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Approval Sheet

Novel Systems to Investigate Interactions between Functional RNA Elements and Candidate *Trans*-factors

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B.Sc., Pennsylvania State University, 1994

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An abstract of
A dissertation submitted to the Faculty of the Graduate School of Emory University in partial fulfillment of the requirements for the degree of
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Abstract

Novel Systems to Investigate Interactions between Functional RNA Elements and Candidate *Trans*-factors

By Chad Michael Kitchen

Cells regulate immediate early gene expression levels by adjusting the rates of gene transcription and mRNA decay. Initially, significant emphasis was placed on the role of transcriptional regulation in these responses following cell signaling, but recent evidence increasingly suggests regulation also occurs at the post-transcriptional level. Determining what processes contribute to experimentally observed changes in RNA stability presents a significant challenge. The goal of this work was to provide an improved understanding of how cells couple extracellular signaling events to changes in RNA metabolism. The first phase of this research set out to evaluate the ability of existing methods to identify *trans*-acting proteins that interact specifically with known functional *cis*-acting RNA regulatory elements. Several methods were stringently tested including multiple RNA immunoprecipitation techniques and a yeast 3 hybrid screen. For all methods, results of specificity assessment experiments revealed serious limitations. In a first effort to address this specificity problem, I chose to take a more functional approach by testing whether the *S. cerevisiae* GPCR-controlled mating pathway makes use of post-transcriptional regulation to alter steady-state RNA levels. The ability to model this type of regulated stability in yeast would not only allow for the identification of novel post-transcription factors, but it would also expand the repertoire of approaches to discover the specific processes targeted by signaling to effect the observed changes in RNA turnover. Since my data indicate that *S. cerevisiae* apparently lacks this mode of

regulation, it was feasible to build a novel system that could be used to identify new proteins that interact with functional signal-regulated RNA elements. I therefore developed and deployed a novel yeast-based screening system that is capable of detecting the interaction between functional RNA *cis*-elements and candidate RNA binding proteins. The true value and novelty of this system is that it can read out a genetic interaction without prejudice to the mechanism of post-transcriptional regulation. Using this system, I have identified a novel interacting protein for the COX2 mRNA and have contributed to our understanding of how cells couple extracellular signaling events to changes in RNA metabolism.

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List of Abbreviations

Abbreviation	Definition
5 FOA	5-Fluoro Orotic Acid - used to "cure" *S. cerevisiae* of plasmids that possess that compensate for Uracil auxotrophy
AKAP	A-Kinase Anchoring Protein
AMD	ARE-meditated decay
Ang II	Angiotensin II - ligand for Gaq-coupled Angiotensin Receptor
ARE	Adenosine-rich Element
CDS	Coding Sequence
Cox 1/2	Cyclooxygenase1/2 - enzymes that catalyze the committed step in prostaglandin synthesis
CSE	cAMP Stability Element
CUGBP2	CU-rich Binding Protein 2 - mRNA binding protein implicated as SMART factor for Cox2 mRNA stability
CURE	CU-rich element
DAG	Diacylglycerol
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ER	Endoplasmic Reticulum
ERK 1/2	Extracellular regulated kinase 1/2
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GPCR	G-Protein Coupled Receptor - e.g. Angiotensin II receptor, Type I
HEK	Human Embryonic Kidney
hnRNP	Hetero-nuclear ribonucleoprotein
IEG	Immediate Early Gene - gene whose mRNA levels increase in absence of de novo protein synthesis
IP3	Inositol Triphosphate
IRE	Iron Response Element
LDH	Lactate Dehydrogenase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MAPKAP2 / MK2	Mitogen-activated protein kinase activator protein 2

MEK	Map kinase that directly activates Erk1/2
MEK-sensitive element	*cis*-acting mRNA stability control element that is regulated by MEK
NFAT	Nuclear Factor of Activated T-Cells - calcium / calcineurin dependent transcription factor
Orphan *cis*-element	Regulated mRNA stability control element for which no specific SMART factor has been identified
PAI	Plasminogen Activator Inhibitor
PCSR	PKC Stability Region
PDGF	Platelet-derived growth factor - activates PDGF homo and hetero-dimeric receptors
PDGFR	Platelet-derived growth factor receptor
PIP2	Phophotidyl Inositol Bisphosphate
PKA	Protein Kinase A - also known as cAMP-dependent protein kinase
PKC	Protein Kinase C - also known as calcium dependent protein kinase
PMA	Poly Myristic Acid - a phorbol ester diacyl glycerol mimetic compound used to help activate PKC
RASMC	Rat Aortic Smooth Muscle Cell
RBP	RNA-binding protein
REMSA	RNA mobility shift assay
RIP-CHIP	RNA Immunoprecipitation followed by microarray gene CHIP analysis
RPA	RNAse Protection Assay
RTK	Receptor Tyrosine Kinase - e.g. Platelet-derived growth factor receptors
SMART Factor	Signal Modulated Altered mRNA Turnover factor
SMC	Smooth Muscle Cell
SOCS3	Suppressor of Cytokine Signaling - also called STAT-induced STAT inhibitor
SR	Sarcoplasmic Reticulum
SPHK1	Sphingosine Kinase 1
TNF	Tumor Necrosis Factor
tPA	Tissue Plasminogen Activator
TTP	Tristetraprolin - RNA-binding protein
VSMC	Vascular Smooth Muscle Cell
α Factor	*S. cerevisiae* alpha mating pheromone

Chapter 1: Introduction

Identification of Immediate Early Gene Signal-Modulated

Altered mRNA Turnover (SMART) Factors

A section of this chapter is being submitted for publication.

Abstract

Eukaryotic cells sense and respond to a wide variety of extracellular cues largely through the activity of several classes of membrane bound receptors, including G-protein coupled receptors (GPCRs), receptor tyrosine kinases (RTKs), cytokine receptors, and various ion channel-based receptors. Activated receptors initiate intracellular signaling pathways that regulate gene expression primarily by altering rates of the synthesis and decay of mRNA and protein. At one time, signal-regulated changes in gene expression were thought to be accomplished primarily through adjustment of the rate of mRNA synthesis. Higher levels of mRNA transcripts would lead to higher levels of the cognate proteins, and mRNA decay processes were considered a cellular housekeeping activity. It is now understood that steady-state mRNA levels, much like mRNA transcription, are precisely regulated by the interaction of *cis*-acting regulatory elements with *trans*-acting factors, and that post-transcriptional processes play an equally significant role in the regulation of gene expression (Audic and Hartley, 2004; Bevilacqua et al., 2003; Ramsay et al., 2003). This chapter sets the stage for the experiments described in subsequent chapters, highlighting our current knowledge of how cells adjust gene expression in response to extracellular signals at the level of mRNA stability control. Particular emphasis will be placed on the *cis*-elements and *trans*-factors that allow cells to couple extracellular signaling events to changes in immediate early gene mRNA stability. The term Signal-Modulated Altered mRNA Turnover (SMART) factor will be used throughout this chapter to refer to the *trans*-acting factors that mediate regulated changes in mRNA metabolism.

Introduction

Changes within the extracellular environment of eukaryotes invariably alter gene expression patterns. Cell signaling pathways control mRNA transcription through the post-translational modification and regulation of *trans*-acting factors that recognize discrete *cis*-acting elements within gene promoters (Ptashne, 1986; Ptashne, 1988; Ptashne and Gann, 2003). The simplest conceivable model for the regulation of post-transcriptional gene expression is built upon an analogous framework, wherein signaling pathways modulate *trans*-acting factors that in turn recognize discrete elements within mRNA transcripts, thereby altering their metabolism. This view is overly simplistic, but a significant body of evidence indicates that 1) signaling pathways do control mRNA metabolism; 2) many *trans*-acting factors have been implicated in this metabolism; and 3) specific elements within mRNA transcripts appear to be necessary for this regulation.

Considerable evidence shows that cells adjust steady-state mRNA levels through synergistic transcription and post-transcription control mechanisms following activation of signal transduction cascades. There are numerous examples that together comprise a compelling body of data to support the assertion that signal transduction systems influence mRNA expression patterns by modulating post-transcriptional processes. Immediate early genes (IEG), those genes whose levels can be increased by signaling events in the absence of de novo protein synthesis, have been used to model both transcriptional and post-transcriptional gene regulation. The main advantage to this approach is that changes in IEG expression are most likely to represent direct effects, such as the signal-regulated post-translational modification of *trans*-acting DNA- or mRNA-binding proteins, rather than secondary effects, such as new protein-mediated

transcriptional or post-transcriptional regulation. The purpose of this chapter is to summarize and consolidate this large and disparate field, with particular emphasis placed upon how extracellular signals influence IEG expression using post-transcriptional mechanisms.

Transcriptional regulation vs. post-transcriptional regulation

While transcription factors activated downstream of signaling pathway activation typically interact with discrete, symmetrical DNA *cis*-elements to regulate mRNA synthesis, Signal-Modulated Altered mRNA Turnover (SMART) factors can interact with linear mRNA sequences and also complicated three-dimensional mRNA structures to regulate mRNA stability (Fernandez et al., 2001; Khaladkar et al., 2008). This facet of mRNA / protein interaction has effectively turned a consensus nucleotide sequence search into a consensus shape search. Several groups have used genome-wide studies to globally assess consensus RNA-binding protein *cis*-regulatory elements (Liu et al., 2005). Furthermore, compared to transcription regulators, SMART factors must engage a highly mobile nucleic acid target. Messenger RNA transcripts are processed (McKee and Silver, 2007; Moore and Silver, 2008; Wang and Burge, 2008), transported to the nuclear pore & exported to the cytoplasm (Rougemaille et al., 2008; Stewart, 2007), translated (Shyu et al., 2008), and finally degraded (Ibrahim et al., 2008; Shyu et al., 2008). A growing body of evidence suggests that the cytoplasmic fate of an mRNA is predetermined according to the protein "signature" it acquires in the nucleus (Giorgi and Moore, 2007). Therefore, post-transcriptional regulation of mRNA levels likely reflects the combined control over *several* discrete processes, occurring in multiple cellular locations, which together

contribute to the longevity, localization, and translatability of a given mRNA or populations of functionally related mRNA transcripts.

Taken together, these processes contribute to the innate stability of mRNA transcripts, but understanding how signaling pathways change the default cellular stability of mRNA transcripts presents a significant challenge. For example, signaling pathway activation significantly stabilizes many basally unstable mRNA transcripts, but the responsible mechanisms remain largely unclear (Kitchen et al., in preparation). The regulation could occur at any one or several of the numerous mRNA processing steps. Figure 1-1 schematically illustrates the fundamental difference between transcription and mRNA stability control regulation.

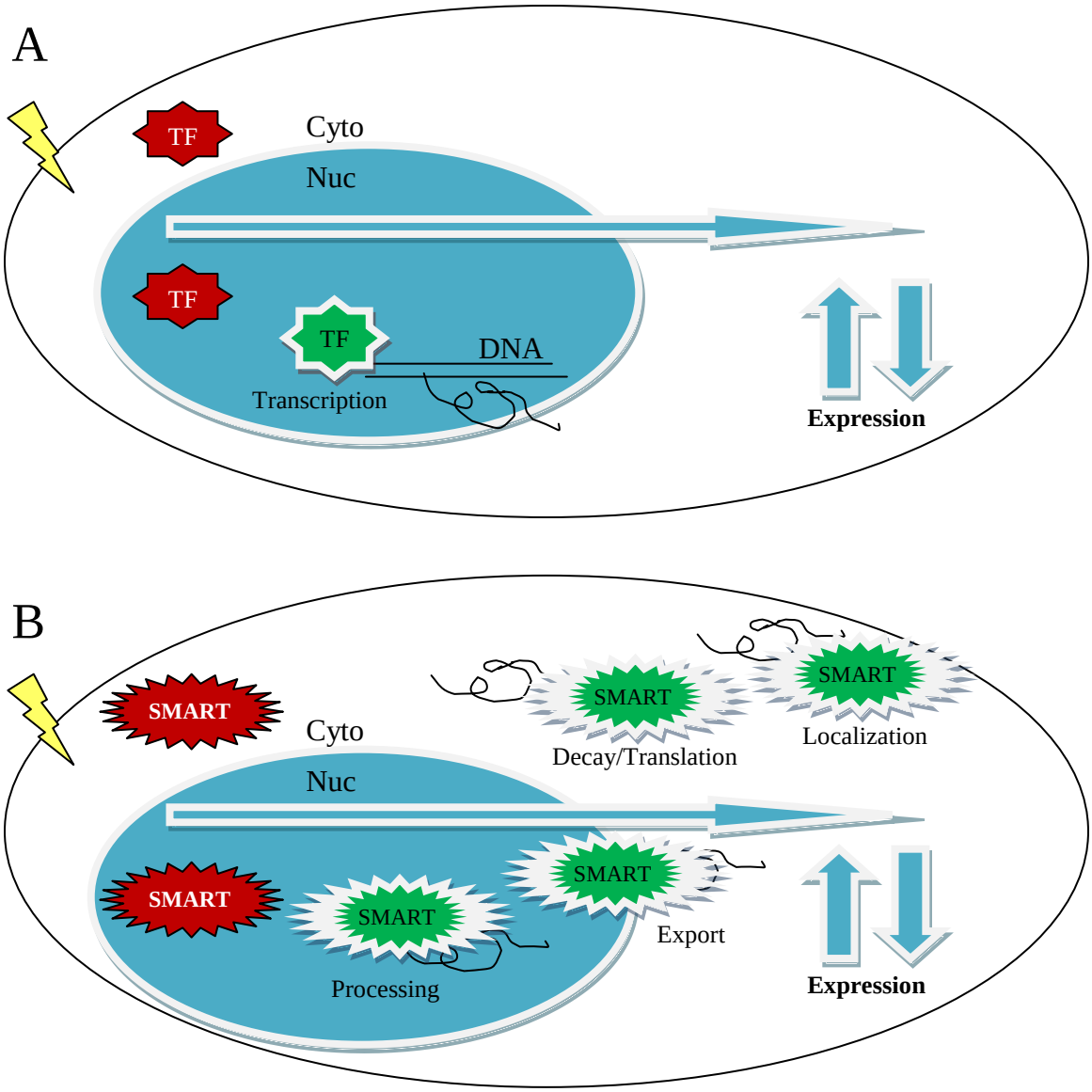
Much has been discovered about how transcription is controlled by signal transduction through understanding the function of the large family of transcription factor proteins and their cognate *cis*-acting gene regulatory elements. This paradigm currently drives discovery of post-transcriptional gene regulation. RNA binding proteins exist that appear to be modulated in any of several ways by changing cellular status, and these proteins interact with specific mRNA transcripts or even specific sequences on these mRNA transcripts and are associated with changes in gene expression.

