA levels was observed (Figure 5b), confirming that the cells were passing through the cell cycle synchronously, and further illustrating the observation that relatively speaking, EF-1α mRNA levels change little during the cell cycle.

Synthesis and Purification of Recombinant GST-EF-1α

In order to facilitate further *in vitro* biochemical studies, we decided to express EF-1α in *E. coli* as a GST fusion protein. The ORF of the EF-1α clone obtained from the library screen was amplified by PCR to create a 5' EcoRI restriction site that maintained reading frame with the portion of glutathione-*S*-transferase expressed from pGEX-2T. This also removed an in-frame stop codon present in the 5' UTR of the full length EF-1α cDNA. The PCR product was first ligated into pGEM-3Zf(+)

digested with SmaI, to produce the plasmid pGEM-EF1α. Subsequently the complete cDNA was excised as an EcoRI fragment and ligated into pGEX-2T that had been digested with EcoRI. Clones containing the EF-1α cDNA in the sense direction were selected. Purification of the GST-EF-1α fusion protein by glutathione-Sepharose affinity chromatography was carried out as outlined in Materials and Methods. SDS-PAGE was used to qualitatively analyze the fractions predicted to contain GST-EF-1α.

After purification, a major species migrating at 77 kDa was observed. This was the size expected for a full-length GST-EF-1α fusion protein given that the GST tag is 27 kDa and the EF-1α cDNA is predicted to encode a 50 kDa protein. The yield of this species was approximately 60 μg per 50 ml bacterial culture processed or 1.2 mg protein per litre of bacterial culture processed. The concentration of the GST-EF-1α fusion protein utilized in subsequent experiments was approximately 200 ng/μl as determined by visual comparison to a concentration series of purified BSA (Promega) analyzed by SDS-PAGE. The GST-EF-1α represented an estimated 50% of the total protein eluted. In addition, there were several other bands representing protein species ranging in size from 30 - 75 kDa. The identities of these species are not known, though they may represent degradation fragments of GST-EF-1α, or protein species which bound non-specifically to the glutathione beads or to EF-1α during purification.

Casein Kinase II Assays

The β subunit of the EF-1 holoenzyme, EF-1β, is phosphorylated and maybe regulated by casein kinase II (reviewed in Merrick, 1992). Analysis of the *S. pombe* EF-1α amino acid sequence demonstrated the presence of 5 casein kinase II phosphorylation consensus sequences, Ser/Thr-X-X-Asp/Glu. Therefore, *in vitro* assays were performed in order to determine whether EF-1α is a substrate for casein

kinase II . The results of this experiment (Figure 6) demonstrate that EF-1α is not efficiently phosphorylated by casein kinase II *in vitro*.

Calmodulin Kinase II Assays

To date the role of CaM in protein translation has not been fully elucidated. Interestingly, the translation factor EF-2, which mediates the translocation of the newly synthesized polypeptide chain from the A site to the P site of the ribosome, is the sole substrate for CaM dependent protein kinase III (Nairn and Palfrey, 1987). EF-1α has been found associated with the mitotic spindle (Ohta et al., 1990). Since CaM dependent protein kinase II (CaMKII) has been found to be a dynamic component of the mitotic apparatus (Ohta et al., 1990) and CaM has been previously shown to sensitize microtubules to the depolymerizing effects of Ca^{2+}, we considered that EF-1α might be a substrate for CaMKII. To test this hypothesis, the ability of CaMKII to phosphorylate EF-1α was determined.

Figure 7, lanes 1 and 2 show that the GST-CaMKII was rapidly autophosphorylated in a Ca^{2+}/CaM-dependent manner, indicating that the kinase was active. This autophosphorylation was used as a direct measure of CaMKII activity for all subsequent experiments. Since the GST-EF-1α and GST-CAMKII used in these assays were similar in size, we expected that phosphorylation of EF-1α by CaMKII would lead to an increase in the appearance of a radio-labeled species of 77 kDa. Not only was this not observed, but the inclusion of GST-EF-1α lead to a decrease in autophosphorylation of CaMKII (Figure 7, lane 5). Since autophosphorylation of CaMKII requires an initial activation by Ca^{2+}/CaM, these results suggested that the presence of EF-1α in the reaction was competitively inhibiting the binding of CaM to CaMKII and subsequent activation of the kinase. To further investigate this,

increasing amounts of GST-EF-1α were added to a reaction containing constant amounts of CaM and CaMKII. The results show that as the EF-1α concentration increases, CaMKII autophosphorylation progressively decreases (Figure 8). GST alone had no effect on CaMKII activity, so the effects are specific to EF-1α. In a separate experiment, increasing the concentration of CaM in the reaction, while maintaining a constant level of GST-EF-1α was able to at least partially recover CaMKII autophosphorylation activity (Figure 9).

Overexpression of EF-1α in S. pombe

In order to determine whether the overexpression of EF-1α *in vivo* might produce an interesting phenotype, the *S. pombe* expression vector, pREP1 (Maundrell, 1990), containing the full-length EF-1α cDNA was constructed. The parent vector, pREP1, contains the promoter from the *nmt* gene of *S. pombe* and allows for regulated expression (Maundrell, 1990). In the presence of thiamine, expression from the *nmt* promoter is repressed, while in the absence of thiamine, expression occurs. Briefly, the complete cDNA encoding EF-1α was excised from pGEM-EF1α as an EcoRI fragment and overhangs filled in with the Klenow fragment of DNA polymerase I and dNTPs. This was then ligated into pREP1 which had been digested with NdeI, and treated with the Klenow enzyme and dNTPs. A clone containing the EF-1α cDNA in the sense direction, pREP1-EF1α, was selected. *S. pombe* strain Q358 was transformed with the pREP1-EF1α construct, and a leu$^+$ colony selected for use in subsequent manipulations. For laboratory identification purposes this strain was named YEG43.

For studying the effect of EF-1α overexpression on cell proliferation, YEG43 cells were cultured either in the absence or presence of thiamine as described in

Materials and Methods. As shown, in the absence of thiamine, cells grew more slowly than the control cultures containing thiamine (Figure 10). The calculated generation time was 3.6 hr for cells grown in the presence of thiamine (repressing conditions), and 7.2 hr for those grown in the absence of thiamine (non-repressing conditions). This suggests that overexpression of EF-1α has a significant growth inhibitory effect.

Figure 1. Nucleotide Sequence of *S. pombe* EF-1α cDNA

A full length *S. pombe* EF-1α cDNA was isolated from a λZap cDNA library using CMTH-A6 as a hybridization probe. The cDNA was mapped by digestion with restriction endonucleases, and various subfragments subcloned for sequencing. The complete cDNA (Genbank Accession No. U42189) and deduced amino acid sequence of *S. pombe* EF-1α is shown. The initiation methionine is encoded by ATG at nucleotide 10. A stop codon upstream of the initiating methionine is underlined. The termination codon is at nucleotide 1395. The encoded protein is comprised of 461 amino acids, and has a predicted molecular mass of 49.7 kDa and a pI of 9.2.

```
   1 TTGTAAAAAAATGGGCAAGGAAAAGGGACATATTAACGTCGTTGTTATCGGTCACGTCGAT    60
   1          M  G  K  E  K  G  H  I  N  V  V  V  I  G  H  V  D     20

  61 TCTGGTAAGTCTACCACCACTGGTCACCTGATTTACAAGTGCGGTGGTATTGACAAGCGT    120
  21  S  G  K  S  T  T  T  G  H  L  I  Y  K  C  G  G  I  D  K  R     40

 121 ACCATTGAAAAATTCGAGAAGGAAGCCACCGAGTTGGGTAAGGGTTCCTTCAAGTACGCC    180
  41  T  I  E  K  F  E  K  E  A  T  E  L  G  K  G  S  F  K  Y  A     60

 181 TGGGTTTTGGACAAGCTCAAGGCCGAGCGTGAACGTGGTATCACTATCGACATTGCCCTT    240
  61  W  V  L  D  K  L  K  A  E  R  E  R  G  I  T  I  D  I  A  L     80

 241 TGGAAGTTCGAGACTCCCAAGTACAATGTTACTGTCATTGATGCCCCCGGTCACCGTGAT    300
  81  W  K  F  E  T  P  K  Y  N  V  T  V  I  D  A  P  G  H  R  D    100

 301 TTCATCAAGAACATGATTACCGGTACTTCTCAAGCTGACTGTGCTGTCCTTATTATTGGT    360
 101  F  I  K  N  M  I  T  G  T  S  Q  A  D  C  A  V  L  I  I  G    120

 361 GGTGGTACTGGTGAGTTCGAGGCTGGTATCTCCAAGGATGGTCAAACCCGTGAGCACGCT    420
 121  G  G  T  G  E  F  E  A  G  I  S  K  D  G  Q  T  R  E  H  A    140

 421 TTGCTTGGTTACACCTTGGGTGTCAAGCAACTTATTGTTGCCGTCAACAAGATGGACACC    480
 141  L  L  G  Y  T  L  G  V  K  Q  L  I  V  A  V  N  K  M  D  T    160

 481 ACTGGCTGGTCCCAAGCTCGTTTCGAGGAAATCGTTAAGGAGACCTCCAACTTCATCAAG    540
 161  T  G  W  S  Q  A  R  F  E  E  I  V  K  E  T  S  N  F  I  K    180

 541 AAGGTCGGTTTCAACCCCAAGACCGTTCCTTTCGTCCCTGTCTCCGGTTTCCAAGGTGAT    600
 181  K  V  G  F  N  P  K  T  V  P  F  V  P  V  S  G  F  Q  G  D    200

 601 AACATGATTGAGCCCACCACCAACATGCCCTGGTACCAAGGCTGGCAAAAGGAGACCAAG    660
 201  N  M  I  E  P  T  T  N  M  P  W  Y  Q  G  W  Q  K  E  T  K    220

 661 GCTGGTGTCGTCAAGGGTAAGACTCTTTTGGAGGCTATTGACTCCATTGAGCCCCCTGCA    720
 221  A  G  V  V  K  G  K  T  L  L  E  A  I  D  S  I  E  P  P  A    240

 721 CGTCCCACTGACAAGCCTCTTCGTCTCCCCCTTCAAGATGTTTACAAGATCGGTGGTATT    780
 241  R  P  T  D  K  P  L  R  L  P  L  Q  D  V  Y  K  I  G  G  I    260

 781 GGTACAGTTCCCGTCGGTCGTGTTGAGACTGGTGTTATCAAGCCCGGTATGATTGTCACC    840
 261  G  T  V  P  V  G  R  V  E  T  G  V  I  K  P  G  M  I  V  T    280

 841 TTCGCTCCCGCTGGTGTCACTACCGAAGTCAAGTCTGTTGAGATGCACCACGAGTCTCTC    900
 281  F  A  P  A  G  V  T  T  E  V  K  S  V  E  M  H  H  E  S  L    300

 901 GACGCTGGTCTTCCCGGTGACAACGTTGGTTTCAACGTTAAGAACGTTTCCGTCAAGGAT    960
 301  D  A  G  L  P  G  D  N  V  G  F  N  V  K  N  V  S  V  K  D    320

 961 ATTCGCCGTGGTAACGTCTGTGGTGACTCCAAGAACGATCCTCCCATGGGTTGTGCTAGC   1020
 321  I  R  R  G  N  V  C  G  D  S  K  N  D  P  P  M  G  C  A  S    340

1021 TTCACCGCTCAAGTCATCATTCTTAACCACCCCGGTCAAATCTCTGCTGGTTACTCTCCT   1080
 341  F  T  A  Q  V  I  I  L  N  H  P  G  Q  I  S  A  G  Y  S  P    360

1081 GTTTTGGATTGCCACACTGCTCACATTGCTTGCAAGTTCGCTGAGCTCATTGAGAAGATT   1140
 361  V  L  D  C  H  T  A  H  I  A  C  K  F  A  E  L  I  E  K  I    380

1141 GACCGTCGTTCCGGTAAGAAGATTGAGGAGTCCCCTAAGTTTGTCAAGTCTGGTGATGCT   1200
 381  D  R  R  S  G  K  K  I  E  E  S  P  K  F  V  K  S  G  D  A    400

1201 TGCATTGCTAAGATGGTTCCTTCCAAGCCCATGTGTGTTGAAGCTTTCACTGACTACGCT   1260
 401  C  I  A  K  M  V  P  S  K  P  M  C  V  E  A  F  T  D  Y  A    420

1261 CCTCTTGGTCGTTTCGGCGCTGTCCGTGACATGCGTCAAACCGTCGCTGTCGGTGTCATC   1320
 421  P  L  G  R  F  G  A  V  R  D  M  R  Q  T  V  A  V  G  V  I    440

1321 AAGGCTGTCGAGAAGGTTGCTCCTGGTGCCGCCAAGGTCACCAAGGCCGCTGTTAAGGCT   1380
 441  K  A  V  E  K  V  A  P  G  A  A  K  V  T  K  A  A  V  K  A    460

1381 GGTGCCAAGAAGTAATTTCATAGTAATTTATGAAATTTGCATTTAGAATTG             1431
 461  G  A  K  K  *                                                 477
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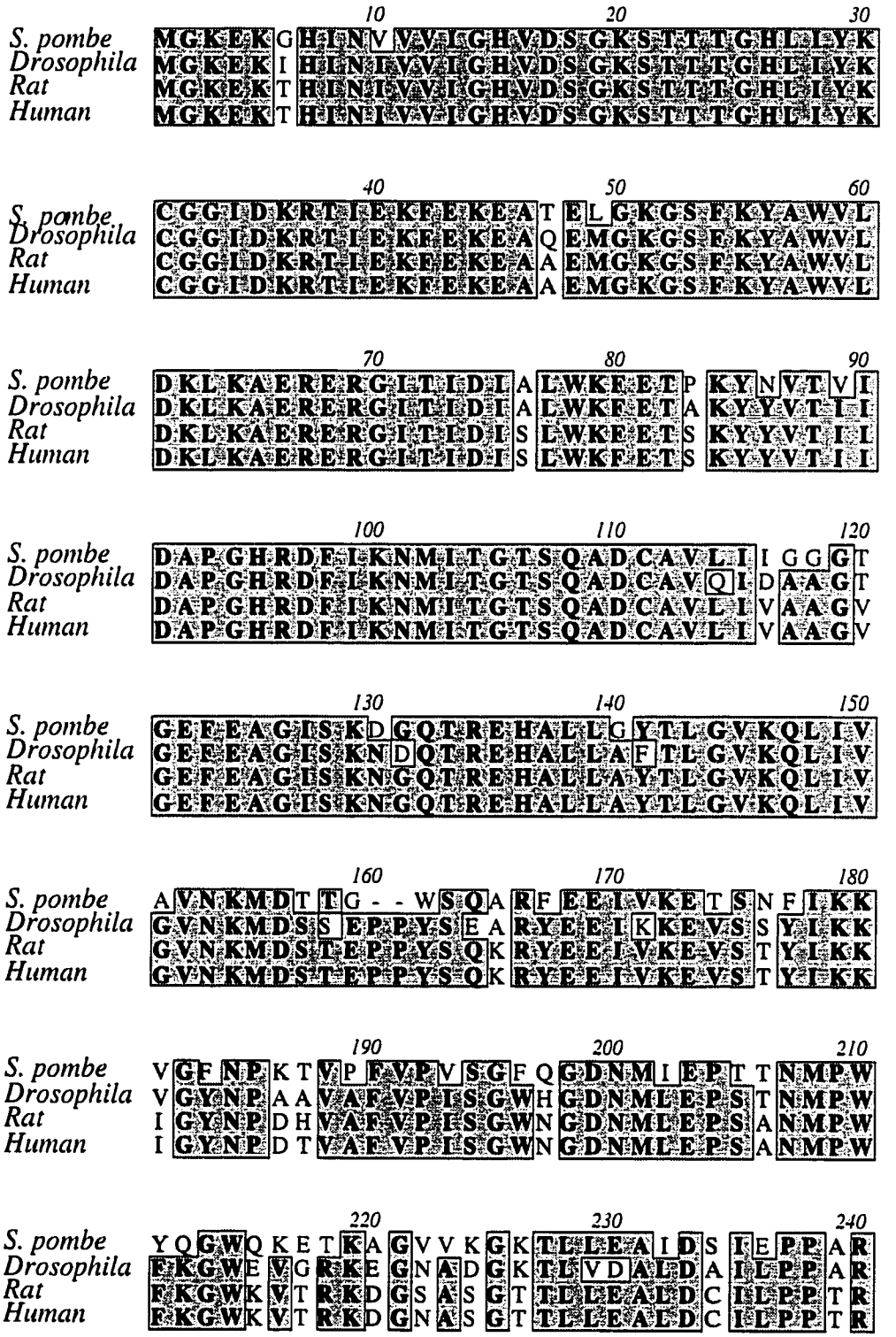
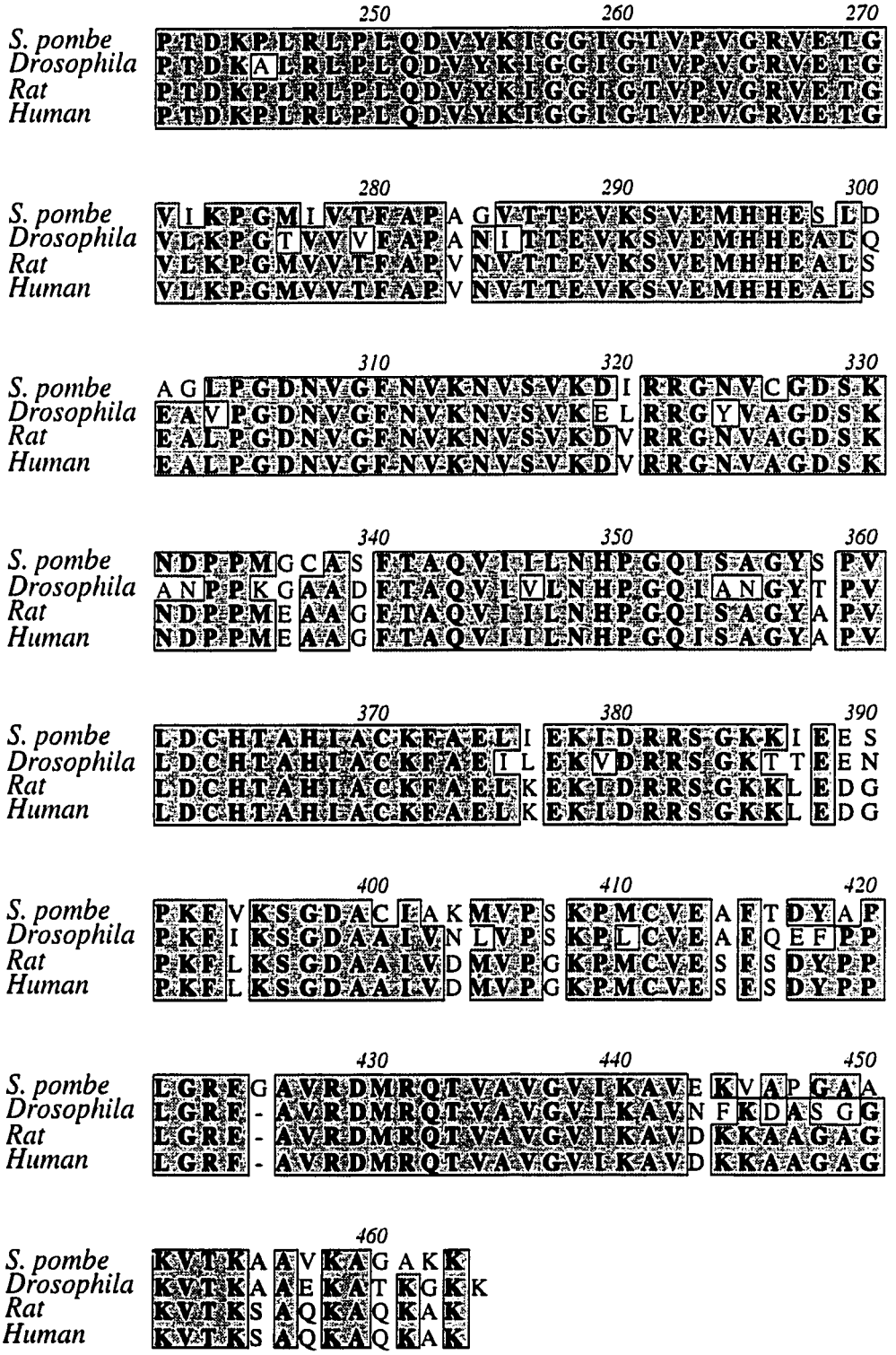
Figure 2. Comparison of *S. pombe* EF-1α with EF-1α from other species

The deduced amino acid sequence of *S. pombe* EF-1α (Genbank Accession No. U42189) is compared with EF-1α from *Drosophila melanogaster* (X06869), rat (L10339) and human (X16869). Identical amino acids are bold-faced and enclosed in a shaded box. Similar amino acids are surrounded by light shading.


10 20 30
S. pombe MGKEKGHINVVVIGHVDSGKSTTTGHLIYK
Drosophila MGKEKIHINIVVIGHVDSGKSTTTGHLIYK