Messenger RNA decay mechanisms

A particularly strong contributor to experimentally observed changes in mRNA turnover that occur in response to signaling pathway activation is the process of mRNA decay. Cells possess specialized machinery to selectively degrade both normal and aberrant mRNA transcripts. The key cellular mRNA decay components include

deadenylases, 5'- decapping enzymes, 5'-3' exonucleases, and 3'-5 exonucleases (Coller and Parker, 2004; Fritz et al., 2004; Tucker and Parker, 2000). In yeast, deadenylation precedes decapping, followed by rapid 5'-3' exonucleolytic degradation of mRNA transcripts (Tucker and Parker, 2000). In contrast, mammalian cells appear to preferentially use 3'-5' exonucleases to degrade messenger RNA (Wang and Kiledjian, 2001). RNA transcripts that contain premature termination codons, or that are 3'-extended due to a mutation in the poly adenylation signal, are each degraded according to the non-sense mediated decay (NMD) surveillance pathway (Gonzalez et al., 2001; Muhlrad and Parker, 1999; Sheth and Parker, 2006). NMD appears to recognize and respond to transcripts whose poly A signal is not positioned properly with respect to its translation terminator. NMD serves to prevent the production of truncated proteins that might otherwise have a negative effect on cells. Finally, micro RNA (miRNA) is increasingly implicated in the modulation of mRNA transcript decay and translation (Shyu et al., 2008; Wang et al., 2007; Wu and Belasco, 2008; Wu et al., 2006). miRNAs are 21-23 nucleotide single-stranded RNAs that can inhibit translation or direct the rapid destruction of mRNA transcripts. The role of miRNA appears to be to provide cells with a very precise mechanism to fine-tune protein expression. Each of these cellular mRNA decay mechanisms has been shown to be influenced, to varying degrees, by signaling pathway activation (Garneau et al., 2007).

Figure 1-1. Schematic representation of regulated mRNA stability vs. regulated transcription. Signaling controls mRNA synthesis using transcription factors (A) and controls mRNA stability using SMART factors (B). Unlike transcription factors that act primarily at fixed locations (i.e. gene promoters or regulatory regions on nuclear DNA), SMART factors act on moving mRNA targets throughout multiple metabolic processes. **RED** = inactive *trans*-acting factor; **GREEN** = active *trans*-factor; "lightning bolt" = active signaling



Methods to Study Post-transcriptional Regulation

Direct and indirect methods are used together to establish evidence that an mRNA is post-transcriptionally regulated. Historically, when stimulation of cells caused a change in the level of expression of a given gene, this effect was assumed to reflect solely changes in the rate of transcription (Wilson and Cerione, 2000). The method of nuclear run-on was devised to determine the rate of transcription (Llopis et al., 1981). The procedure involves arresting on-going transcription by isolating cell nuclei, washing away ribonucleotide triphosphates, adding back ribonucleotide triphosphates (typically with one radio-labeled), and allowing loaded RNA Polymerase II complexes to resume transcription and "run-on". Radiolabeled RNA is isolated and transferred via slot blot apparatus to nitrocellulose or nylon that has been previously bound by gene specific cDNAs. The levels of new RNA produced are directly proportional to the number of Pol II complexes transcribing the gene at the time of transcription termination. If more radioactive signal is detected by the run-on assay in stimulated cells versus unstimulated cells, the stimulus has caused an increase in the rate of transcription. If the stimulus increases steady-state mRNA levels without increasing the rate of transcription, as measured by nuclear run-on assay, the stimulus likely regulates gene expression post-transcriptionally. Hence, the nuclear run on assay has proven to be an essential method in determining whether an mRNA is post-transcriptionally regulated (Tenenbaum et al., 2003).

Nuclear run-on does have several drawbacks, such as requiring a large number of nuclei and the need for relatively high levels of radioactivity. Transcripts may also terminate pre-maturely, due to damage occurring during the nuclear isolation process,

leading to underestimation of transcription rate increases. By comparing the changes in the rate of transcription to changes in the steady state levels of mRNA transcripts, one can infer that changes in expression are due to post-transcriptional regulation if the run on method shows transcription alone cannot account for the difference. For example, the COX2 mRNA is robustly induced in many cell types by cellular adhesion, mitogens and inflammatory mediators (Ramsay et al., 2003). In cultured vascular smooth muscle cells, steady state COX2 mRNA can be induced 100-fold over its baseline expression levels by mitogens that only cause a modest 4-5-fold increase in transcription, as assessed by nuclear run-on assay. This relatively modest transcription rate increase, relative to the more robust steady-state mRNA increase is indicative of post-transcriptional regulation (Xu et al., 2000).

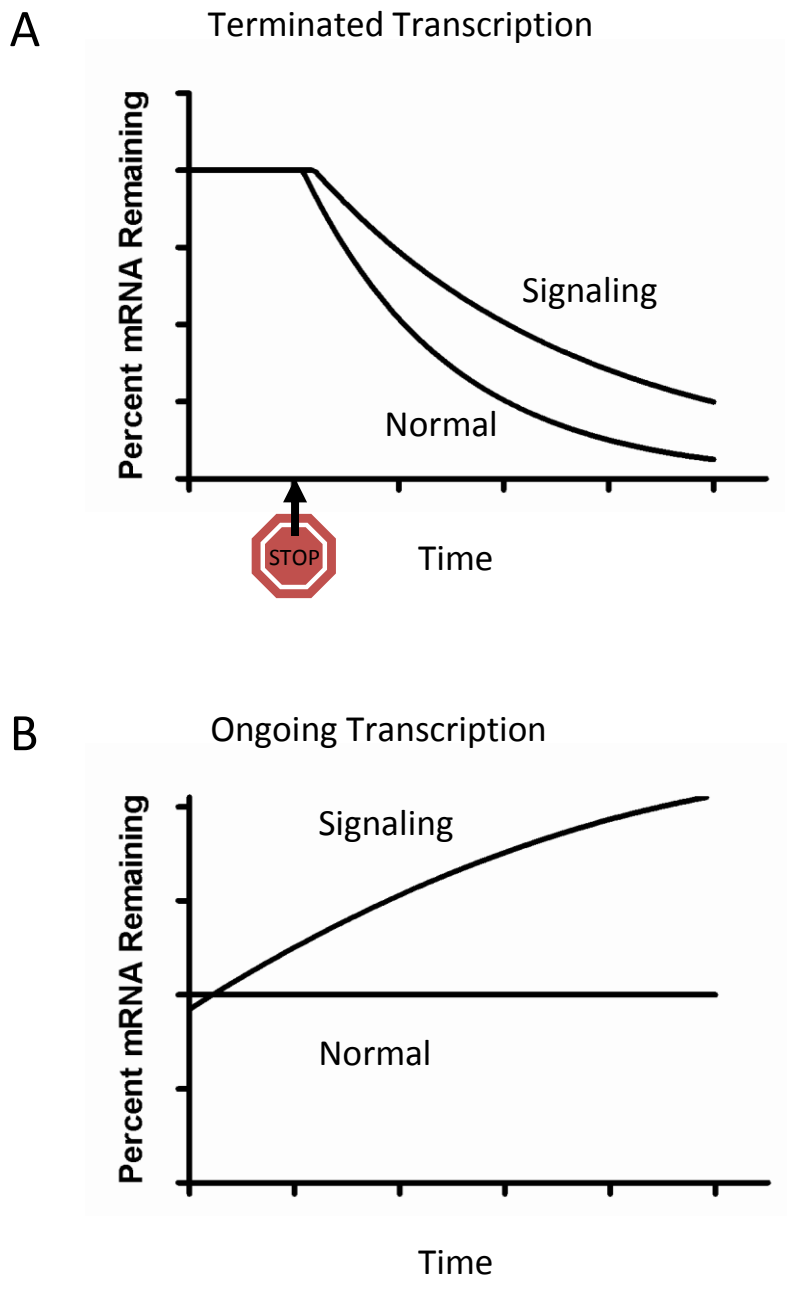
Another historical limitation of the nuclear run-on assay has been that it provides a snapshot of transcriptional activity for a single gene at one time. The advent of cDNA microarrays has at least allowed the possibility of surveying multiple genes simultaneously, in the form of *enmasse* nuclear run-ons (Tenenbaum et al., 2003). When one compares the global nuclear run-on data to the global steady state mRNA expression level data, one can determine which mRNA transcripts a stimulus is likely to regulate post-transcriptionally (Narsai et al., 2007). Another useful method to measure changes in transcription is RNA immunoprecipitation-chip (RIP-CHIP). This non-radioactive technique may be more compatible with today's global microarray studies, but both nuclear run-on and RIP-CHIP have been applied recently to assess genome-wide transcription rate changes (Sandoval et al., 2004).

A more direct test for examining regulated mRNA stability involves determining how signaling influences mRNA half-lives. RNA half-life determination requires measuring the time-dependent decrease in RNA level. To understand whether signaling influences mRNA stability, transcript half-life is measured in the absence or presence of signaling when transcription is terminated. This can be achieved either non-selectively using a transcriptional poison, or more selectively using a genetic mechanism, or a regulated promoter/mRNA reporter system. RNA is detected at several time points thereafter, and the time-dependent RNA decay is measured. If the mRNA half-life is relatively higher for the stimulated versus the unstimulated cells, then the signaling pathway has stabilized the mRNA of interest. Figure 1-2 illustrates graphically how mRNA half-life can change in response to signaling.

Some transcriptional poisons include pharmacological inhibitors such as 5,6-dichlororibofuranosylbenzimidazole (DRB) (Dreyer and Hausen, 1978), actinomycin D (Sobell, 1985), thiolutin (Khachatourians and Tipper, 1974), or alpha amanitin (Lindell et al., 1970; Seifart and Sekeris, 1969). DRB is a kinase inhibitor that prevents phosphorylation of the CTD domain of the large subunit of RNA polymerase II and therefore arrests transcriptional elongation (Dreyer and Hausen, 1978).

The use of DRB to measure signaling-regulated mRNA half-life changes is inherently problematic due to its lack of selectivity for the kinases that phosphorylate the Pol II subunit. For instance, data analysis can be confounded if DRB also inhibits signaling pathway kinases. Actinomycin D is a DNA intercalator that binds to the relatively open initiation complex on DNA templates.

Figure 1-2. Representative plot for mRNA decay where half-life is increased due to activation of a signaling pathway. If signaling is initiated when transcription is terminated, a stabilized transcript will have a relatively longer half-life, A. Similarly, if signaling is initiated in the presence of ongoing transcription, a stabilized mRNA will continue to increase to a new steady-state level, B.



Similar to the other transcription poisons, actinomycin D is a non-selective inhibitor of RNA Polymerases I, II, and III (Sobell, 1985). Finally, Alpha Amanitin is comparatively selective for RNA Polymerase II, but is extremely toxic (Lindell et al., 1970). A general lack of specificity is the common limitation of these poorly selective drugs. Consequently, post-transcriptional processes can be disrupted in a number of ways. For example, they impair nuclear cytoplasmic shuttling of several RNA-binding proteins requires ongoing RNA Polymerase II transcription (Pinol-Roma and Dreyfuss, 1992). Such shuttling may be a critical component of a signal-regulated process involved in the specification of the half-life of an mRNA under study, and so inhibiting this process can influence mRNA steady-state and confound interpretation of the results. Finally, thiolutin is a powerful antibiotic that targets bacterial and yeast RNA polymerases. Its utility in determining signal-regulated mRNA half-life changes may be somewhat limited. For example, recent experiments reveal that thiolutin not only affects transcription, but it also variably affects poly A-independent RNA decay (Pelechano and Perez-Ortin, 2008).

Other methods of stopping transcription that may suffer from similar lack of selectivity include temperature sensitive Pol II mutants and metabolically regulated promoters. For example, global transcription is dramatically impaired when *rpb1-1* temperature sensitive mutant *S. cerevisiae* cells are shifted to the non-permissive temperature. Using this allele produces results comparable to those obtained using thiolutin (Herrick et al., 1990). Regulated promoter systems offer the advantage of turning off a single gene and assessing its transcript stability. For example, in yeast, several metabolically regulated promoters under control of trace metals, amino acids, and carbon source have been or could be used to drive the expression of recombinant mRNA

transcripts to determine their half lives (Kuo et al., 2005). The most commonly used of these regulated promoter for mammalian cells is the tet operator system (Gossen and Bujard, 1993). Transcription of a recombinant gene of interest driven by a constitutively expressed tet operator is suppressed rapidly by addition of a tetracycline analog. Of these regulated promoter systems, the tet-regulated version seems to be the most specific because the tetracycline analogs rarely, if ever, interfere with endogenously expressed genes or other cellular machinery that might be involved in the specification of signaling regulated mRNA stability. The principle advantage of the tet-system is that it allows for the conducting of structure-activity studies, e.g. deletional and mutational analyses to discover mRNA elements that are required for signal regulation. For example, if the removal of a discrete sequence changes the mRNA pattern of response to a signal, or if an element confers regulation upon a heterologous transcript, it is very likely that the *cis*-element is mediating the observed signal-regulated changes in mRNA metabolism. A disadvantage is that they do not allow for a complete reconstitution of the entire transcription, and so, processing/splicing factors might be overlooked.

Several of these approaches need to be deployed to make the determination that an mRNA transcript is subject to signal-regulated stability changes. Crucial information is in the determination of how a change in extracellular status affects transcription of a gene. In this regard, the nuclear run-on is unavoidable. Awareness is growing of the many confounding non-specific consequences of these transcriptional poisons have and they should be avoided where possible. The regulated promoter systems, particularly the tet-based systems, appear to provide the best means for assessing how dynamic signaling can affect an mRNA post-transcriptionally, but are limited because they only allow the

study of one gene at a time. Even so, the regulated promoter systems depend upon assaying the behavior of recombinantly expressed mRNA transcripts that may not recapitulate fully all of the properties of the endogenously expressed mRNA that it seeks to mimic. Therefore, it seems crucial to carefully evaluate steady state mRNA levels, run-on transcription, and use recombinant systems in their combination.

Signaling pathways and the mRNA transcripts they regulate

The following section summarizes the most recent understanding of how the major signaling pathways exert changes in the metabolism of target mRNA transcripts. For each signaling pathway, mechanisms of activation are discussed, including relevant kinases, as well as inhibitor means that are typically used to implicate the dependence of mRNA stability on the actions of these kinases. Transcripts and putative **S**ignal **M**odulated **A**ltered m**R**NA **T**urnover (SMART) *trans*-factors will discussed and summarized in table form. Particular emphasis will be placed on direct actions of regulated mRNA stability, such as direct phosphorylation of mRNA-binding proteins resulting in immediate early-like changes in mRNA metabolism. For the purposes of this review, indirect signal-regulated mRNA stabilization or destabilization is hereby defined as mRNA stability regulation that depends upon compensatory effects following immediate-early signaling time-frame, and for the most part are beyond the scope of this review. Figure 1-3 illustrates a general mechanism by which signaling pathways might deploy immediate early-like Signal Modulated Altered mRNA Turnover (SMART) factors to control mRNA stability.

Figure 1-3. Schematic representation of regulated mRNA stability mediated by post-translational modification of SMART factors. When cells are at rest, certain stabilizing SMART factors (shown in **GREEN**) remain nuclear, allowing their target mRNA transcripts to bind cytoplasmic-resident destabilizing SMART factors (shown in **RED**), resulting in rapid mRNA decay (A). Signaling can cause several SMART factors to become phosphorylated, with the net consequence of increased mRNA stability due to increased association of transcripts with stabilizing factors and reduced association of transcripts with destabilizing factors (B).

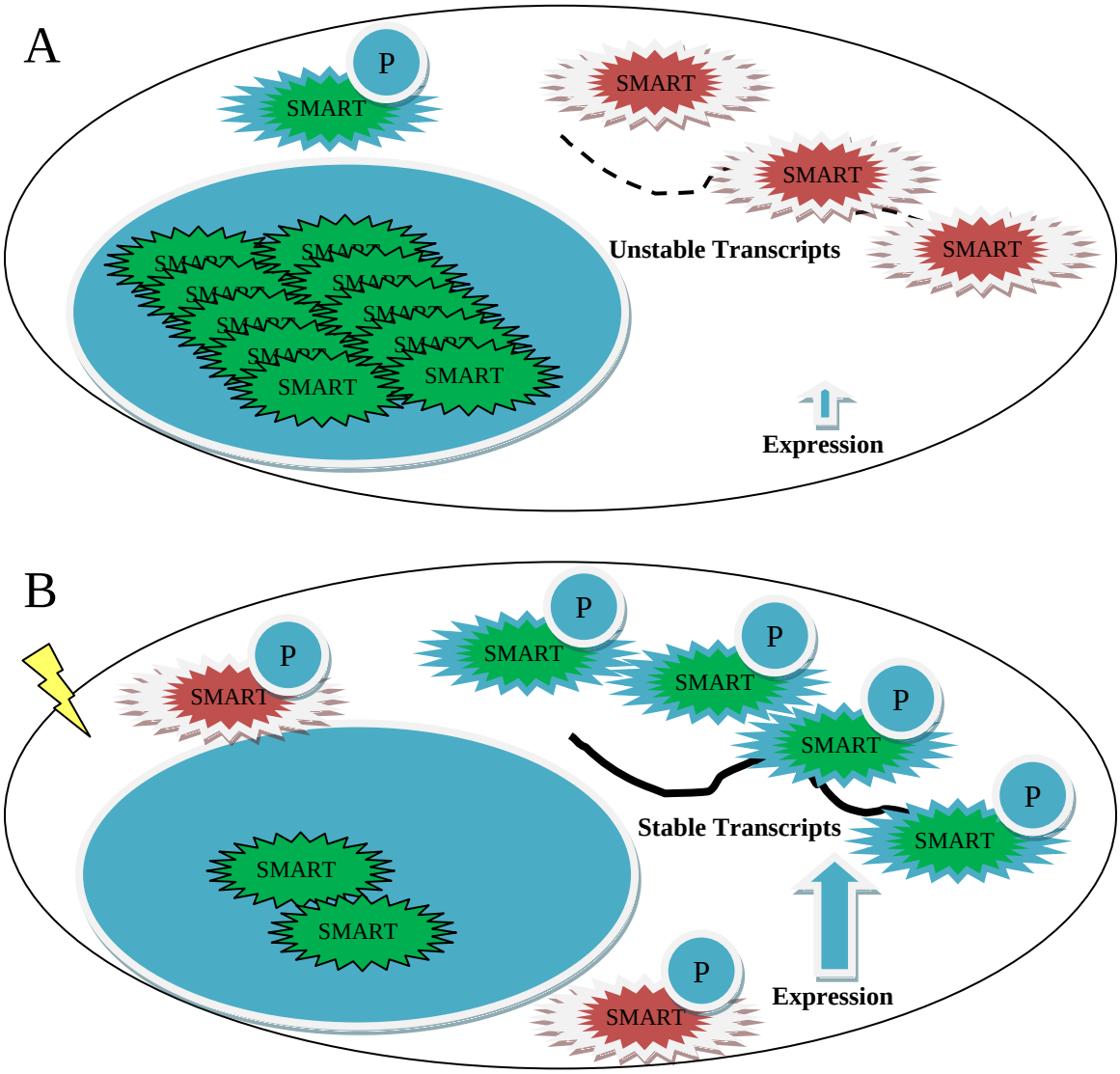


Table 1-1. PKA regulated mRNA transcripts and candidate SMART effectors.

Putative SMART *Trans*-Factor	**Target mRNA**	**Reference**
Unknown	↓AT(1)R	(Xu and Murphy, 2000)
AUF1	↓βAR	(Tholanikunnel et al., 1995)
	↓CRFR1	(Moriyama et al., 2005)
CSR binding proteins	↑LDH-A	(Jungmann and Kiryukhina, 2005; Tian et al., 1998a; Tian et al., 1998b)
AUF1	↑PEPCK	(Dhakras et al., 2006)
	↑SGLT1	(Lee et al., 2000; Loflin and Lever, 2001)
	↑GLUT5	(Gouyon et al., 2003)
PTB	↑Insulin	(G. Fred et al., 2006; Knoch et al., 2006; Ma et al., 2007; Xie et al., 2003)
	↑IP3R	(Lee and Laychock, 2000)
HuR	↑↓Renin	(Morris et al., 2004)
AKAP121 (scaffold role)	↑MnSOD	(Ginsberg et al., 2003)
PAI1BP	↑PAI1	(Heaton et al., 2003)
	↑COX2	(Tamura et al., 2002)

Post-transcriptional regulation by PKA

Protein kinase A (PKA) is a broad specificity kinase that has multiple cellular effects (Skalhegg and Tasken, 1997). PKA is activated when cAMP binds to the inhibitory subunits, liberating the catalytic subunits to phosphorylate serines and threonines on downstream target proteins. Therefore, extracellular stimuli that lead to increased cAMP concentration, activators of adenylate cyclase or inhibitors of phosphodiesterases, have the potential to activate PKA signaling. PKA has long been known to activate cAMP-response element binding (CREB)-dependent transcription, but several studies show PKA also plays a role in the mRNA stability control of an increasing number of transcripts. Table 1-1 highlights the most prominent examples of PKA-regulated mRNA transcripts, linked to candidate or suspected SMART factors where known.

PKA signaling has been implicated in regulating the stability of several specific mRNA transcripts, including AT(1)R (Xu and Murphy, 2000), CRF1 (Moriyama et al., 2005), LDH (Jungmann and Kiryukhina, 2005; Tian et al., 1998a; Tian et al., 1998b), PEPCK (Dhakras et al., 2006), SGLT1 (Lee et al., 2000; Loflin and Lever, 2001), GLUT5 (Gouyon et al., 2003), Insulin mRNA (Knoch et al., 2006), IP3R (Lee and Laychock, 2000), Renin mRNA (Morris et al., 2004), MnSOD (Ginsberg et al., 2003), PAI1 (Heaton et al., 2003), and COX2 (Tamura et al., 2002) (stabilization). Most of the PKA-stabilized mRNA transcripts possess one or several AU-rich elements (AREs), the presence of which is generally predictive of a basally unstable mRNA transcript (Bolognani and Perrone-Bizzozero, 2008). Activation of PKA signaling appears to increase the stability of ARE-containing transcripts by inducing the relocalization of

stabilizing AU-rich binding proteins (AUBP), such as poly pyrimidine tract binding (PTB) protein (Knoch et al., 2006; Ma et al., 2007).

Recently, it has been established that PKA regulates CU-rich element containing mRNA transcripts post-transcriptionally via serine phosphorylation of the PTB protein (Knoch et al., 2006; Xie et al., 2003). Serine 16 phosphorylation is accompanied by PTB movement from the nucleus to the cytoplasm where it binds to and stabilizes mRNA transcripts such as those coding for insulin (Knoch et al., 2006; Ma et al., 2007). This translocation is explained by the fact that phosphorylation at Serine 16 appears to obscure the nuclear localization signal (NLS) within PTB, allowing the protein to accumulate in the cytoplasm. In neurons, PKA causes PTB to exert a stabilizing influence over the β-actin transcripts, allowing them to localize to sites of neurite outgrowths (Ma et al., 2007).

PKA activation can destabilize transcripts via direct action, such as appears to be the case for plasminogen activator inhibitor 1 (PAI1) mRNA destabilization, or by indirect mechanisms, whereby PKA stabilizes the mRNA that codes for an instability SMART factor. To date, several *trans*-factors such as PTB (Knoch et al., 2006; Ma et al., 2007; Xie et al., 2003), AUF1 / hnRNP D (Dhakras et al., 2006; Tolnay et al., 2002), HuR (Kloss et al., 2004; Loflin and Lever, 2001; Subbaramaiah et al., 2003), and CNBP (Lombardo et al., 2007) have been identified as phosphorylated in response to PKA pathway activation. This phosphorylation can have varying effects, most prominent being the subcellular relocalization of the *trans*-factor.

PKA can also cause stabilization of ARE-containing mRNA transcripts presumably via AUF1/hnRNP D-dependent mechanisms (Dhakras et al., 2006). Forskolin, a specific activator of adenylyl cyclase, stabilizes synthetic phosphoenolpyruvate carboxykinase (PEPCK) mRNA, but direct evidence for AUF1 phosphorylation by PKA or PKA-dependent kinases has yet to be determined. Other groups have implicated PKA activity in the stabilization of the lactate dehydrogenase (LDH) mRNA, providing strong evidence for a 22-nucleotide cAMP-Stabilizing Region (CSR) within a U-rich region of the 3 ’UTR (Tian et al., 1998b). Subsequent work suggests PKA-dependent phosphorylation of at least four CSR-binding proteins is necessary for the observed mRNA stabilization (Jungmann and Kiryukhina, 2005; Tian et al., 1998a; Tian et al., 1998b).

Though not typically considered *trans*-acting factors, the scaffolding A Kinase Anchoring Proteins (AKAPs) participate in PKA-mediated modulation of LDH mRNA stability by serving as an mRNA-binding protein assembly complex (Jungmann and Kiryukhina, 2005). One thought on how this functions in mRNA stabilization is that the CSR/AKAP95/PKA complex may encourage a relatively more closed ribonucleoprotein configuration, thus shielding complexed mRNA transcripts from exosome recruitment factors (Chen CY, 2001; van Hoof and Parker, 2002). Other AKAPs, like the KH-domain-containing AKAP121, also link PKA signaling to mRNA stability regulation. AKAP121 binds to the 3’UTR of both the mATP-synthase-Fo-f subunit mRNA and the MnSOD mRNA in a PKA-dependent manner (Ginsberg et al., 2003). In response to cAMP, PKA/AKAP121 binds to these mRNA transcripts leading to their mitochondrial

translocation. The net effect is to increase MnSOD protein levels in the mitochondria through PKA-mediated mRNA post-transcriptional regulation.

PKA signaling can also destabilize mRNA transcripts. One system that involves putative SMART factors regulates plasminogen activator inhibitor 1 mRNA stability (Heaton et al., 2001). Plasminogen activator inhibitor 1 mRNA binding protein (PAIBP) occurs in 4 splice forms, each of which possesses a putative PKA phosphorylation site. Upon PKA stimulation, PAI-1BP associates with the U-rich sequences in the PAI-1 mRNA 3'UTR, causing reduced transcript stability. Since PAI-1 protein normally antagonizes the levels of tissue plasminogen activator (tPA), tPA-dependent activities such as fibrinolysis and wound healing increase markedly when PAI-1 is down-regulated and so this mechanism of mRNA stability may have physiologic consequences (Heaton et al., 2003). AT_1-R mRNA down regulation represents yet another PKA-modified mRNA destabilized process. In this instance, the 5'UTR of the AT_1-R mRNA functions as a *cis*-acting sensor of PKA, resulting in AT_1-R down-regulation during PKA signaling. The 5'UTR can confer PKA-mediated destabilization when placed upon a heterologous mRNA, suggesting a dynamic cis/trans process is involved, though putative SMART mediators are unknown.

There is now clear evidence that cells regulate gene expression at the level of mRNA stability in response to PKA signaling pathway activation. There is evidence that mRNA transcripts can be stabilized or destabilized, implying significant complexity in SMART factors and the processes involved. Strong evidence exists for direct PKA-mediated mRNA stability control, as when PKA causes a SMART factor such as PTB to move from the nucleus to the cytoplasm to stabilize a target mRNA via specific

mRNA/protein interactions. There are clear unmet needs when it comes to identifying all the SMART factors that PKA signaling mobilizes to exert mRNA stability control, but for most examples to date, a strong case has been built for the existence of discrete, *cis*-acting regulatory elements within PKA-regulated mRNA transcripts without knowledge of the SMART factors involved. The clear task ahead is to determine what cellular mediators cooperate with these mRNA regulatory motifs.

Table 1-2. PKC regulated mRNA transcripts and candidate SMART effectors.

Putative SMART *Trans*-Factor	**Target mRNA(s)**	**Reference**
	↑p21	(Akashi et al., 1999; Park et al., 2001)
HuR	↑NRAMP1 (SLC11A1)	(Lafuse et al., 2000; Xu et al., 2005)
hnRNP K	↑VEGF	(Chabannes et al., 2001; Feliers et al., 2007; Sataranatarajan et al., 2008)
ACF	↑ApoB editing	(Lehmann et al., 2007)
	↓NAV1.7α	(Wada et al., 2004)
AUF1	↓Serca2A	(Blum et al., 2005)
HuD/nELAV	↑Nova1	(Ratti et al., 2008)
HuD/nELAV	↑GAP43	(Mobarak et al., 2000; Pascale et al., 2005b)
HuR	↑COX2	(Doller et al., 2007b)
IRP1	↑IRE containing	(Fillebeen et al., 2005)
	↑LDH	(Short et al., 2000)
	↑iNOS	(Carpenter et al., 2001)
HuD & HuR	↑MARCKS	(Wein et al., 2003)
14-3-3β / TTP	↑β4GalT1	(Gringhuis et al., 2005)
	↑GM-CSF	(Kanda and Watanabe, 2004)
	↑TNFα	(D'Addario et al., 2000; Wilson et al., 2003b)
	↑IL1β	(Wilson et al., 2003b)
p37AUF1	↓PP2A-B56α	(Glaser et al., 2006)
PTB/PTB-T	↑↓CD154	(Hamilton et al., 2003)

Post-transcriptional regulation by PKC

Protein kinase C (PKC) refers to a collection of at least 12 different serine/threonine kinase isoforms that are activated by a wide variety of extracellular stimuli (Battaini and Mochly-Rosen, 2007; Mellor and Parker, 1998). Unstimulated PKC is retained largely in the cytoplasm where the regulatory domain pseudosubstrate sequence binds to the PKC active site, thus denying access to bona fide PKC substrates. Pharmacological or physiological ligands that activate certain membrane bound receptors mobilize PLCγ or PLCβ to produce diacyl glycerol (DAG) and inositol triphosphate (IP_3) from membrane phospholipids such as phosphatidyl inositol bisphosphate (PIP_2). IP_3 binds to the ER/SR IP_3 receptor causing a massive efflux of calcium stores into the cytoplasm. Calcium exposes the PKC DAG-binding site, allowing for direct activation of PKC. Other compounds, such as ionophores (e.g. ionomycin), can penetrate cells and cause calcium release, but they do not increase levels of DAG. One can co-administer certain phorbol esters (e.g. PMA), to mimic the effects of cellular DAG in order to fully activate PKC (Yamanishi et al., 1983). Several novel isoforms of PKC depend on neither calcium nor DAG, but collectively, these kinases are all referred to as Protein Kinase C and transcriptionally/post-transcriptionally regulate the expression of many downstream genes (Ventura and Maioli, 2001). Table 1-2 summarizes PKC-regulated mRNA transcripts.