Rat MGKEKTHINIVVIGHVDSGKSTTTGHLIYK
Human MGKEKTHINIVVIGHVDSGKSTTTGHLIYK
40 50 60
S. pombe CGGIDKRTIEKFEKEATELGKGSFKYAWVL
Drosophila CGGIDKRTIEKFEKEAQEMGKGSFKYAWVL
Rat CGGIDKRTIEKFEKEAAEMGKGSFKYAWVL
Human CGGIDKRTIEKFEKEAAEMGKGSFKYAWVL
70 80 90
S. pombe DKLKAERERGITIDLALWKFETPKYNVTVII
Drosophila DKLKAERERGITIDIALWKFETAKYYVTIII
Rat DKLKAERERGITIDISLWKFETSKYYVTIII
Human DKLKAERERGITIDISLWKFETSKYYVTIII
100 110 120
S. pombe DAPGHRDFIKNMITGTSQADCAVLIIGGGT
Drosophila DAPGHRDFIKNMITGTSQADCAVQIDAAGT
Rat DAPGHRDFIKNMITGTSQADCAVLIVAAGV
Human DAPGHRDFIKNMITGTSQADCAVLIVAAGV
130 140 150
S. pombe GEFEAGISKDGQTREHALLGYTLGVKQLIV
Drosophila GEFEAGISKNDQTREHALLAFTLGVKQLIV
Rat GEFEAGISKNGQTREHALLAYTLGVKQLIV
Human GEFEAGISKNGQTREHALLAYTLGVKQLIV
160 170 180
S. pombe AVNKMDTTG--WSQARFEEIVKETSNFIKK
Drosophila GVNKMDSSEPPYSEARYEEIKKEVSSYIKK
Rat GVNKMDSTEPPYSQKRYEEIVKEVSTYIKK
Human GVNKMDSTEPPYSQKRYEEIVKEVSTYIKK
190 200 210
S. pombe VGFNPKTVPFVPVSGFQGDNMIEPTTNMPW
Drosophila VGYNPAAVAFVPISGWHGDNMLEPSTNMPW
Rat IGYNPDHVAFVPISGWNGDNMLEPSANMPW
Human IGYNPDTVAFVPISGWNGDNMLEPSANMPW
220 230 240
S. pombe YQGWQKETKAGVVKGKTLLEAIDSIEPPAR
Drosophila FKGWEVGRKEGNADGKTLVDALDAILPPAR
Rat FKGWKVTRKDGSASGTTLLEALDCILPPTR
Human FKGWKVTRKDGNASGTTLLEALDCILPPTR


250
260
270
S. pombe
Drosophila
Rat
Human
280
290
300
S. pombe
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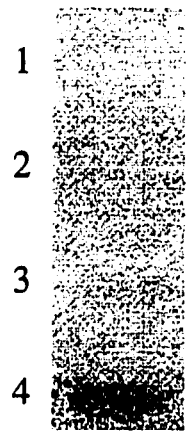


Figure 3. Yeast Two-Hybrid Interaction Between CaM and EF-1α

Constructs encoding either CaM (pGBT9-SpCaM / pGBT10-SpCaM), EF-1α in the forward (pGAD424-EF1α(+)) or reverse (pGAD424-EF1α(-)) orientation, or "bait" vector (pGBT9) alone were cotransformed into the yeast reporter host strain SFY526 and co-transformants were selected by plating on media lacking leucine and tryptophan. *In vivo* protein interactions were determined by monitoring the expression of β-galactosidase (β-gal) using a filter assay as described in the Materials and Methods. SFY526[pCL1] which expressed the wild type GAL4 protein was used as a positive control for the filter assay. Results from co-transformants containing the following combinations of plasmids: (1) pGBT9-SpCaM + pGAD424-EF1α(+); (2) pGBT9-SpCaM + pGAD424-EF1α(-); (3) pGBT9 + pGAD424-EF1α(+); (4) pGBT10-SpCaM + pGAD424-EF1α(+) are shown. Blue colouration is indicative of β-gal activity.

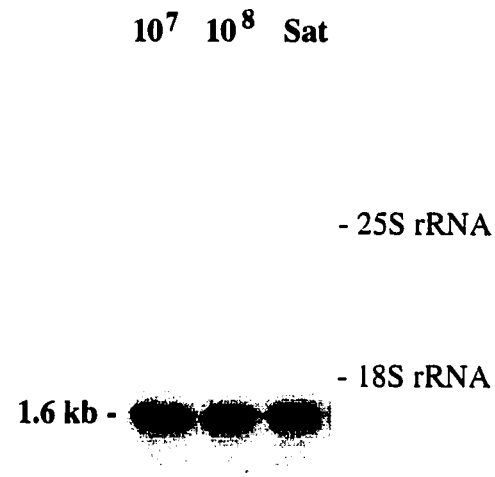
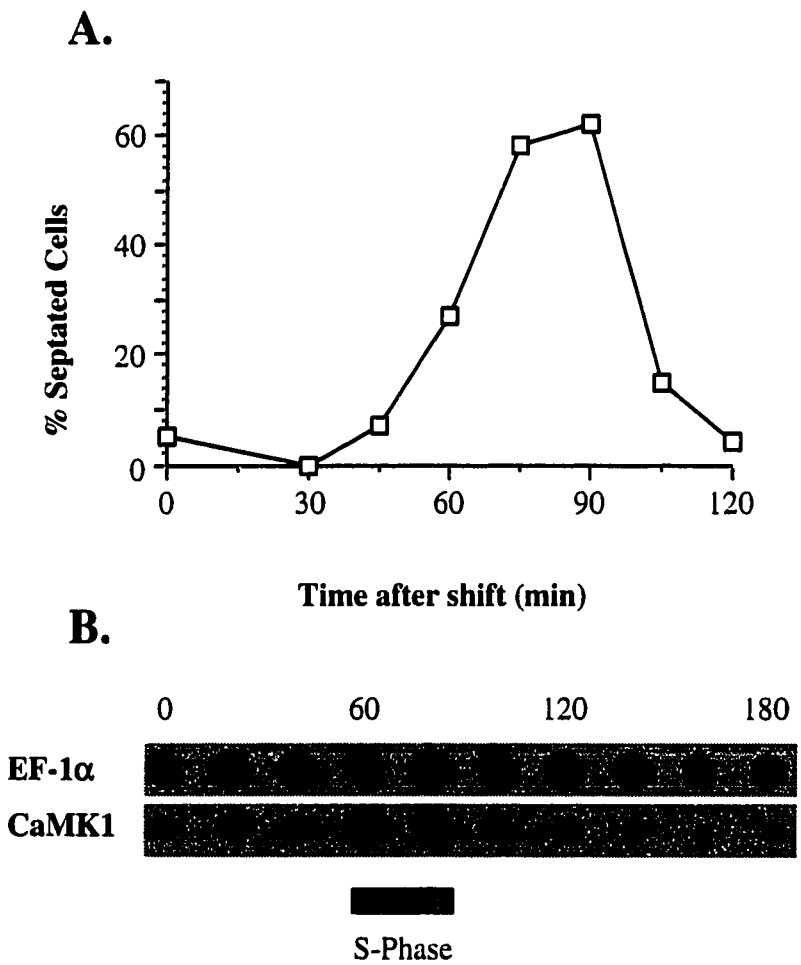


Figure 4. Expression of EF-1α mRNA

Total RNA from exponentially growing *S. pombe* Q358 cells (10^7), cells just prior to stationary phase (10^8), and cells from saturated cultures (Sat) was prepared and Northern blotted using ^{32}P-labeled EF-1α cDNA as a hybridization probe.

Figure 5. Cell Cycle Regulation of EF-1α Expression

Northern blot analysis was used to analyze EF-1α mRNA levels during the cell cycle. A *S. pombe cdc25* strain was synchronized by shifting a mid-exponential phase culture to 35°C, incubating for 4.25 h, and shifting back to 25°C. Samples were taken every 20 min and total RNA prepared for Northern blotting. (A) Percent of septated cells as determined by phase-contrast microscopy at indicated times following release from the *cdc25* block. Equal amounts of RNA (determined by A_{260}) were resolved by formaldehyde-containing agarose gel electrophoresis, transferred to Magna nylon membrane and probed first for EF-1α mRNA and then for CaM kinase I mRNA (B).


A.
% Septated Cells
0
20
40
60
0
30
60
90
120
Time after shift (min)
B.
0
60
120
180
EF-1α
CaMK1
S-Phase

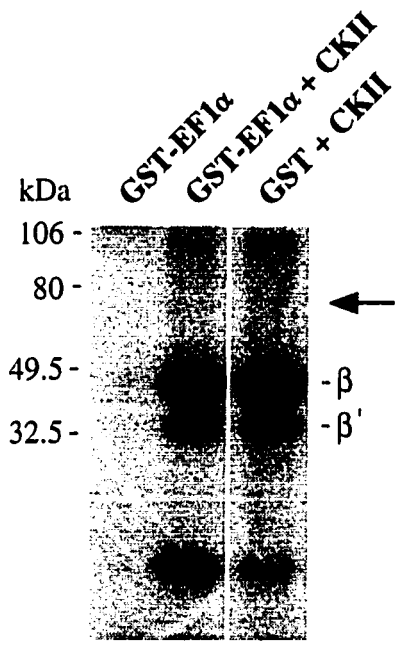


Figure 6. EF-1α as a Possible Substrate for Casein Kinase II

For each assay approximately 80 ng of casein kinase II holoenzyme and 1 μL [γ-^{32}P]ATP (3000Ci/mM) were used. Approximately 1μg of GST and GST-EF-1α were tested respectively. Reactions proceeded at 30°C for 40 min and were terminated by the addition of SDS sample buffer and incubation at 95°C for 5 min. Samples were resolved by SDS-PAGE, and following electrophoresis the gel was dried and exposed to Kodak X-OMAR AR film. The results of the reactions are as follows: Lane 1: GST-EF-1α only; Lane 2: casein kinase II + GST-EF-1α; and Lane 3: casein kinase II + GST. The β and β' subunits of the kinase are indicated.

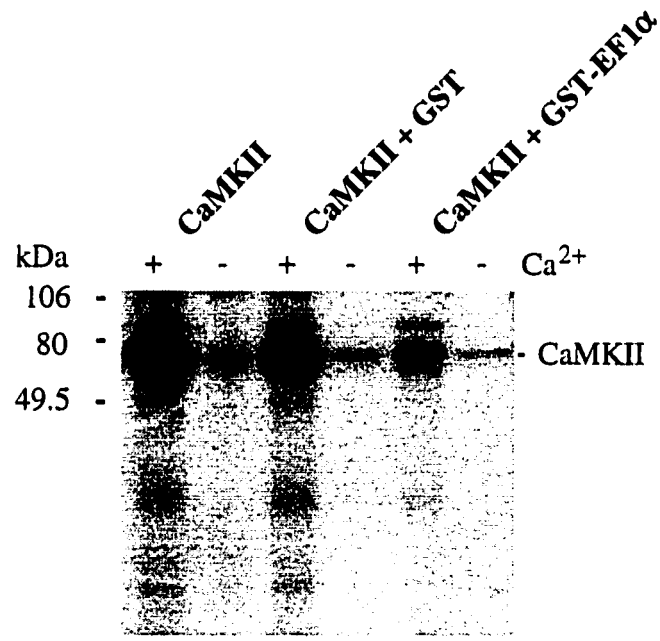


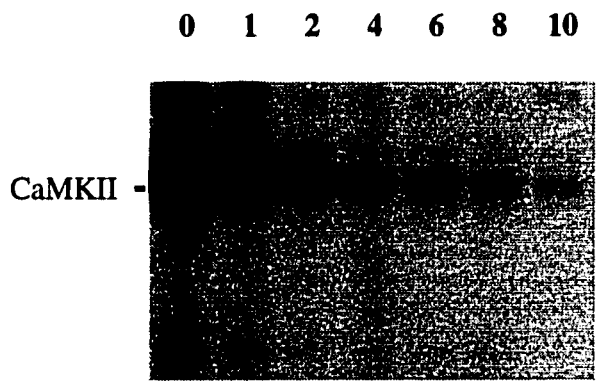
Figure 7. EF-1α as a Possible Substrate for Calmodulin Kinase II

For each assay approximately 2 μg CaM, 1 μg CaMKII and 1 μL [γ-^{32}P]ATP (3000Ci/mM) were used. Approximately 1μg of GST and GST-EF-1α were tested respectively in the presence of 10 mM $CaCl_2$ (Lanes 1, 3, 5) or 10 mM EGTA (Lanes 2, 4, 6). Reactions proceeded at 30°C for 60 min and were terminated by the addition of SDS sample buffer and incubation at 95°C for 5 min. Samples were resolved by SDS-PAGE, and following electrophoresis the gel was dried and exposed to Kodak X-OMAR AR film. The results of the reactions are as follows: Lanes 1, 2: CaMKII only; Lanes 3, 4: CaMKII + GST; and Lanes 5, 6: CaMKII + GST-EF-1α.