PKC signaling has been implicated in the post-transcriptional stabilization of numerous basally unstable mRNA transcripts, including IL-2 (Sanchez-Lockhart and Miller, 2006), TNFα (Nagy, 2004), Msi1 (Ratti et al., 2006), VEGF (Feliers et al., 2007; Sataranatarajan et al., 2008), AT(1)R (Mueller et al., 2008), IRE (Schalinske et al., 1997),

ApoB (Lehmann et al., 2007; Sidiropoulos et al., 2007), Serca2A (Blum et al., 2005), GAP43 (Mobarak et al., 2000; Pascale et al., 2005b), COX2 (Doller et al., 2008; Doller et al., 2007b), LDH (Short et al., 2000), iNOS (Carpenter et al., 2001), eNOS (Marsen et al., 1999), and many other, largely AU-rich element (ARE) containing transcripts. PKC is also associated with the destabilization of a small handful of mRNA transcripts such as the one that encodes the $NA_V1.7$ sodium channel (Wada et al., 2004), but it appears to stabilize many more mRNA transcripts than it destabilizes.

PKC appears to control mRNA stability through multiple SMART factors including nELAV proteins (Pascale et al., 2005b; Ratti et al., 2006), HuD (Mobarak et al., 2000; Wein et al., 2003), p40 AUF1 (Blum et al., 2005), iron response protein/IRP (Eisenstein and Blemings, 1998; Schalinske et al., 1997; Thomson et al., 2000), HuR (Doller et al., 2008; Doller et al., 2007b; Wein et al., 2003), the p68 RNA helicase (Rosenberger et al., 2002), PSF (Rosenberger et al., 2002), hnRNP K (Feliers et al., 2007; Sataranatarajan et al., 2008), hnRNP A3 & L (Rosenberger et al., 2002), calreticulin (Mueller et al., 2008), ACF (Lehmann et al., 2007), and CUGBP1 (Kuyumcu-Martinez et al., 2007).

Activators of PKC stabilize ARE-containing mRNA transcripts such as those encoding TNFα, GM-CSF, VEGF and COX2 by causing nucleocytoplasmic shuttling of SMART stability factors like HuR (Amadio et al., 2008; Doller et al., 2008; Doller et al., 2007a; Nagy, 2004). PKC-dependent phosphorylation of HuR appears to only affect its cytoplasmic accumulation without altering its affinity for ARE sequences (Doller et al., 2008). Similarly, PKCα activity induces the nuclear to cytoplasmic translocation of HuD, resulting in stabilization of ARE-containing transcripts Msi1, GAP43, and Nova1

(Mobarak et al., 2000; Pascale et al., 2005b; Ratti et al., 2008; Ratti et al., 2006; Wein et al., 2003). The precise mechanism of the cytoplasmic / cytoskeletal redistribution of HuR and HuD has yet to be elucidated, though the movement of HuB/C/D is accompanied by increases in their expression levels, and increased PKCα-dependent threonine phosphorylation (Pascale et al., 2005a). HuD and HuR, when artificially overexpressed, also bind to a PKC-regulated CU-rich mRNA instability element in the MARCKS mRNA 3'UTR, leading to an increase in the half-life of the transcript (Wein et al., 2003). One model suggests that a PKC-regulated destabilizing SMART factor may preferentially occupy this instability element, but excess HuR or HuD may compete for binding, thereby stabilizing the MARCKS mRNA. In human mesangial cells, PKCα activation leads to dual serine phosphorylation (pS158/pS221) of HuR, resulting in the stabilization of ARE-containing mRNA transcripts such as those encoding COX2 (Doller et al., 2008; Doller et al., 2007b). Non-hydrolyzable ATPγS, as well as PKCα overexpression, induces the nuclear to cytoplasmic translocation of HuR, with concomitant increases in both HuR binding to COX2 mRNA and secretion of the COX2 product, PGE2 (Doller et al., 2007b). In general, the regulation of Hu/ELAV proteins by PKC influences mRNA stability both directly, by causing SMART factor subcellular relocalization, and indirectly, by leading to long term increases in SMART factor abundance.

Studies using the phorbol ester TPA have revealed PKC SMART factors that control the stability of mRNA transcripts encoding cytokines and inflammatory mediators (Wilson et al., 2003b). In resting cells, the dually serine-phosphorylated p40 AUF1 is associated with ARE-containing mRNA transcripts, such as TNFα, in polysomes. Upon treatment with TPA, the polysomes preferentially associate with the unphosphorylated

form of p40 AUF1. Evidence suggests that association with the dually phosphorylated p40 AUF1 is coincident with a more open, less stable mRNA configuration (Wilson et al., 2003a; Wilson et al., 2003b).

PKC can also regulate lactate dehydrogenase (LDH) mRNA stability via a *cis*-acting AU-rich sequence named the PKC stability region (PCSR) in the 3' UTR of the transcript (Short et al., 2000). This element appears to be distinct from the PKA-regulated cAMP-stability element (CSE) (refer to PKA section of this chapter) though there does appear to be a 13-nucleotide AU-rich stretch that marks a point of intersection between PKC and PKA mediated mRNA stability control. Independent of one another, PKC and PKA increase the LDH mRNA half-life by 5- and 8-fold, respectively, but when they act in concert, they synergistically increase the half-life by 21-fold over the unstimulated half-life. The evidence for a discrete *cis*-regulatory element is solid, but it remains to be determined whether PKC signaling modifies these SMART factors the same way that PKA signaling modifies the four CSE SMART factors.

PKC signaling can also stabilize iron response element (IRE)-containing transcripts, including those that encode the amyloid precursor protein (APP) and the transferrin receptor (Christova and Templeton, 2007; Eisenstein and Blemings, 1998; Thomson et al., 2000). Phorbol esters increase phosphorylation of iron response protein at serine 711, and this phosphorylation is concomitant with ER localization and with stabilization of IRE-containing mRNA transcripts.

PKCδ (as well as IκB kinase-β) is required for stabilization of the β4GalT1 gene in HUVECs (Garcia-Vallejo et al., 2005; Gringhuis et al., 2005). In unstimulated cells,

the mRNA is relatively unstable owing to its association with a destabilizing 14-3-3β / tristetraprolin (TTP) complex. TNFα stimulation promotes 14-3-3β phosphorylation at two critical serine residues resulting in the dissociation of the proteins from the mRNA. Upon release, TTP translocates to the nucleus, wherein it becomes sequestered, resulting in the stabilization of the β4GalT1 mRNA.

In general, PKC-regulated SMART factor phosphorylation occurs concomitantly with subcellular translocation. This can be nuclear to cytoplasm, as with nELAV/HuD or cytoplasm to nucleus, as with TTP. In the case of nELAV/HuD, PKC signaling increases transcript stability by recruiting a *stabilizing* SMART factor. In the case of TTP, signaling increases transcript stability by removing a *destabilizing* SMART factor. PKC can act independently in regulating mRNA stability control, but it often acts in concert with other kinases, such as PKA and IκB kinase-β. Overall, discrete *cis*-acting elements have been identified, though it is unclear whether PKC simply causes stabilizing factors to move into proximity to their target mRNA transcripts, and destabilizing factors to move away from target mRNA transcripts, or whether the phosphorylation changes cause actual changes in affinity of post-transcription factors for their target mRNA transcripts. Of the PKC SMART factors identified to date, nELAV and iron response protein are the best examples of direct phospho accepting substrates. The PKC SMART factor PTB appears as an exception to this phosphoprotein / translocation model (Hamilton et al., 2003; Kosinski et al., 2003; Rosenberger et al., 2002). PKC pathway activators, PMA and calcium, cause the cytoplasmic accumulation of a novel PTB isoform, PTB-T, and this translocation is coincident with increased CD154 mRNA stability (Hamilton et al., 2003).

Table 1-3. p38 MAPK regulated mRNA transcripts and candidate SMART effectors.

Putative SMART *Trans*-Factor	**Target mRNA**	**Reference**
NRAMP1	↑NRAMP1	(Lafuse et al., 2002)
HuR	↑GCSh	(Song et al., 2005)
	↑IFNγ-Inducible	(Sun and Ding, 2006)
	↑CyclinD1/↑cMYC	(Marderosian et al., 2006)
	↑BMP-2	(Fukui et al., 2006)
PAI-1BP	↑PAI-1	(Norata et al., 2004)
	↑TSP1	(Okamoto et al., 2002)
AKAP121	↑MnSOD, ↑MASPIN	(Davis et al., 2001a; Ginsberg et al., 2003)
	↑SOCS3	(Ehlting et al., 2007)
	↑c-fos	(Winzen et al., 2004; Winzen et al., 1999)
	↑E47	(Frasca et al., 2007)
	↑CFTR	(Baudouin-Legros et al., 2005)
	↑MMP1/↑MMP3	(Saklatvala et al., 2003)
	↓MMP9	(Liu et al., 2006)
HuR	↑COX2	(Di Mari et al., 2007; Doller et al., 2008; Faour et al., 2003; Subbaramaiah et al., 2003)
	↑VEGF	(Pages et al., 2000)
MK2 Targets	↑uPA	(Han et al., 2002; Montero and Nagamine, 1999)
	↑IFNγ	(Mavropoulos et al., 2005)
AUF1	↑GROα & ↑IL-1β	(Chen et al., 2006; Sirenko et al., 1997; Wang et al., 1999)
TTP	↑CXCL1	(Datta et al., 2008)
	↑CXCL10	(Dhillon et al., 2007)
	↑MCP-1	(Waterhouse et al., 2001)
	↑MKP1	(Kuwano et al., 2008; Lasa et al., 2002)
MK2 Targets	↑IL-2, ↑IL-3, ↑IL-6	(Stoecklin et al., 2001; Winzen et al., 1999)
TTP, KSRP	↓↑IL-8	(Holtmann et al., 1999; Ma et al., 2004; Suswam et al., 2008; Winzen et al., 2007)

TTP, HuR	↓↑TNFα	(Chung et al., 2003; Deleault et al., 2007; Fotheringham et al., 2004; Kotlyarov et al., 1999; Rajasingh et al., 2006; Stoecklin et al., 2001)
	↓↑TTP	(Hitti et al., 2006; Tchen et al., 2004)
	↑Tis11B	(Busse et al., 2008)
	↑TNFα/↑IL-6/↑MIP1α	(Wang et al., 1999)
	↑iNOS	(Fechir et al., 2005)
	↑MIP2	(Numahata et al., 2003)
	↑CD38	(Tirumurugaan et al., 2007)
	↑GM-CSF	(Frevel et al., 2003; Winzen et al., 1999)
Nucleolin	↑Nucleolin regulated	(Turck et al., 2006; Yang et al., 2002)
	↑CTGF/CCN2	(Chowdhury and Chaqour, 2004)
BRF1	↑ARE transcripts	(Dean et al., 2004; Maitra et al., 2008)
	↑Myogenic transcripts	(Briata et al., 2005; Busse et al., 2008)
KSRP	↓KSRP regulated	(Winzen et al., 2007)
	↑Catalase	(Sen et al., 2005)
	↑Collagen-α	(Tsukada et al., 2005)
	↑TLR4/↑HuR	(Lin et al., 2006)
	↓Kv4.3	(Zhou et al., 2006)
	↑p75(NTR)	(Quann et al., 2007)
	↓MKK6	(Ambrosino et al., 2003)
	↑NFE2, ↑BCL2, ↑BCL-XL, ↑CA2	(Frevel et al., 2003)
	↑BCL-XL	(Bachelor and Bowden, 2004)
	↑TIMP1	(Cao et al., 2006)
	↓Endothelin-1	(Farhat et al., 2008)

Post-transcriptional regulation by p38 MAPK

Of the signaling pathways known to regulate mRNA stability, the p38 MAP kinase pathway is the most involved or implicated in this form of gene expression control (Dean et al., 2004; Khabar, 2005; Nagy, 2004; Saklatvala et al., 2003; Winzen et al., 2004). p38 MAPK activators include stress stimuli such as heat shock, osmotic stress, UV light, cytokines, or bacterial polysaccharides (e.g. LPS) (Dean et al., 2004). The traditional view of how p38 MAPK effects changes in gene expression is by mobilizing transcription factors to enter the nucleus to alter rates of transcription. It is becoming clear that p38 MAPK, largely by acting through one of its proximal downstream kinases, MAPKAP2, can also regulate gene expression at the level of mRNA stability (Ehlting et al., 2007; Kotlyarov and Gaestel, 2002; Winzen et al., 1999). Table 1-3 summarizes the most prominent p38 MAPK SMART factor / mRNA pairings.

The p38 MAPK signaling controls the stability of numerous transcripts, many of which contain ARE sequences and therefore basally unstable (Dean et al., 2004). Some p38 MAPK mRNA-stability regulated gene classes include cytokines, such as interleukins (Quante et al., 2008; Stoecklin et al., 2001; Winzen et al., 1999) and chemokines (Dhillon et al., 2007), transcription factors, such as c-fos (Winzen et al., 2004; Winzen et al., 1999) and E47 (Frasca et al., 2007). p38 MAPK signaling also has a significant influence on the stability of mRNA transcripts that code for the mRNA binding SMART factors themselves, such as increases or decreases in TTP transcript levels (Frasca et al., 2007; Hitti et al., 2006; Tchen et al., 2004) and increases in Tis11B transcript levels (Busse et al., 2008). Subsequent targeting of mRNA transcripts by these SMART factors is a prime example of indirect p38 MAPK mRNA stability regulation.