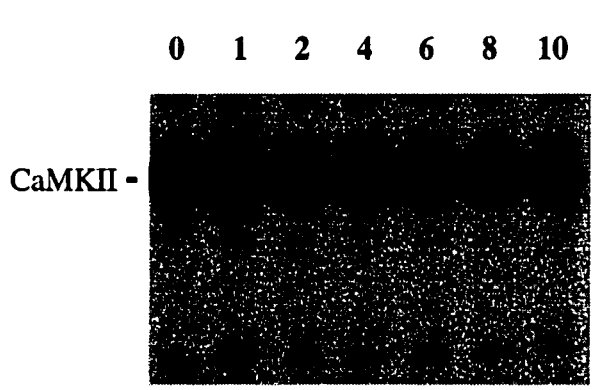
Figure 8. Competitive Inhibition of Calmodulin Kinase II Activity by EF-1α

For each assay approximately 2 μg CaM, 1 μg GST-CaMKII and 1 μL[γ-^{32}P]ATP (3000Ci/mM) were used, with increasing amounts of GST-EF-1α (Panel A) or GST (Panel B) where 5 μl is equivalent to approximately 1 μg of protein. Reactions proceeded at 30°C for 15 min and were terminated by the addition of SDS sample buffer and incubation at 95°C for 5 min. Samples were resolved by SDS-PAGE, and following electrophoresis the gel was dried and exposed to Kodak X-OMAR AR film. For densitometric analysis, the dried gel was exposed to a FUJI Imaging Type BAS-IIIS phosphoimager plate, and the data analyzed using the FUJIX BAS1000 system and MacBAS software (Panel C).

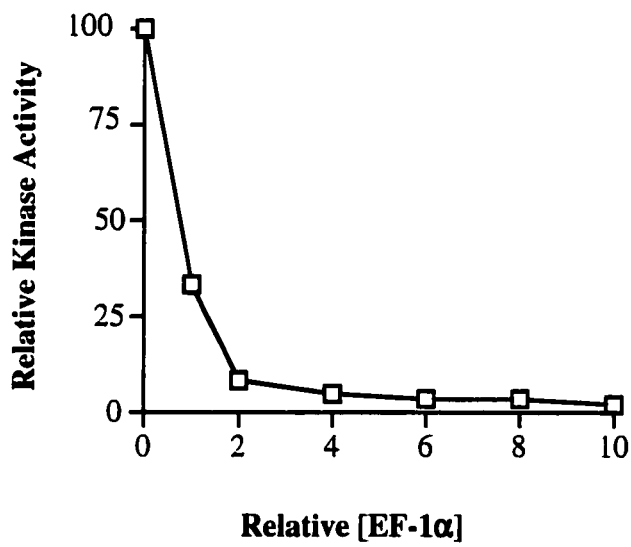
A.


0 1 2 4 6 8 10 µl GST-EF1α
CaMKII

B.


0 1 2 4 6 8 10 µl GST
CaMKII

C.


Relative Kinase Activity
100
75
50
25
0
0 2 4 6 8 10
Relative [EF-1α]

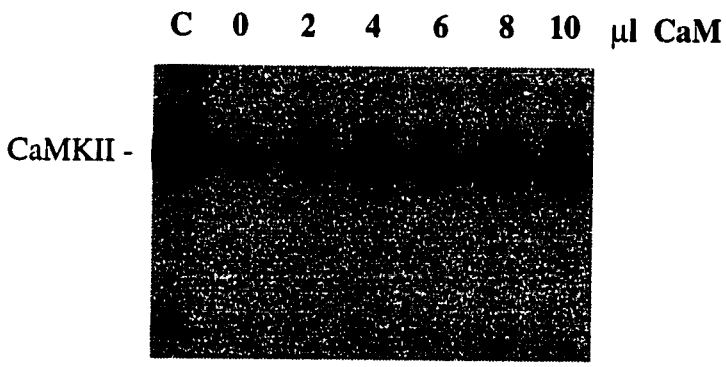


Figure 9. Rescue of Calmodulin Kinase II Activity

For each assay approximately 2 µg CaM, 1 µg GST-CaMKII, 1 µg GST-EF-1α, and 1 µL[γ-^{32}P]ATP (3000Ci/mM) were used. CaM was added in excess at increasing concentrations where 1 µl is approximately equivalent to 1 µg. Reactions proceeded at 30°C for 15 min and were terminated by the addition of SDS sample buffer and incubation at 95°C for 5 min. Samples were resolved by SDS-PAGE, and following electrophoresis the gel was dried and exposed to Kodak X-OMAR AR film. EF-1α is absent in the control lane (C).

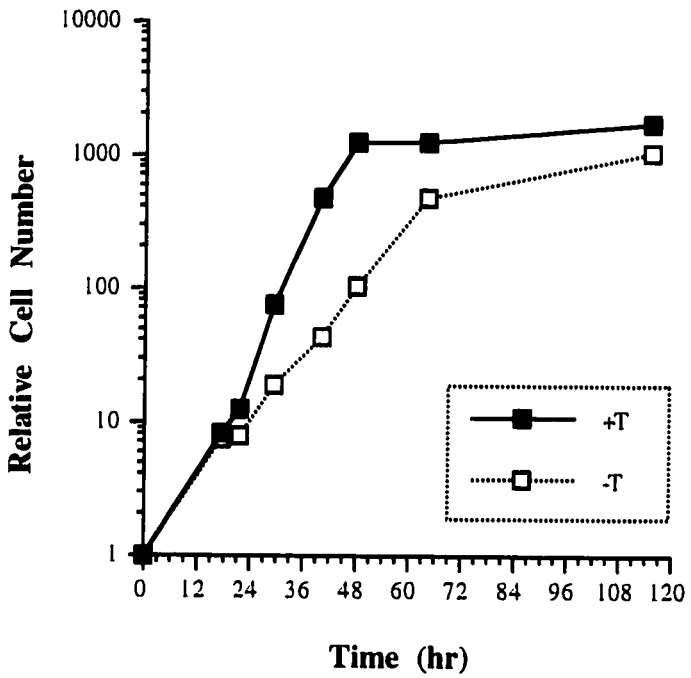


Figure 10. Growth of Cells Overexpressing EF-1α

YEG43 cells were incubated in the presence of 2 μM thiamine overnight at 30°C. Cells were then diluted to a density of 10^5 cells/ml and incubated in the presence and absence of 2 μM thiamine at 30°C for 4 - 5 days. At various time intervals indicated, aliquots were removed for microscopic analysis and for determination of culture density.

4. DISCUSSION

Cloning of an S. pombe EF-1α cDNA by Two-Hybrid Selection

Our laboratory decided to employ the yeast two-hybrid system to screen for CaM-binding proteins. To our knowledge there has been no other reports of utilizing the two-hybrid system to screen for CaM-binding proteins. From an initial screen, a partial cDNA predicted to encode a portion of an *S. pombe* EF-1α homolog was isolated. Upon investigation of the literature it was noted that another group had previously isolated an EF-1α homolog from *Trypanosoma brucei* in a biochemical screen for CaM-binding proteins, thus confirming the observation that EF-1α is a CaM-binding protein (Kaur and Ruben, 1994). The two-hybrid system was also used to confirm that full-length *S. pombe* EF-1α interacts with CaM. Significantly, this work demonstrates that CaM and EF-1α are able to interact *in vivo*.

EF-1α is one of the most widely studied protein translation factors and has been cloned and sequenced from a multitude of organisms including human, rabbit, mouse, *Xenopus*, *Drosophila*, tomato, carrot, wheat, various filamentous fungal and yeast species (Merrick, 1992). In eukaryotic cells, EF-1α is one of the most abundant proteins, constituting between 3-10% of total soluble protein. EF-1α can be isolated from the cell both as a monomer, and also as a component of the holoenzyme, EF-1H which is composed of 3 subunits, α, β, γ. During the course of this project, an inquiry into the Genbank database led to the discovery that sequences for three genes encoding EF-1α in *S. pombe* had been deposited. In *S. cerevisiae* two genes encoding EF-1α have been identified (Nagata et al., 1984; Schirmaier and Philippsen, 1984). Although the gene dosage may affect translation fidelity (Song et al., 1989),

budding yeast can grow normally with either of the two EF-1α genes deleted (Cottrelle et al., 1985). In human cells EF-1α and its homolog S1 are encoded by multiple genes referred to as the EF-1α-S1 gene family (Lee et al., 1996).

In protein translation EF-1α functions as a ternary complex, binding GTP and an aminoacyl tRNA (aa-tRNA), with its role being to mediate the transfer of the aa-tRNA to the activation site of the ribosome adjacent to the growing nascent polypeptide chain. Upon transfer of the aa-tRNA, GTP is hydrolyzed to GDP which remains tightly bound to EF-1α after this reaction. The β subunit (and possibly the γ subunit) of EF-1H stimulates nucleotide exchange to regenerate EF-1α-GTP for the next elongation cycle (reviewed in Merrick, 1992).