For example, stabilization of TTP mRNA and increased abundance of cognate protein can lead to the destabilization of TTP SMART factor target mRNA transcripts (Tchen et al., 2004).

p38 MAPK controls mRNA stability through multiple SMART factors including MK2 (Han et al., 2002; Montero and Nagamine, 1999), Nramp1 (Lafuse et al., 2002), HuR (Song et al., 2005), PAI1BP (Norata et al., 2004), AUF1(Chen et al., 2006; Mifflin et al., 2004; Sirenko et al., 1997; Vasudevan and Peltz, 2001; Wang et al., 1999), TTP (Chen et al., 2006; Mifflin et al., 2004; Sirenko et al., 1997; Vasudevan and Peltz, 2001; Wang et al., 1999), BRF1 (Dean et al., 2004; Maitra et al., 2008), Nucleolin (Turck et al., 2006; Yang et al., 2002), and KRSP (Winzen et al., 2007). MK2 is an intermediate kinase that relays the mRNA stability control signal to downstream SMART factors, but other SMART factors interact directly with target mRNA transcripts to mediate p38 MAPK regulated mRNA stability control. Several mRNA-binding SMART factors, such as BRF1 (Dean et al., 2004; Maitra et al., 2008), TTP (Sun et al., 2007a), and AUF1 (Chen et al., 2006; Mifflin et al., 2004; Sirenko et al., 1997; Vasudevan and Peltz, 2001; Wang et al., 1999) are phosphorylated directly by either p38 MAPK or MK2, and so represent the best examples of direct p38 MAPK regulated mRNA stability control.

Direct p38 MAPK pathway mRNA stability control is typically exerted by SMART factors interacting with *cis*-acting regulatory elements. In one of the more mechanistically complete examples, the inflammatory cytokine TNF-α activates p38 MAPK, which activates MAPKAP2, leading to stabilization of the suppressor of cytokine signaling (SOCS3) mRNA via a 120-nt ARE *cis*-acting element (Ehlting et al., 2007). The destabilizing SMART factor TTP may also be involved. MK2 can phosphorylate

TTP at serines 52 and 178, allowing TTP to complex with 14-3-3 (Sun et al., 2007a). Since unsequestered TTP is associated with ARE-dependent mRNA degradation in stress granules, p38 MAPK may increasingly stabilize messages by reducing their association with TTP (Gringhuis et al., 2005). Whatever the proximate cause of p38 MAPK-mediated mRNA stability, the phosphorylation status of both TTP and 14-3-3 appears to be critically important.

p38 MAPK signaling may even control ARE-mediated mRNA decay (AMD) after a SMART factor has bound target transcripts and recruited decay enzymes. Recent evidence reveals that the ARE-binding decay factor BRF1 is phosphorylated by MK2 (Maitra et al., 2008). MK2 phosphorylation of BRF1 inhibits ARE-mediated decay, but the mechanism by which ARE-containing mRNA transcripts are stabilized by BRF1 phosphorylation remains to be clarified. Phospho-BRF1-bound mRNA transcripts could become part of a P-body (Parker and Sheth, 2007) where they may stored for some period of time prior to entering translation and degradation.

Oxidative stress-activated p38 MAPK, acting through its SMART factor HuR, regulates stabilization of the gGCSh mRNA (Song et al., 2005). As is the case for other instances involving HuR, cytoplasmic accumulation of HuR is the proximate apparent cause of increased mRNA stability. During monocyte adherence, a precursor to atherosclerotic plaque formation, inflammation and tissue repair, p38 MAPK signaling increases the stability of GROα and IL-1β mRNA transcripts (Wilson et al., 2003b). A related study demonstrated that addition of exogenous AUF1(hnRNP D) alters cytoskeletal arrangement, leading to unexpected IL-1β mRNA stabilization (Sirenko et al., 2002). Recent evidence indicates that the chaperone HSP27 binds to several ARE sequences and

cooperates in AUF1 ubiquitination / proteasomal destruction (Sinsimer et al., 2008). The extent to which destruction of destabilizing SMART factors is important indirectly to p38 MAPK-mediated stability control mechanism remains to be determined. Other ARE-containing mRNA transcripts are similarly affected, but the possible role of proteasomal degradation of AUF1/hnRNP D in these responses has not been tested (Alford et al., 2007; Lasa et al., 2000).

p38 MAPK can also indirectly control mRNA stability of target genes by changing levels of SMART factors. For instance, p38 MAPK can increase the level of p37 AUF1 protein, coincident with decreased stability of the mRNA encoding the PP2A regulatory subunit B56α (Glaser et al., 2006). Destabilization of B56α transcripts has the effect of tuning down the level and activity PP2A phosphatase, which can lead to prolonged activation of pathways otherwise attenuated by dephosphorylation of activated signaling molecules.

Table 1-4. ERK 1/2 regulated mRNA transcripts and candidate SMART effectors.

Putative SMART *Trans*-Factor	**Target mRNA**	**Reference**
Nucleolin, hnRNP C	↑↓APP, ↑Nucleolin	(Westmark and Malter, 2001)
	↑iNOS	(Bergeron and Olivier, 2006)
DAZAP (ARE-binding)	↑ARE-transcripts	(Morton et al., 2006)
	↓Tropoelastin	(DiCamillo et al., 2006)
	↑Mmp2	(Zhao et al., 2004)
	↑MIP-2	(Numahata et al., 2003)
	↑Nramp1	(Lafuse et al., 2002)
	↑COX2	(Cao et al., 2007; Moon and Pestka, 2002; Tamura et al., 2002; Xu et al., 2000; Zhang et al., 2000)
TTP	↑TNFα	(Deleault et al., 2007)
	↑Type I Collagen	(Sato et al., 2004)
	↑StAR	(Zhao et al., 2005)
	↓p27	(Sakakibara et al., 2005)
HuR	↑p21	(Donadelli et al., 2006; Esposito et al., 1997; Yang et al., 2008; Yang et al., 2004a)
	↓Nav1.7 (indirect)	(Wada et al., 2004; Yanagita et al., 2003)
	↓Chemoattractants	(Pastore et al., 2005)
	↑βAR	(Headley et al., 2004)
	↑PAI-1	(Takeda et al., 2001)
	↑CFTR	(Baudouin-Legros et al., 2005)
hnRNP K, hnRNP E2/E1	↑Multiple CU-rich mRNA, ↑LDH	(Wang et al., 2006)
	↑MKP-1	(Casal et al., 2007)
hnRNP-E2	↑C/EBPα	(Chang et al., 2007)
	↑CD38	(Tirumurugaan et al., 2007)
	↑Endothelin-1	(Farhat et al., 2008)

Post-transcriptional regulation by ERK1/2

The extracellular regulated kinase (1/2) (ERK1/2) signaling pathway is activated by 2 main receptor groups: $G_{\alpha q}$-coupled receptors and growth factor receptors. In the case of the former, the signal proceeds by mobilization of Ca^{2+} and DAG to activate PKC. This activation then leads to cross activation of ERK1/2. For receptor tyrosine kinase receptor, the signal generally proceeds from receptor, to a Grb family member, to SOS, to Ras, to Raf, and finally to ERK1/2. Recent findings indicate that ERK1/2 not only plays a role in regulating transcription, but also plays a significant role in regulating mRNA stability. Table 1-4 summarizes the most prominent ERK1/2-regulated transcripts and identifies SMART factors implicated as acting downstream of ERK1/2 activation.

The *cis*-acting regulatory elements are varied, ranging from unknown to usual suspects. Some ERK1/2-regulated transcripts contain ARE sequences, like TNFα (Deleault et al., 2007), p21 (Donadelli et al., 2006; Esposito et al., 1997; Yang et al., 2008; Yang et al., 2004a), and COX2 (Cao et al., 2007; Moon and Pestka, 2002; Tamura et al., 2002; Xu et al., 2000; Zhang et al., 2000). Other ERK1/2-regulated mRNA transcripts contain CU-rich stretches, such as that coding for the Amyloid Precursor Protein (APP) (Westmark and Malter, 2001). Though there are relatively few details available regarding how ERK controls mRNA stability, this kinase appears to do so via multiple SMART factors including HuR (Yang et al., 2004a), DAZAP1 (Morton et al., 2006), hnRNP K (Habelhah et al., 2001), TTP (Deleault et al., 2007), Sam68 (Paronetto et al., 2006), and PABP (Ma et al., 2006). However, as compared to the p38 MAPK/MK2 pathway, very few direct ERK1/2 mRNA-binding protein substrates have been identified.

There are several examples of transcripts clearly regulated by ERK, but for which no putative SMART factor has been identified. For instance, COX2 mRNA is regulated both transcriptionally and post-transcriptionally by a host of extracellular stimuli (Doller et al., 2008; Ramsay et al., 2003; Xu et al., 2007). Activation of P2Y receptors dramatically increases COX2 mRNA stability, and effect conferred in part through an ~130 nucleotide sequence within its distal 3'UTR, that specifically senses ERK1/2 relative to other MAP kinases (Xu et al., 2000). The SMART factor responsible remains to be identified.

Another activator of the ERK1/2 pathway is growth factors such as PDGF, which decreases the stability of p27 mRNA, though the corresponding SMART factor has yet to be identified (Sakakibara et al., 2005). G-protein coupled prostaglandin receptor activation can promote ERK1/2 activation, leading to p21 mRNA stabilization (Donadelli et al., 2006; Esposito et al., 1997; Yang et al., 2008; Yang et al., 2004a). This stability effect depends upon ARE sequences and HuR, but it is unclear precisely how ERK1/2 regulates p21 mRNA stability. In adrenal chromaffin cells, both ERK1/2 and PKCε destabilize the mRNA encoding $NA_V1.7$ sodium ion channel alpha and beta subunits (Wada et al., 2004; Yanagita et al., 2003).

There are several examples where the mechanistic details of ERK1/2 mediated mRNA stability are clearly understood, but the precise consequences for target mRNA transcripts remain elusive. For instance, the ARE-binding protein DAZAP1 is phosphorylated by ERK2 at two sites, T269 & T315, reducing the association of DAZAP1 with a binding partner DAZ (Morton et al., 2006). DAZAP1 can bind to the poly A binding protein PABP, and binds preferentially when it is not associated with

DAZ. This dual binding modality suggests that ERK2 phosphorylation of DAZAP1 may control mRNA stability by promoting DAZAP1/PABP/mRNA complex formation, yet this remains to be shown for any target mRNA.

ERK1/2 can indirectly regulate the stability of several ARE containing mRNA transcripts, like that encoding the amyloid precursor protein (APP), by promoting the exchange of a destabilizing factor, nucleolin, for a stabilizing factor, hnRNP C (Westmark and Malter, 2001). This stability control example appears to depend upon new nucleolin synthesis, and so represents an indirect ERK1/2 stabilization event. Importantly, the production of increased nucleolin protein appears to result from increased stabilization of its encoding mRNA. The kinetics of ERK1/2-mediated nucleolin transcript induction suggests one of the nucleocytoplasmic shuttling mRNA binding proteins may involved.

ERK1/2 can also influence the stability of non-ARE containing mRNA transcripts. For example, ERK1/2 promotes increased stability and represses the translation of CU-rich element-containing mRNA transcripts (Wang et al., 2006). One suspect CU-rich element interacting factor is hnRNP K whose nuclear to cytoplasmic translocation is favored by under conditions wherein ERK is activated. By stabilizing mRNA transcripts and repressing translation, activated hnRNP K may promote the storage of mRNA transcripts, as it appears to do in xenopus embryos (Iwasaki et al., 2008). Overall, ERK1/2 appears to positively regulate hnRNP K binding to target mRNA transcripts. Precisely how it does so remains to be elucidated. One clue may come from a study that demonstrates that epidermal growth factor (EGF) stabilizes gastrin mRNA in a manner that depends upon hnRNP K, PCBP1, and nucleolin (Lee et al., 2007). EGF

increases hnRNP K / PCBP1 / nucleolin interaction, increases gastrin mRNA-binding activity of nucleolin, and increases gastrin mRNA stability. Since activation of growth factor receptors can activate ERK1/2 via the RAS-RAF-MEK pathway, ERK1/2 phosphorylation may be responsible for the observed interactions. Studies are ongoing to reveal the precise molecular details of EGF-regulated gastrin mRNA stability control.

Typically, mRNA stability control effects due to ERK appear to be tightly coupled to other signaling pathways, and so the effects that are solely attributable to ERK remain largely uncharacterized. ERK may regulate ARE-containing transcripts both directly (e.g. via DAZAP1, (Morton et al., 2006)) and indirectly (e.g. via HuR / AUF1, (Annabi et al., 2006; Liu et al., 2006)), but a clear cut "transcription factor-like" phosphorylation/subcellular redistribution of a SMART factor to control mRNA stability has yet to be described. CU-rich element-containing transcripts are likely directly regulated by ERK, but the precise details of the SMART factors remain to be worked out. For instance, the role of hnRNP K in controlling mRNA stability is not completely clear, though serine / threonine phosphorylation (by ERK or other kinases) is related to increased hnRNP K association with its target mRNA transcripts. The most recent evidence indicates a strong role for hnRNP K in increasing gastrin mRNA stability, potentially by fostering the recruitment of nucleolin (Lee et al., 2007). Since ERK-induced nucleolin has previously been associated with mRNA destabilization (i.e. APP mRNA, WILL ADD REF), it is likely that signaling influences both SMART factor / mRNA interactions and SMART factor / SMART factor interactions.

Table 1-5. Src family kinase regulated mRNA transcripts and candidate SMART Effectors.

Putative SMART *Trans*-Factor	**Target mRNA(s)**	**Reference**
QKI	↓MBP	(Lu et al., 2005; Zhang et al., 2003)
Calreticulin	↓AT(1)R	(Mueller et al., 2008)
CUGBP2	↑COX2	(Xu et al., 2007)
AU binding proteins	↑Multiple IEG transcripts	(Bromann et al., 2005)
AUF1	↑Sphingosine Kinase 1	(Sobue et al., 2008)
	↑eNOS	(Davis et al., 2001b)

Post-transcriptional regulation by Src family kinases

Src family kinases are non-receptor tyrosine kinases that include Src, Abl, Fyn, Yes, Lck, Hck, Blk, Fgr, Lyn, and Yrk (Robinson et al., 2000). Several known activators of these kinases include protein tyrosine phosphatases (PTP), $G_{\alpha q}$-coupled GPCRs, T-cell antigen receptors, and receptor tyrosine kinases (RTK) (Bjorge et al., 2000). Some of these signaling pathways are sensitive to specific inhibitors providing useful tools to test whether Src family kinases influence mRNA stability control (Hernandez-Boluda and Cervantes, 2002). Table 1-5 summarizes prominent regulated mRNA transcripts.