An independent group has recently cloned all three EF-1α genes, EF-1αA, EF-1αB, and EF-1αC) from *S. pombe* (Mita et al., 1997) and the nucleotide sequences deposited into the GenBank database with the accession Nos. D82571, D82572, and D82573 respectively. Sequence analysis of the cDNAs show that the predicted amino acid sequences are nearly identical, however the 3' untranslated regions (UTRs) are completely different. Examination of the 3' UTR nucleotide sequence of the cDNA that was isolated in our lab suggests that it is likely the same clone as EF-1αC.

Sequence alignment of the predicted amino acid sequence encoded by the *S. pombe* EF-1α cDNA with sequences from other organisms, namely, human, rat, and *Drosophila*, showed greater than 80% identity suggesting that the requirement for EF-1α in eukaryotic protein translation and possibly its other functions leaves little room for the evolution of divergent protein structure. The interactions of EF-1α with GTP, aa-tRNA molecules and the ribosome components around the tRNA acceptor site most likely prevent significant alterations in the primary structure of EF-1α. Thus it is not

surprising that the amino acid sequences or derived sequences of the eukaryotic EF-1αs reported to date exhibit such a high degree of homology in the primary sequence.

Additional Roles for EF-1α in vivo

Recent evidence suggests that EF-1α may have a variety of other functions in the cell which may, in part, explain the high levels of this protein as compared to the levels of other translation factors. This idea is based on the observations that EF-1α is found associated with the valyl-tRNA synthetase complex, messenger ribonucleoprotein particle (mRNP) complexes, the mitotic apparatus, actin, and that it appears to function in phosphoinositide signaling, and protein degradation (Merrick, 1992).

Numerous studies have provided evidence for interactions between the cytoskeleton and the eukaryotic protein translation machinery (Bassell et al., 1994; Cervera et al., 1981; Howe and Hershey, 1984; Lenk et al., 1977; Liu et al., 1996; and reviewed in Bassell and Singer, 1997; Hesketh and Pryme, 1991; Liu et al., 1996). The physiological relevance is unclear, though possible roles include mRNA localization, transport, anchoring, and spatial and/or temporal regulation of protein translation. The cytoskeleton may provide a mechansim to segregate specific translational components to distinct compartments in order to localize protein synthesis.

In addition, accumulating evidence suggests that EF-1α may influence cytoskeletal function. EF-1α has been identified in putative centrosome precursor particles from CHO cells (Marchesi and Ngo, 1993), and in MT-organizing centers of sea urchin mitotic spindles (Ohta et al., 1990), suggesting that EF-1α binds to the

minus ends of MTs and functions in MT nucleation. The injection of monoclonal antibodies against EF-1α into sea urchin eggs caused shortening of the mitotic spindle (Ohta et al., 1990), demonstrating the importance of EF-1α in regulating the integrity of the mitotic spindle. Conversely, in Xenopus egg extracts EF-1α was found to possess microtubule severing activity. EF-1α was reported to sever MTs devoid of MAPs *in vitro*, and in fibroblasts induced to overexpress EF-1α *in vivo*, depolymerization of MT arrays was also observed (Shiina et al., 1994). If EF-1α functions *in vivo* to sever MTs, this activity must somehow be regulated so that severing occurs only as the cell transits from interphase into mitosis. Consistent with this idea is the finding that Ca^{2+}/CaM modulates the MT-bundling activity of carrot EF-1α *in vitro* (Durso and Cyr, 1994). Thus, CaM could be involved in the signal transduction mechanism that results in EF-1α-mediated MT depolymerization. The fact that CaM has been previously shown to sensitize MTs to the depolymerizing effects of Ca^{2+} is also supportive of this hypothesis (Marcum et al., 1978).

In *Tetrahymena*, EF-1α was identified as a 14-nm filament associated protein, found to associate with the 14-nm filament protein even during the polymerization and depolymerization steps that are part of the 14-nm filament protein purification protocol (Kurasawa et al., 1992). However, electron microscopy studies have shown that purified 14-nm filament protein was capable of forming filaments without EF-1α (Takeda et al., 1995). Therefore, EF-1α appears not to regulate the polymerization state of 14-nm filament protein, at least *in vitro*.

Several groups have found EF-1α to be associated with actin. EF-1α has been identified as an actin-bundling protein in *Dictyostelium* (Yang et al., 1990) and *Tetrahymena* (Numata, 1996). Immunofluorescence studies have demonstrated that EF-1α is present in filopodia and other cortical regions that contain actin filament