As indicated in Table 1-5, there are fewer well-characterized examples of Src-family mRNA stability control regulated transcripts than there are for the other signaling pathways discussed in this chapter. Src family kinases appear to play a significant role in mRNA translation, acting via *trans*-factors such as hnRNP proteins (Ostareck-Lederer et al., 2002; White et al., 2008), but their role in controlling mRNA stability is less well understood. Src phosphorylation of SMART factors appears to reduce their mRNA binding affinity (Ostrowski et al., 2000). It logically follows from this general observation that Src activity can promote mRNA stability by reducing the binding of destabilizing SMART factors. This may be the case for Src mediated stabilization of ARE-containing transcripts, like that coding for sphingosine kinase 1 (SPHK1) (Sobue et al., 2008), as well as for a host of IEG transcripts induced by PDGF/Src signaling (Bromann et al., 2005). When active Src is present, the SMART factor AUF1 is tyrosine phosphorylated and less associated with SPHK1. At the same time, the SMART factor HuR is serine/threonine phosphorylated, and as previously mentioned, phospho-HuR accumulates in the cytoplasm where it stabilizes certain ARE-containing mRNA

transcripts. This type of stability regulation has good potential to represent direct mRNA stability control by Src kinases. Figure 1-4 presents a possible model. Finally, Src may directly affect mRNA decay via the endonuclease PMR1 (Peng et al., 2008; Peng and Schoenberg, 2007). Specific target mRNA transcripts have yet to be identified, but when PMR1 is tyrosine phosphorylated, it associates with polysomes and is associated with accelerated mRNA decay.

Table 1-6. SMART Factor Hall of Fame.

SMART Factor	Pathway	mRNA Stability Effect (⬆ or ⬇)	Reference
AU-Rich Factor 1 Instability factor Ubiquitous	c-Src	⬆	(Sirenko et al., 1997; Sobue et al., 2008)
	ALK	⬆	(Fawal et al., 2006)
	p38 MAPK	⬆	(Sirenko et al., 1997)
	PKA	⬇	(Dhakras et al., 2006)
	GSKβ	⬆⬇	(Tolnay et al., 2002)
Polysomal Ribonuclease 1 Instability factor	c-Src	⬇	(Yang and Schoenberg, 2004)
Tristetraprolin Instability factor Stress granules	p42 MAPK	⬆	(Deleault et al., 2008)
	MK2, 14-3-3	⬆	(Chrestensen et al., 2004)
	PP2A	⬇	(Sun et al., 2007b)
KH-Signal Regulated Instability factor	Wnt	⬆	(Briata et al., 2003)
	p38 MAPK	⬆	(Fechir et al., 2005; Linker et al., 2005)
	AKT	⬆	(Gherzi et al., 2006; Ruggiero et al., 2007)
HuR Stability factor Ubiquitous	p38 MAPK	⬆	(Jin et al., 2007; Subramaniam et al., 2008; Wang et al., 2008)
	PKC	⬆	(Doller et al., 2008)
	T-cell antigen	⬆	(Yarovinsky et al., 2006)
HuD Stability factor Neuronal expression	PKCα	⬆	(Pascale et al., 2005b)
	Heatshock	⬆	(Burry and Smith, 2006)
	ATP	⬆	(Chen et al., 2007)

SMART Factor	Pathway	mRNA Stability Effect (⬆ or ⬇)	Reference
CU-rich Binding Protein 2 Stability factor	PDGFR, Src	⬆	(Xu et al., 2007)
	Gamma Irradiation	⬆	(Mukhopadhyay et al., 2003a)
Calreticulin Instability factor	PKC	n/a	(Rendon-Huerta et al., 1999)
	High glucose	⬇	(Totary-Jain et al., 2005)
	AT1-R	⬇	(Mueller et al., 2008)

Perspectives

Signaling pathways influence Immediate Early Gene mRNA levels by both transcription and mRNA stability control mechanisms. The historically more rapid identification and characterization of regulated transcription mediators led to an initial disproportionate view that cells rely exclusively upon transcription to alter steady-state mRNA transcript levels, and that cells degrade mRNA transcripts simply as a house keeping function. Better understanding and better experimental tools have provided strong evidence that post-transcriptional mechanisms, regulated by signaling, play an important role in determining steady-state mRNA levels. Like regulated transcription, regulated mRNA stability appears to be controlled by the action of *trans*-acting factors interacting with *cis*-acting regulatory elements. Unlike regulated transcription, control of mRNA stability is likely to involve multiple processes, such as export, localization, and translation. We present the descriptive phrase "Signal Modulated Altered mRNA Turnover" (SMART) factor to describe any *trans*-acting factor that is deployed by cellular signaling pathways to effect changes in mRNA metabolism, operating at a post-transcriptional level.

Overall, SMART factors can influence the stability of an mRNA transcript at many points along the journey from site of transcription to eventual site of decay in the cytoplasm. This "non-discrete" nature of mRNA metabolism has provided a significant experimental challenge to understanding the mechanistic details of regulated mRNA stability. To date, evidence strongly indicates that *cis*-elements are necessary for signal mediated changes in mRNA stability. Some *cis*-elements are discrete, and can be moved to heterologous constructs to confer sensitivity to specific signaling pathways. Other *cis*-

elements are not so easily dissected away from the mRNA sequences that they regulate, perhaps predictive of a need to co-assemble with several co-factors to achieve biological effects. In either case, signaling pathway deployed SMART *trans*-factors interact with *cis*-elements to control transcript abundance at the level of mRNA metabolism. Future experiments will focus upon determining the mechanistic details of how these *cis* / *trans* interactions influence specific mRNA (metabolism?) processes. Looking forward, mRNA stability is just one indicator showing that regulatory processes are engaged by signaling. Better assays for localization, transport, processing, etc. are likely to uncover additional regulatory complexity.

Appendix

Table 1-7. Pharmacological Kinase Inhibitors.

Kinase	Pharmacological or Genetic Inhibitor
PKA	H-89, HA-1004, H-7, H-8, HA-100, PKI, staurosporine
PKC	Bisindolylmaleimide IX, edelfosina, ET18OCH3, H-7, HA-100, H89, HA-1004, Ro 31-8220, rottlerin, staurosporine, quercetin
ABL	PP1, PP2
Akt/PKB	Akt1/2 Inhibitor, 10-DEBC hydrochloride, Triciribine (TCN, NSC-154020, API-2)
AMPK	Indirubin-3'-oxime, 5-Iodotubercidin, H-89
BCR-ABL	Adaphostin, Tyrphostin AG 957, Gleevec
EGF-R kinase	Erbstatin analog, gefitinib, PD 153035, Tyrphostin AG 490, Tyrphostin AG 825, PP1, PP2
ERK kinase	PD 98059, SL327, olomoucine, 5-Iodotubercidin
FYN	PP1, PP2, SU6656
GSK3	Aloisine, aloisine A, indirubin-3'-oxime, kenpaullone
JNK	Aloisine A, $SP_{600}125$
MAPK	SB202190, SB203580
MAPKK	Arctigenin, PD 98059, SL327, U0126
MEK	PD 98059, SL327, U0126
MKK	Arctigenin, PD 98059, SL327, U0126
P38 MAPK	SB202190, SB203580
PDGF-R kinase	DMPQ
PI3K	LY294002, wortmannin, quercetin
RAF-1	GW 5074
RHO kinase	HA-1077, hydroxyfasudil

Chapter 2: Evaluation of methods used to study interactions between mRNA and proteins

Using Yeast 3 Hybrid to Identify SMART trans-Factors for the MEK-Sensitive cis-Acting Regulatory Element

Abstract

Higher eukaryotes use *cis*-acting mRNA stability control elements and *trans*-acting SMART factors as part of a strategy to control gene expression in response to extracellular signaling. For several IEG transcripts, regulated mRNA stabilization is coordinated with increased rates of transcription to elevate steady-state mRNA levels. We have recently characterized a novel MEK-sensitive *cis*-acting mRNA stability control element located within the distal 3'UTR of the COX2 transcript. To date, neither we nor others have identified a binding partner for this MEK element, one that might explain its regulatory characteristics. The MEK element sequence is highly conserved and appears to be restricted to mammalian lineages, suggesting conservation of function. We also applied in silico MFold analysis to see what types of secondary structures the element might form. On the basis of these analyses, in addition to functional binding and experimental decay data, we hypothesized that the MEK element might operate by interacting with specific SMART factors. To search for these, we placed the element into a yeast 3 hybrid bait vector and screened a murine E11 fibroblast library. To assess the mRNA-dependence and binding specificity of candidate SMART factors arising from this screen, we developed and executed a stringent battery of tests for RNA specificity and dependence. As read out by these assays, a positive control interaction comprised of IRE and IRE-BP produced up to 40 fold more specific reporter activity than did the interaction of any candidate protein with the MEK element bait mRNA. Unfortunately, no candidate SMART factors initially isolated in the 3 hybrid screen yielded a specificity ratio comparable to that produced by the IRE/IRE-BP interaction. In a final effort, activation of the yeast mating factor signaling pathway, which is analogous to the

mammalian MEK signaling pathway, did not have a significant effect on interactions between the MEK element and a selected group of clones identified through the screen.

Introduction

Like multicellular organisms, *S. cerevisiae* appears capable of regulating gene expression at the level of both transcription and mRNA stability, since mammalian gene-derived AU-rich element containing (ARE) mRNA transcripts are stabilized downstream of p38 MAPK / HOG1 stress pathway activation (Vasudevan and Peltz, 2001). This pathway is highly evolutionarily conserved and appears to control mRNA stability largely by causing stabilizing SMART factors to move from the nucleus to the cytoplasm (Dean et al., 2004; Doller et al., 2008; Ma et al., 2007). Our recent experiments provide evidence that another *S. cerevisiae* signaling pathway, the GPCR-controlled mating cascade, does not appear to exploit regulated mRNA stability as a means to control gene expression (Kitchen et al., 2009a). However, several higher eukaryotes are known to exploit a homologous signaling pathway (ERK1/2 MAPK) to regulate mRNA stability via SMART *trans*-factors and *cis*-acting mRNA regulatory elements (Moon and Pestka, 2002; Tamura et al., 2002; Westmark and Malter, 2001; Xu et al., 2000).

We previously identified a 134nt novel *cis*-acting regulatory element within the COX2 3'UTR (Xu et al., 2000). This element functions to destabilize the COX2 mRNA under conditions where the p42/p44 MAPK pathway is not active. However, when the p42/p44 MAPK pathway is activated, this discrete *cis*-acting mRNA regulatory element mRNA contributes to COX2 mRNA stabilization. This element confers this regulatory property when placed in heterologous reporter transcripts. RASM cell nuclear extracts contain several unidentified proteins that crosslink to this element. The simplest

hypothesis to explain these functional data is that this element can be occupied by some SMART factor, which can be regulated by the p42/p44 signaling pathway, leading to conditional stabilization of the COX2 mRNA.

Since mRNA *cis*-acting regulatory elements tend to be longer and more dependent upon their ability to form specific secondary structures, we used Mfold to examine possible structure of this 134 nt element (Khaladkar et al., 2008; Pesole et al., 2001; Zuker, 2003). We were encouraged to see that similar IEG transcripts, Mkp1 and c-fos, with similar regulatory properties had sequences that formed Mfold like structures that were similar to that formed by the MEK element (Figure 2-1) (Farhana et al., 2000; Kuwano et al., 2008; Xu et al., 2000). Hypothetically, this common cruciform structure may bind to a factor that is operates to enhance mRNA stability in response to MEK signaling pathway activation. Furthermore, the distal 3'UTR of the COX2 mRNA is highly conserved among mammals (Figure 2-2). Cross-species conservation suggests that the MEK element may be an invention of mammals, similar to how the NFAT transcription factor appears to be an invention of vertebrates (Wu et al., 2007). The platypus is the only non-mammalian species to show significant sequence identity in this region, perhaps reflecting its unique assortment of mammalian and reptilian features.

To find a possible interacting partner for the MEK regulatory element, we chose to exploit the *S. cerevisiae* 3 Hybrid Assay (Hook et al., 2005). This works in much the same way as a yeast 2 hybrid screen, except that a third hybrid, the RNA bait construct, is added to the assay design. One significant caveat to taking this approach is that adding a third hybrid can increase the number of false positives, as compared to those obtained during a traditional 2 hybrid screen. Even so, yeast 3 hybrid has been used successfully to

identify new binding partners for existing RNA elements (Hook et al., 2005; Jaeger et al., 2004; Riley et al., 2006), new RNA elements for existing proteins (Seay et al., 2006), and even new RNA partners for existing RNA elements (Piganeau et al., 2006).

Taken together, these observation suggested that it was reasonable to test the hypothesis that the MEK element may regulate COX2 mRNA stability by interacting with MEK-dependent SMART factor(s) and that a 3-hybrid screen would be a suitable and potentially powerful discovery vehicle for this objective.

Figure 2-1. MFold predicted secondary structures for the distal-most 3'UTR sequence of three unstable immediate early gene mRNAs. The consensus distal COX2 3'UTR MEK element, the MKP1 distal 3'UTR, and the c-fos distal 3'UTR sequences were submitted to MFold RNA secondary structure analysis. The most thermally stable structures are presented below.