bundles, and have shown that the association with actin is regulated during chemotaxis (Demma et al., 1990; Dharmawardhane et al., 1991). In addition, co-sedimentation and electron microscopy have been used to investigate the interactions between EF-1α, actin, and CaM, all separately purified from *Tetrahymena* (Kurasawa et al., 1996). Ca^{2+}/CaM was found bound to the EF-1α/F-actin complex. Co-sedimentation experiments demonstrated that Ca^{2+}/CaM directly bound to EF-1α without affecting EF-1α/F-actin complex formation. However, Ca^{2+}/CaM inhibited formation of F-actin bundles by EF-1α. These studies suggest that the influence of EF-1α on the actin cytoskeleton is mediated by a Ca^{2+}/CaM-sensitive mechanism.

In carrot cells EF-1α has been identified as an activator of phosphatidylinositol 4-kinase (PI4K) (Yang et al., 1993; Yang and Boss, 1994). PI4K regulates the levels of phosphatidylinositol 4-phosphate (PI4P) and phosphatidylinositol 4,5-bisphosphate (PI4,5BP). PI4,5BP is a source of the second messengers inositol 1,4,5-triphosphate and diacylglycerol. PI4P has been shown to regulate actin polymerization by binding actin-severing proteins (Yang et al., 1993). Thus, it is possible that EF-1α may regulate the actin cytoskeleon via effects on components of the phosphoinositide signaling pathway. Another possibility is that EF-1α facilitates the association of PI4K with the cytoskeleton, thus increasing the synthesis of PI4P in discrete regions of the membrane at the points of cytoskeletal attachment.

Interestingly, in mouse fibroblasts, constitutive overexpression of EF-1α causes increased susceptibility to transformation induced by 3-methylcholanthrene and UV light (Tatsuka et al., 1992). In this case, EF-1α may render cells competent for growth by serving as a downstream component of growth signaling pathways (Taniguchi et al., 1991; Tatsuka et al., 1992). It is also hypthesized that EF-1α may mediate an alteration in actin filament organization observed in the G_0 to G_1 transition

which may be required in order for cells to respond to a progression factor that is required to initiate DNA replication (Herman and Pledger, 1985; Tatsuka et al., 1992). Alternatively, constitutive expression may render cells competent for growth simply by increasing the translation rate of genes which are essential for growth.

Gonen *et al.* (1994) have demonstrated a role for EF-1α in ubiquitin-mediated degradation of certain *N*-acetylated proteins. Previous work had identified a specific protein, factor Hedva (FH), which acted with the 26S protease complex to degrade ubiquitin-conjugated *N*-acetylated proteins such as histone H2A, actin, and α-crystallin (Gonen et al., 1991). Recently this group has demonstrated that FH is EF-1α (Gonen et al., 1994). EF-1α does not function in the ubiquitin-conjugation pathway *per se*, but stimulates degradation of the conjugated proteins, probably interacting with them prior to degradation. The role of EF-1α in this interaction might either be enzymatic, cleaving ubiquitin moieties from certain poly-ubiquitin chains, or as a chaperone, possibly facilitating the interaction of the protein to be degraded with the 26S protease complex. Together, it seems clear that EF-1α may have multiple roles in cells, ranging from protein synthesis, protein degradation, phosphinositide signaling, to regulating cytoskeletal architecture. However, the precise nature of EF-1α's role in these processes remains to be elucidated. The characterization of its interaction with CaM may provide valuable clues as to the nature of EF-1α's multi-tasking role within the cell.

Characterization of Calmodulin Binding to EF-1α

In our lab, using the CaM-overlay technique proved inconclusive in showing a Ca^{2+}/CaM interaction with EF-1α. In one instance, using biotinylated CaM, we observed a Ca^{2+}-dependent interaction of CaM with GST-EF-1α. However, the same

interaction was observed between CaM and GST, the negative control. In other experiments using radiolabeled CaM, if binding was detected it was only a very weak or faint signal and was observed both in the presence and absence of Ca^{2+}. Under these conditions, binding of CaM to GST was not observed (unpublished observations). These findings suggest that perhaps the three dimensional structure of EF-1α is important for the interaction with CaM, and that the overlay technique, which utilizes denaturing PAGE to resolve proteins, precludes efficient detection of CaM binding to EF-1α.