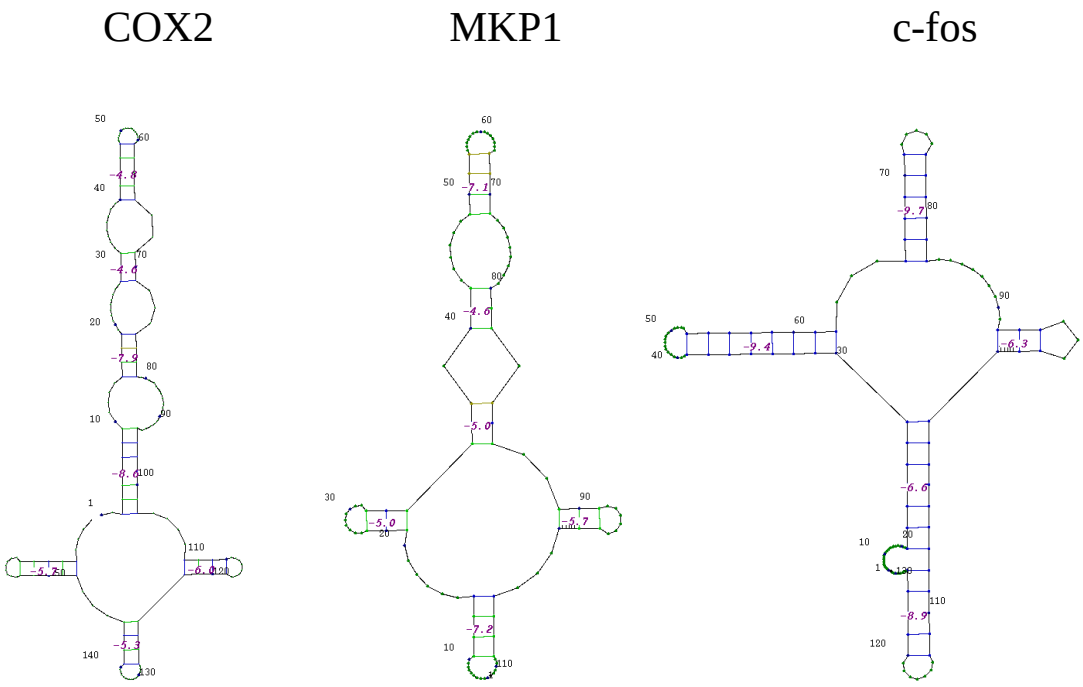
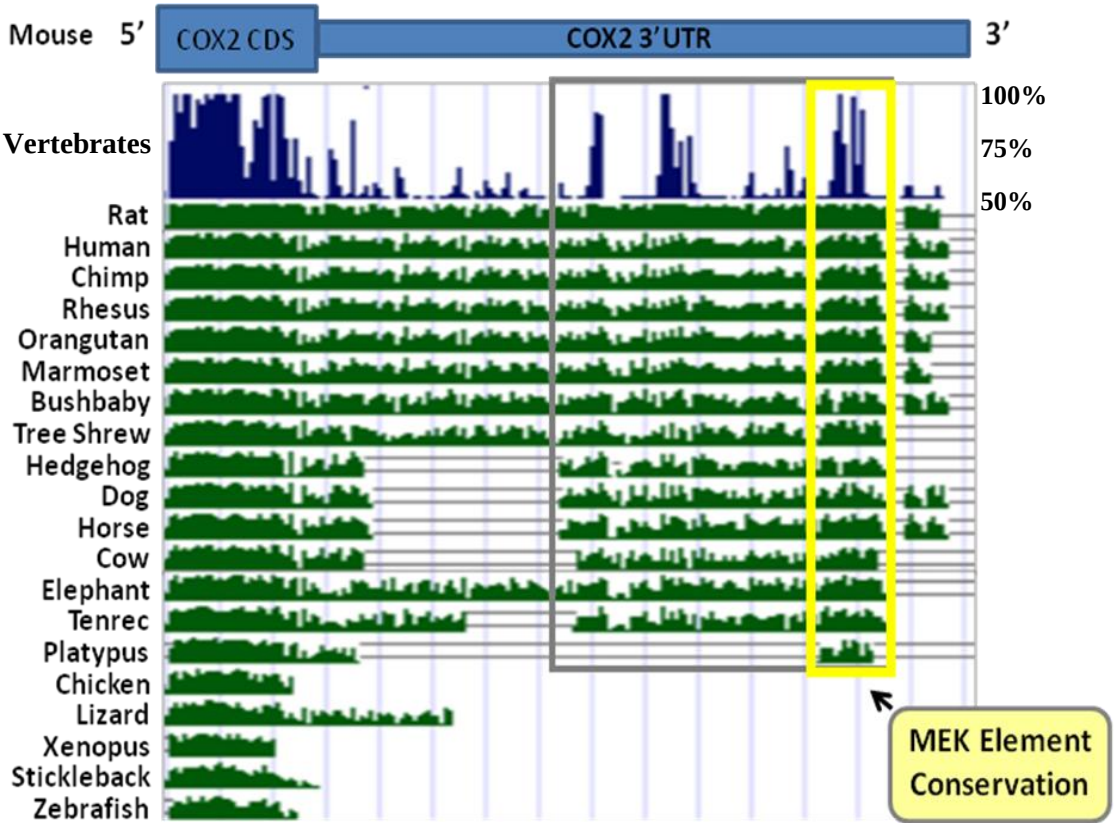


Figure 2-2. MultiZ multiple species alignment of the COX2 (PTGS2) exon, intron, and proximal intergenic DNA sequence. The UCSC genome browser was used locate the Mouse COX2 genomic sequence. The taller rectangle indicates the final COX2 mRNA exon 6 coding sequence and the shorter rectangle indicates COX2 mRNA 3'UTR sequence. The overall vertebrate sequence homology is indicated by the height of the blue vertical trace lines. The sequence homology between the Mouse and each species is indicated by the height of the green vertical trace lines. The grey rectangle highlights regions that contain significant vertebrate homology. The heavy yellow rectangle highlights the MEK element sequence conservation across multiple species.



Results

Design of the system

In order to directly test the hypothesis that a protein SMART factor can bind to the MEK mRNA stability control element the enhanced yeast 3 hybrid system was used to screen for proteins that bind to it (Hook et al., 2005). The key component of the system is the YBZ1 yeast 3 hybrid reporter strain, which has HIS3 and β-galactosidase as LexA-driven reporters and a genomically-encoded LexA DNA binding domain (DBD) fused to the MS2 coat protein. Vectors used include a hybrid RNA bait plasmid comprised of the MS2 stem loop fused to the MEK element, and a Gal4 activation domain (AD) prey plasmid. As depicted in Figure 2-3, the DBD-MS2 coat fusion protein binds to the MS2-stem loop allowing the MEK element RNA to be presented as bait for a potential interacting SMART factor. If a SMART factor interacts with the MEK element, the two halves of the transcription factor are brought in close proximity to activate transcription of the reporter genes HIS3 and β-galactosidase. The plasmids used are shown in Figure 2-4. The MEK element was cloned behind the dyad repeat of the MS2 stem loop binding element (MBE). Several control vectors were also produced, including a reversed MEK element (pCMK71) and a negative control vector that produces no hybrid RNA.

YBZ1 bait cells express the MS2-MEK hybrid mRNA

To ensure that the bait mRNA was being expressed in the YBZ1 cells, cells transformed with the plasmids were grown to mid-log then isolated the total RNA as previously described. As a control, total RNA was isolated from YBZ1 cells transformed with the IRE bait plasmid, or no bait plasmid at all. The levels of MEK-MS2 mRNA

were detectable and determined to be about one-third of the levels of the IRE-MS2 mRNA and deemed sufficient to proceed with the screen (data not shown).

Yeast 3 hybrid library screening produces 500 colonies

6 X 10^8 clones from an E11 mouse library were screened by mating YBZ1 cells transformed with the bait RNA plasmid to Y187 library cells. After 8 days growing on QDO plates at 30 °C, 500 bright white colonies (>2mm in diameter) had been produced. These were transferred to 96-well format and subjected to additional analyses.

To determine which of the 3 hybrid interactions was dependent upon the MEK element, gene reporter activity was measured both before and after the RNA bait plasmid was removed from the original positive clones using 5FOA curing. According to Figure 2-5C, the growth of approximately 1 in 3 initial positive 3 hybrid clones is dependent upon the presence of the MEK element mRNA bait. Of these, PCR analysis and restriction enzyme mapping showed that approximately 100 represented unique clones, which were sequenced (data not shown). The remaining clones appear to be capable of activating the reporter genes independently of the presence of the MEK mRNA, and thus are considered RNA-independent.

Figure 2-3. Schematic representation of the yeast 3 hybrid interaction. YBZ1 cells genomically encode a LexA-DBD:MS2 Coat Fusion protein and two reporter genes. The fusion protein binds to LexA operators upstream of the HIS3 and β-Galactosidase reporter genes via its DNA-binding domain. It also binds to the MS2 Stem Loop RNA structure via its MS2 Coat protein domain. A bait plasmid is prepared such that it expresses the MS2 Stem Loop RNA fused to the bait RNA, in this case, the MEK mRNA stability control element. This plasmid is transformed into the YBZ1 cells in preparation for mating to the library cells. The Y187 cells are pre-transformed with a library of cDNA:Gal4 Activation Domain fusion constructs, so that when they are mated to the RNA bait-transformed YBZ1 cells, the MEK element is presented to the library proteins for possible interaction. If the MEK element interacts with a fusion protein encoded by a library clone, the HIS3 and β-Galactosidase reporter genes are activated.

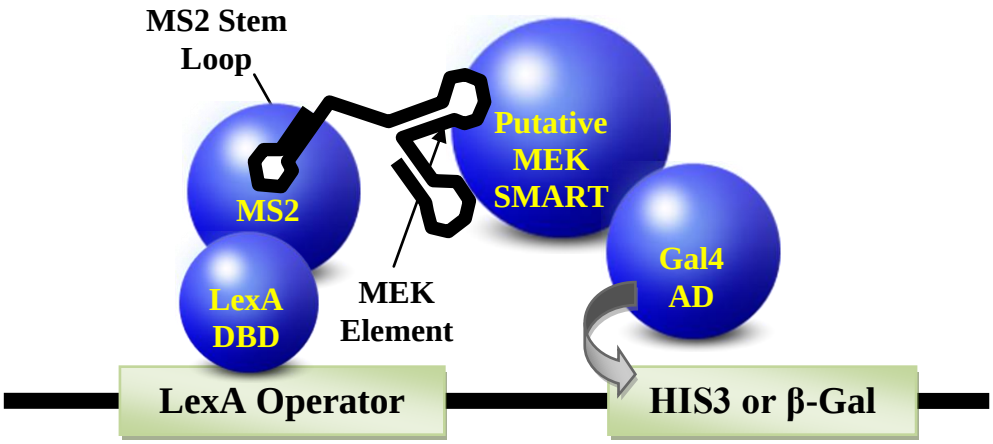
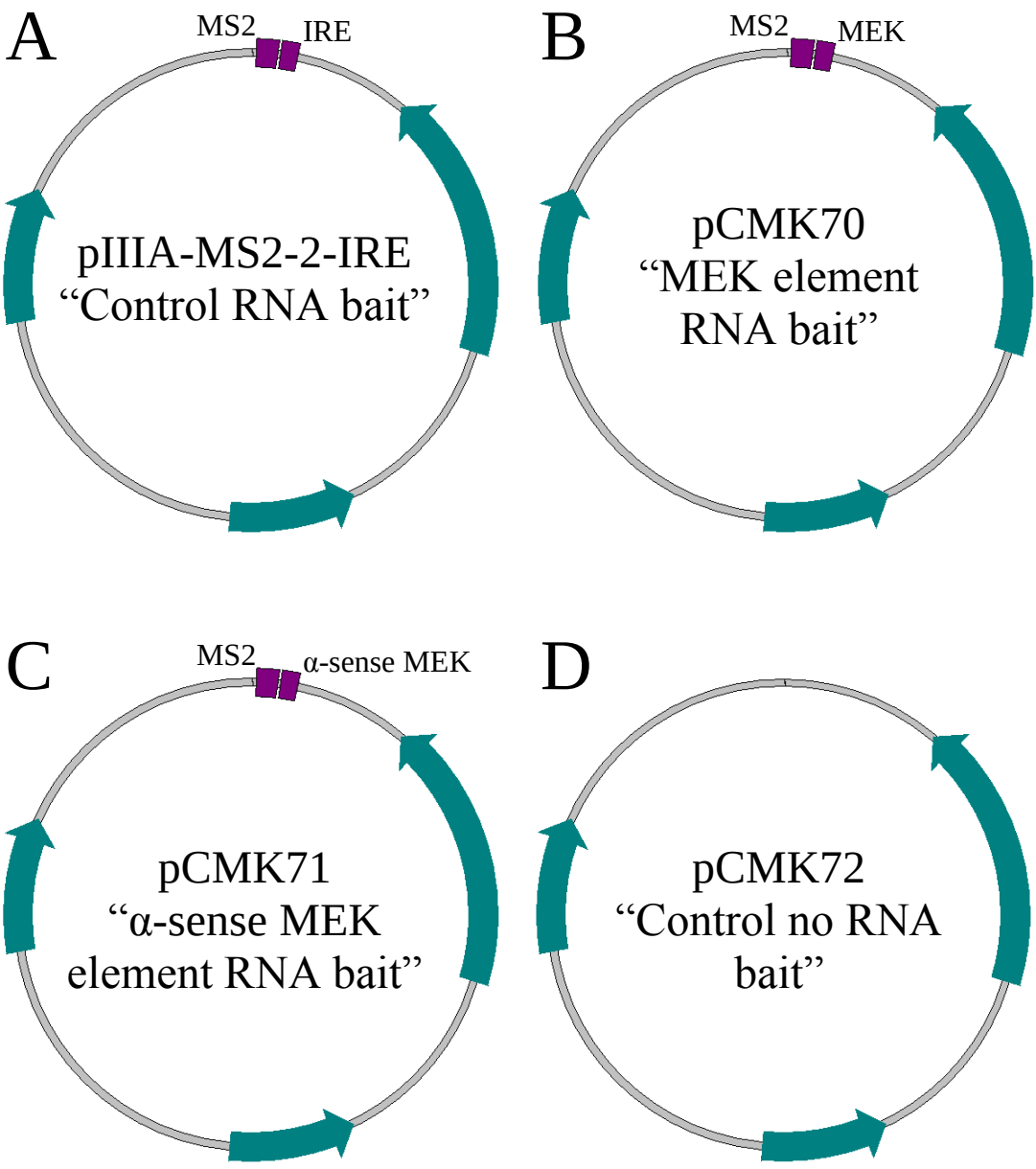


Figure 2-4. Maps for the RNA Bait / Bridge plasmids and the Pol III-null RNA dependence test plasmid. The MS2-2 orientation provides for RNA Polymerase III first transcribing the bait RNA element and then the tandem MS2 stem repeat. A) Control RNA bait plasmid, pIIIA-MS2-2-IRE, B) library screening MEK element bait plasmid, pCK70, C) specificity control antisense MEK element bait plasmid, pCK71, and D) empty control RNA bait plasmid, pCK72.



Several RNA-dependent clones have been linked to signaling

The identity of the RNA-dependent clones were determined by sequencing using a gene-specific primer following rescue of the plasmids from the yeast colonies. Table 2-1 summarizes the longest ORFs isolated during the 3 hybrid screen. Both GRB10 and GRB14 are known to link insulin receptor signaling to changes in gene expression (Holt and Siddle, 2005; Langlais et al., 2005; Nantel et al., 1998), and HSP90 has been implicated in stabilizing signaling proteins, including transcription factors, mRNA localization factors, kinases and phosphatases (Katschinski et al., 2004; Piper et al., 2006; Song et al., 2007; Truman et al., 2006).

Specificity evaluation experiments

The RNA dependence of reporter activity does not necessarily indicate a specific interaction between a candidate clone and the RNA bait. It is formally possible that strong reporter activity can result from an RNA-dependent, yet non-specific interaction. To test this, the specificity of reporter activity was determined for each protein by comparing that with the MEK-element to that with an antisense of the MEK-element, or an irrelevant control element, the IRE. The interaction between IRP and the IRE was used as a positive control in this specificity screen. The data in Table 2-2 show that IRP activity was 20-fold higher in cells expressing the IRE-MS2 hybrid than in either those expressing the MEK or the antisense MEK-MS2 hybrids indicating specificity between the IRP and the IRE. In contrast, none of the RNA-dependent proteins derived from the library screen using the MEK-element bait mRNA showed a similar degree of selectively for the MEK-element compared to either the antisense MEK-element or the IRE-MS2 hybrids.

Figure 2-5. Selected Beta Glo Values for Original and 5FOA-Cured Library Clones. Beta Glo values were measured for the original 3 hybrid positive colonies both before (A) and after (B) 5FOA curing of the RNA bait plasmid. Dividing the relative Beta Glo values in original colonies, (A) by cured colonies, (B) yields RNA dependence ratios, (C). The higher dependence ratios (**bold**) represent clones that depend upon the presence of the MEK-containing hybrid RNA transcripts to activate reporter gene expression.