Using phage display to clone CaM-binding proteins another group has also recently cloned an *S. pombe* EF-1α cDNA (Bachs, 1997). In subsequent experiments they observed that binding to CaM did not appear to be Ca^{2+}-dependent (Bachs, 1997). It should be noted though, that CaM binding to proteins in the absence of Ca^{2+} is not without precedence, and in *S. cerevisiae*, the essential cellular functions of CaM do not require Ca^{2+} binding at all (Geiser et al., 1991). Examples of proteins which have been identified to bind CaM in the absence of Ca^{2+} include neuromodulin and intestinal brush border myosin I (Rhoads and Friedberg, 1997). However, a concern regarding attempts to demonstrate an interaction between EF-1α and CaM using the overlay technique may be potential limitations imposed by the use of bacterially synthesized recombinant protein in these experiments. EF-1α synthesized and purified from bacteria may be missing post-translational modifications that could be necessary for normal functions, including the ability to bind CaM. Whether EF-1α is purified in a GTP- versus a GDP-bound form may also influence its ability to interact with CaM. One way to circumvent this problem would be to synthesize EF-1α as a GST fusion protein using an expression vector designed for *S. pombe* (Maundrell, 1993). In this way, the protein would be subject to all the normal modifications that might be important for its activity.

In experiments showing the competitive inhibition of CaMKII activation by EF-1α we were able to demonstrate an interaction between EF-1α and CaM. However, whether or not the interaction was Ca^{2+}- dependent could not be determined since Ca^{2+} is required for the initial CaM-dependent activation of the CaMKII used in the assay. Nonetheless, several groups have been able to demonstrate that CaM and native EF-1α do interact, and that this interaction is Ca^{2+}-dependent (Durso and Cyr, 1994; Kaur and Ruben, 1994; Kurasawa et al., 1996). These observations seem somewhat paradoxical considering the results of Bachs (personal communication). The observed discrepancy may be the reflection of the technical problems addressed above, or an indication that the CaM-EF-1α interaction in *S. pombe* is different than that of higher organisms.

Expression of EF-1α mRNA

Upon growth to saturation, fission yeast cells respond to nutrient starvation by ceasing growth and entering a non-proliferating state. This exit of the mitotic cycle known as stationary phase is thought to be similar to the mammalian G_0 phase or quiescence. There are many characteristics that distinguish these stationary phase cells from exponentially growing cells. Some of these include a reduction in protein synthesis, a downregulation of transcription, and a significant decrease in the abundance of many mRNAs (Zaragoza, 1997). Our results showed that unlike most mRNAs, the accumulation of EF-1α mRNA during both exponential growth and in stationary phase remained constant. This may not necessarily reflect continued transcription of EF-1α but perhaps a lack of mRNA degradation. The production of certain transcription factors (e.g. Atf1) (Takeda et al., 1995) and stress response proteins (reviewed in Zaragoza, 1997) are important for the entry and survival of cells in stationary phase. Since EF-1α is required for the efficient translation of these

products, the continued presence of EF-1α mRNA during stationary phase is not surprising, and may suggest that EF-1α protein levels may also be stable. Determining if this is the case would require Western blot analysis of EF-1α protein levels using an antibody that is monospecific for *S. pombe* EF-1α. Regardless, even if EF-1α protein levels or activity decreases during stationary phase, maintaining stable mRNA levels would be expected to ensure a more rapid response when the need for increased protein synthesis capacity occurs, as would be the case when the cell is stimulated to re-enter the cell cycle. Whether the maintenance of EF-1α mRNA levels is dependent on the transcription of a specific EF-1α gene is unknown. Based on the high sequence identity among the EF-1α cDNAs isolated from *S. pombe* thus far, the hybridization probe that was used for the Northern blot experiments is likely capable of hybridizing to transcripts from all three EF-1α genes. Therefore if there were any changes in the accumulation of one specific transcript it would have not be possible to detect it without the use of transcript specific probes which were unavailable during the course of these studies.

Northern blot analysis has demonstrated tissue- and stage-specific expression of EF-1α in mammals (Lee et al., 1996), and *Drosophila* (Hovemann et al., 1988) and fungal cells (Linz and Sypherd, 1987) respectively. In *S. pombe* Northern blot analysis, with hybridization probes specific to each 3' UTR, was used to investigate the effect of stressful conditions (UV, heat shock, starvation) on the accumulation of specific EF-1α transcripts (Mita et al., 1997). Results indicate that one of the three EF-1α genes was inducible with UV irradiation, while the level of expression for another was repressed by UV and heat shock treatments (Mita et al., 1997). Under starvation conditions the levels of all three transcripts appeared constant as compared to those under normal conditions.

For unknown reasons, the cessation of RNA synthesis and the resulting reduction in protein synthesis during mitosis that occurs in higher eukaryotes does not appear to happen in *S. pombe* (Creanor and Mitchison, 1982; Creanor and Mitchison, 1984). In studies where rate of incorporation of radiolabeled amino acids reflected the rate of protein synthesis it was found that the average single cell had an increasing rate of protein synthesis for the first 60% of the cell cycle and a constant rate for the remaining 40% (Creanor and Mitchison, 1982). Within the observed pattern for the rate of protein synthesis there was also a sharp increase in rate at an 'acceleration point' (at approximately 90% of the cell cycle) which is linked to nuclear division (Creanor and Mitchison, 1984). Thus EF-1α protein and mRNA levels would be expected to remain relatively constant throughout most of the cell cycle. This is precisely what was observed in the present study. Therefore, if future studies show variations in either EF-1α protein levels or activity, these are likely the result of either post-transriptional or post-translational mechanisms.

Overexpression of EF-1α

It is thought that increased levels of EF-1α enhances protein synthesis by increasing translational efficiency and fidelity (Song et al., 1989, Shepherd et al., 1989). This hypothesis is supported by work on *S. cerevisiae* where a correlation existed between EF-1α levels and translational fidelity (Song et al., 1989). Overexpression of EF-1α rendered mammalian cells more susceptible to oncogenic transformation (Tatsuka et al., 1992) and EF-1α levels were upregulated in cancer cells *in vivo* (Grant et al., 1992; Koch et al., 1990). In *Drosophila* overexpression of EF-1α led to an increase in lifespan (Shepherd et al., 1989). In fibroblasts induced to overexpress EF-1α the microtubule arrays were depolymerized (Shiina et al., 1994). In *S. pombe* our finding that constitutive overexpression of EF-1α led to a reduction

in the growth rate of the cells by nearly 50% was very unexpected. The slower growth rate observed was not simply due to a loss in cell viability. Microscopic analysis of the cells overexpressing EF-1α did not show evidence of cell lysis which would be expected in the event of cellular death. Furthermore, in a preliminary experiment, cells overexpressing EF-1α exhibited equivalent levels of cell viability when compared to control cells with normal EF-1α levels (unpublished results).

One hypothesis for the reduced growth rate is the possibility that an excess of EF-1α will bind and hence sequester CaM, thereby reducing the amount of CaM available for its other normal cellular functions. Conversely, perhaps an excess of EF-1α is interacting directly with components of the cytoskeleton and in turn interfering with normal cellular growth in this manner, or excess EF-1α might competitively inhibit protein synthesis by forming non-productive complexes with other components of the protein synthetic machinery. However, under normal conditions, EF-1α exists in a stoichiometric ratio of 10:1 and 25:1, when compared to other components of the EF-1H complex and ribosome respectively. Thus, it is difficult to imagine how a further increase in EF-1α could affect the protein translational machinery in this way.

In summary, further experimentation is required to determine why an excess of EF-1α is able to retard the growth rate of *S. pombe*. Immunofluorescence studies will be able to determine whether components of the cytoskeleton, namely actin and tubulin exist in normal arrangements when EF-1α is overexpressd. Flow cytometric analysis will be able to determine if the delay in cell division rate is due to an accumulation of cells within a specific cell cycle compartment. Finally, deletion of the CaM binding domain and subsequent expression of this modified EF-1α *in vivo* may eventially yield clues as to the role CaM may play in regulating EF-1α function.

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IMAGE EVALUATION
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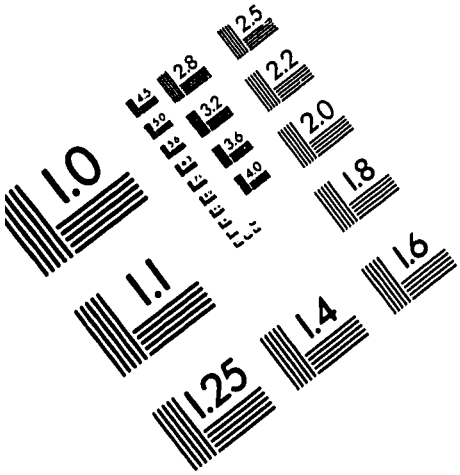

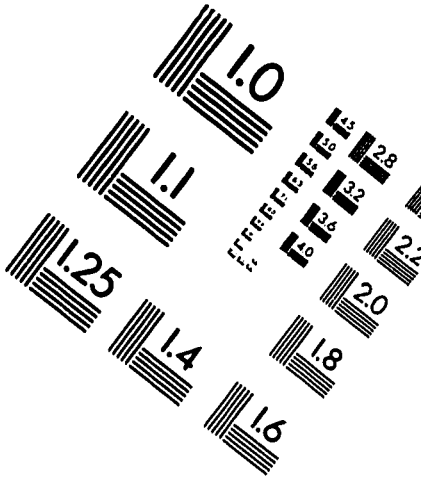

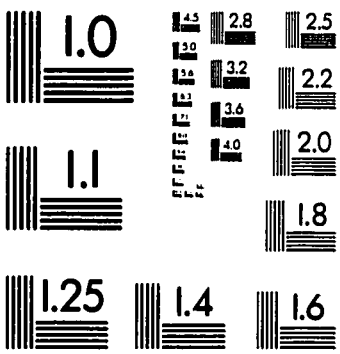

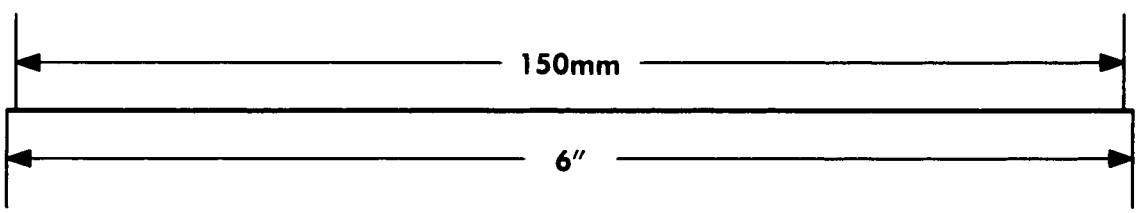

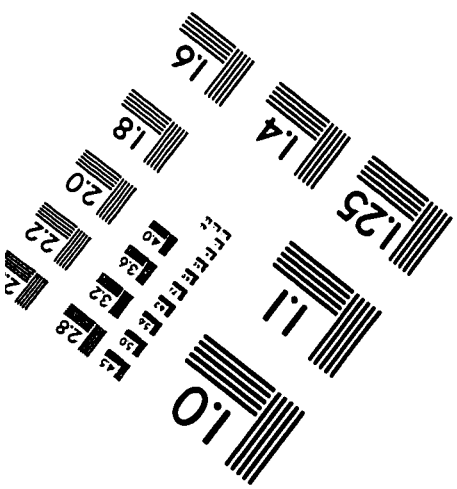

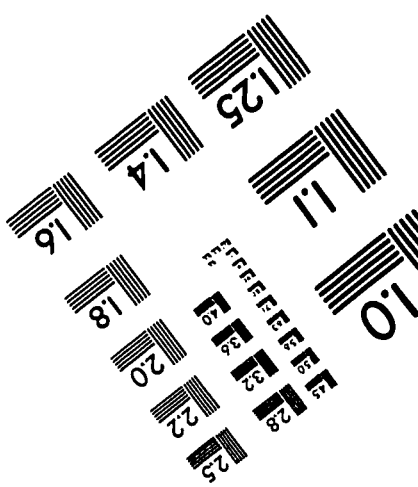

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