A

Positive Clones	1	2	3	4	5	6	7	8	9	10	11	12
A	7051	12691	45	17600	7533	10695	33	5348	3792	15865	9412	17427
B	10946	9161	14825	6463	4883	7665	18934	8625	16874	10865	20976	7370
C	10517	12063	11737	18944	15638	14556	17830	23331	10899	12867	15593	15889
D	13335	11445	11079	30904	11178	24254	18314	68	9840	16939	13584	12983
E	35159	17579	16147	40469	12492	47	10056	17545	32857	143	24218	13967
F	13881	13134	5923	17503	25349	52	11852	14401	16712	8128	13996	7809
G	20534	10391	14170	11951	11126	15960	14738	15841	10144	15288	14142	12591
H	14123	17852	29537	49	30288	23923	8290	18210	39359	27188	15857	9441

B

5FOA Cured	1	2	3	4	5	6	7	8	9	10	11	12
A	8844	11261	20	9354	13321	6943	11116	8074	580	11294	9653	11915
B	12232	9971	13390	8655	22	14216	34	9817	7314	8567	22	13275
C	8906	93	15	30	10882	9067	22	22	16	8476	24	2830
D	11121	31	29	11763	23	5585	9766	7052	13626	7442	7879	6012
E	15	18	9982	11418	4252	8401	8573	7928	10294	23	8625	25
F	9185	6469	35	8304	10125	23	5805	10322	3634	27	14401	13503
G	6040	10391	7709	11565	12670	27	13955	15469	26	22	21	29
H	38	18	28	20	16132	13011	2861	8749	12735	11763	20846	19

Figure 2-5 continued. Values in cells are arbitrary β-glo activity units. Higher values (**bold**) represent RNA-dependent clones.

C

RNA Depend	1	2	3	4	5	6	7	8	9	10	11	12
A	1	1	2	2	1	2	0	1	7	1	1	1
B	1	1	1	1	**223**	1	**550**	1	2	1	**959**	1
C	1	**130**	**807**	**623**	1	2	**819**	**1048**	**663**	2	**638**	6
D	1	**374**	**382**	3	**487**	4	2	0	1	2	2	2
E	**2402**	**952**	2	4	3	0	1	2	3	6	3	**559**
F	2	2	**169**	2	3	2	2	1	5	**299**	1	1
G	3	1	2	1	1	**598**	1	1	**389**	**703**	**670**	**431**
H	**374**	**972**	**1059**	2	2	2	3	2	3	2	1	**485**

Table 2-1. Sequence Identity for Largest Insert Library Clones.

Clone	Clone Identity	ORF Length	Accession Number
A2E	RPL8	269	NM_012053
A7G	Electron Transferring Flavoprotein	253	NM_026695
B2G	Grb10	225	NM_001001555
F2-5D	Grb14	192	NM_004490
F2-9C	G10 Peptide	193	NM_007916
F1-12G	BUD31	189	NM_001008705
F1-12H	HSP90B1	97	NM_011631
A9F	RPL29	88	NM_000992
F1-3B	Fibronectin 1	72	NM_212475
B12A	RPL31	61	NM_053257
A7D	Cytochrome C Oxidase-II	60	AK131586
A6E	RPL41	49	DU636045

Table 2-2. Selected specificity battery assay results. YBZ1 cells were first transformed with MEK element, anti-sense MEK element, no RNA control, or IRE control to produce the battery assay cells. Next, the indicated library clones were transformed into each of the battery cells, to produce doubly transformed YBZ1 cells. The cells were grown to mid-log and their β-Glo activity was assessed as before. All β-Glo values were corrected for cell number and then normalized to the control interaction between IRP and IRE which was set to equal 100%. Values in the cells are the average β-galactosidase activity in arbitrary light units as determined by luminometry. Blank = untransformed YBZ1 cells.

Protein ID	IRE control element	MEK element RNA bait	antisense MEK	No RNA control
IRP	100 ±4	4 ±5	5 ±1	7 ±3
GRB10	594 ±99	541 ±200	105 ±68	79 ±80
GRB14	2784 ±89	3263 ±224	2275 ±68	576 ±54
HSP90	266 ±62	267 ±132	338 ±16	18 ±9
BUD31	469 ±51	528 ±142	812 ±83	916 ±442
Blank	7 ±2	2 ±1	3 ±2	6 ±4

Surprisingly, these clones gave very strong absolute values of reporter activity relative to the positive control IRP/IRE interaction, were RNA-dependent, but this RNA-dependency is non-specific.

Mating factor does not influence the specificity of the interaction between GRB10 and the MEK-element

Analysis of the primary amino acid sequence for library clone GRB10 showed that there is a possible ERK1/2 phosphoacceptor site. Postulating that GRB10 may be a substrate for Fus3, the *S.cerevisiae* ERK1/2 homolog, we tested whether mating factor pathway activation could modulate the specificity of GRB10 for the MEK element. The data in Figure 2-6 show, however, that up to 2.5 hours of alpha-factor stimulation fails to modulate reporter activity in cells expressing both GRB10 and the MEK element MS2 hybrid.

Figure 2-6. Specificity Battery Assay with Mating Factor Stimulation. YBZ1 battery assay cells were sequentially transformed with empty vector, IRP, or GRB10. Cells were grown to early log phase, then stimulated with either water or 50 nM α mating factor. Beta Glo assay was performed at 0, 1.5 hours, and 2.5 hours after stimulation. All Beta Glo values were corrected for cell number and then normalized to the non-alpha factor stimulated control IRP / IRE interaction (set to equal 100%). Values in cells are arbitrary β-glo activity units.

0 Hours	Vector	IRP	GRB10	pACTII+α	IRP+α	GRB10+α
MEK-MS2	0	1	**300**	2	1	**331**
IRE-MS2	2	**100**	**167**	3	**118**	**155**

1.5 Hours	Vector	IRP	GRB10	pACTII+α	IRP+α	GRB10+α
MEK-MS2	0	1	**280**	1	1	**263**
IRE-MS2	2	**100**	**206**	2	**86**	**207**

2.5 Hours	Vector	IRP	GRB10	pACTII+α	IRP+α	GRB10+α
MEK-MS2	1	1	**407**	1	1	**381**
IRE-MS2	2	**100.0**	**238.4**	2	**70**	**257.1**

Discussion

Several signal transduction-regulated *cis*-acting mRNA stability control elements have been identified in the COX2 mRNA 3'UTR (Dixon et al., 2001; Faour et al., 2003; Lasa et al., 2000). In response to environmental cues, SMART factors such as CUGBP2 (Sureban et al., 2007; Xu et al., 2007), HuR (Doller et al., 2008), and TTP (Lin et al., 2008) interact with these *cis*-acting elements to influence COX2 mRNA stability, localization, and translation. For several reasons, a 134nt MEK-sensitive element that is located at the extreme distal 3'UTR was a very attractive candidate to deploy in the 3 hybrid assay to find interacting proteins. First, the element is of a typical size for well-behaved *cis*-acting mRNA regulatory elements, which tend to be larger than DNA regulatory elements. Ultraviolet crosslinking studies show this element specifically interacts with proteins that appear enriched in nuclear fractions over cytosolic fractions. Furthermore the element functions *in trans* in that it can confer regulatory characteristics when placed upon heterologous mRNA sequences (Xu et al., 2000). Additionally the MEK-element is highly conserved in the COX2 mRNA of multiple mammalian species, often unusual for untranslated regions, suggesting conservation of function. Finally, computer-assisted secondary structure suggests that the MEK-element is capable of forming secondary structures which could be recognized by mRNA-binding proteins.

To search for a protein SMART factor that can interact with the MEK mRNA stability control element, we exploited the enhanced yeast 3 hybrid system. We used the MEK element as bait RNA to screen a murine E11 fibroblast cDNA library for candidate SMART interacting partners. Typical of yeast hybrid screens, there are many false

positive interactions. Out of 500 possible interactors, only one-third appeared to be RNA-dependent based upon the ratio of reporter gene expression levels in original clones to that in 5FOA-cured cells (Figure 2-9). Of the RNA-dependent clones, approximately 10% produced proteins greater than 50 amino acids in length. Oddly, the interactors that tended to produce the highest levels of reporter activity were very short arginine and lysine-rich peptides. Strong interactions between arginine-rich peptides and RNA have several precedents in the literature, including one group that demonstrated subnanomolar affinity between a phage arginine-rich motif (ARM) protein and its RNA interacting partner (Austin et al., 2002).

Interestingly, several ribosomal proteins were pulled out of this 3 hybrid screen. Though many of these interactions appeared to depend upon RNA, none of the ribosomal protein interacted specificity with the MEK element versus control bait RNAs. These results may not be surprising, considering that ribosomal proteins must interact with a variety of RNA species. For one of the proteins, GRB10, we chose to test whether activation of the yeast mating pathway might influence its specificity of interaction with the MEK element. We cannot rule out the possibility that the protein was not modulated by the kinase or that some required element may not be present in *S. cerevisiae*.

The robustness of the reporter activity does not predict specificity. In this yeast 3 hybrid screen, all RNA-dependent library clones interacted non-specifically with the MEK-element, even though some had orders of magnitude greater reporter activity than the IRP IRE positive control, a well-known authentic protein RNA interacting pair. There are several factors that may explain this. One of the most important relates to the 3 dimensional presentation of the MEK element to putative interacting proteins. It is

possible for the MS2 stem loop RNA to form secondary structures with the bait RNA, such that the native secondary structure of the bait RNA is altered. Such changes in secondary structure can interfere with the identification of bona fide interacting proteins. It was therefore important to have the IRP / IRE control 3 hybrid interaction to demonstrate how a specific interaction might look when compared to non-specific RNA baits. In no battery assay did we observe any interaction between a candidate protein and the MEK element that exhibited the tight specificity we observed for the interaction between the IRP and the IRE. *In silico* analysis indicates that both the MEK element and the IRE are capable of forming secondary structures whose melting temperatures exceed 50°C. It is therefore unlikely that failure to identify a specific interacting protein was due to the thermal instability of the MEK element secondary structure. However, it is formally possible that formation of the native RNA secondary structure is due to the MEK element's proximity to the MS2 stem loop sequence in the hybrid bait RNA transcript. Such a phenomenon may be an unavoidable limitation of using the yeast 3 hybrid method to identify RNA-binding proteins (Hook et al., 2005).

Chapter 3: S. cerevisiae mating pathway does not regulate genes at the level of mRNA stability

This chapter has been published (Kitchen et al., 2009).

Background

Studying how cells transduce extracellular stimuli into changes in gene expression due to modification of mRNA stability has presented a significant experimental challenge. Several factors contribute to this challenge, including the difficulty of identifying direct-acting *trans*-factors, the large number of proteins that are commonly associated with individual mRNA transcripts, the transient nature of *trans*-factor / mRNA interactions, and the low affinity nature of *trans*-factor / mRNA interactions. Many experimental approaches taken to date have relied upon some type of binding assay, for instance, RNA mobility shift assay (REMSA), immunoprecipitation followed by an identification technique (e.g. mass spectrometry), and most recently, RNA Immunoprecipitation-CHIP assays, to identify / implicate specific *trans*-factors in the regulation of specific mRNA transcripts.

The following text describes the experimental approaches taken to evaluate the feasibility of using the *S. cerevisiae* GPCR-controlled mating pathway as a model for higher eukaryotic regulated IEG mRNA stability control. To our knowledge, these experiments represent the first attempt to formally and directly test the hypothesis that *S. cerevisiae* modulate the steady-state level of mating genes via control of *both* transcription and mRNA stability.

Abstract

Many extracellular signals trigger changes in gene expression by altering the steady-state level of target transcripts. This modulation of transcript levels is typically ascribed to changes in transcription of target genes; however, there are numerous examples of changes in mRNA processing and stability that contribute to the overall change in transcript levels following signaling pathway activation. The α-factor-stimulated mating pathway in *Saccharomyces cerevisiae* is a receptor operated MAP kinase cascade that results in increased levels of a large number of target mRNA transcripts when stimulated acutely. A previous study identified many of the transcripts modulated in response to α-factor and argued based on genetic studies that the response occurred solely at the level of gene transcription [Roberts *et al.* 2000. Signaling and circuitry of multiple MAPK pathways revealed by a matrix of global gene expression. *Science* **287**: 873-880]. We directly examined whether enhanced mRNA stability contributes to the increase in the steady-state level of α-factor target transcripts by exploiting a temperature sensitive RNA Polymerase II mutant, a Ste12 transcription factor import mutant, and tet-regulated synthetic mating factor minigene reporters. Examination of a panel of α-factor responsive transcripts reveals no change in mRNA stability in response to α-factor stimulation, providing direct evidence that this signal transduction pathway in *S. cerevisiae* does not function through modulating transcript stability.

Introduction

Many eukaryotic cell types respond to a variety of extracellular cues by changing gene expression via transcriptional and post-transcriptional mechanisms (Moore, 2005). In the past, gene regulation was thought driven primarily by changes in the rate of mRNA synthesis. However, the regulated induction of certain classes of very unstable mRNA transcripts in mammalian cells, such as those that code for inflammatory mediators and oncogenes, cannot be accounted for solely by changes in the rate of transcription (Ross, 1995; Wilusz et al., 2001). Recent evidence implicates the regulation of RNA stability, and related post-transcriptional mechanisms, in the larger picture of gene regulation (Garneau et al., 2007; Raghavan and Bohjanen, 2004). Genome-wide studies now estimate a significant portion of all alterations in mRNA levels, in response to particular signaling events, are attributable to changes in the rate of mRNA decay (Gingerich et al., 2004; Wilusz and Wilusz, 2004).

Several cell surface receptor-regulated pathways in mammalian cells regulate gene expression by controlling mRNA stability (Cibotti et al., 2000; Mahtani et al., 2001; Rajasingh et al., 2006; Skinner et al., 2008; Xu et al., 2007; Xu et al., 2000). For example, G-protein coupled receptors (GPCRs) and growth factor receptors activate MAPK signaling cascades that simultaneously influence both rates of transcription and rates of decay of target mRNA transcripts (Gingerich et al., 2004; Shim and Karin, 2002; Whitmarsh, 2007; Wilson and Cerione, 2000). MAPK signaling pathways in *S. cerevisiae* become active in response to some environmental cues (Chen and Thorner, 2007), and two of these pathways are known to be regulated by a cell surface receptors; the well-

studied GPCR-regulated mating factor pathway (Schultz et al., 1995) and the less well-understood GPCR-regulated glucose response pathway (Versele et al., 2001).

In the GPCR mating factor-regulated pathway, binding of the mating pheromone peptide to the cell surface Ste2/3 GPCR stimulates the mating MAPK signaling cascade, causing budding yeast to exit the cell cycle and enter the mating process (Blumer et al., 1988; Kim et al., 2004; Oehlen and Cross, 1998; Sprague et al., 1983; Thorner, 1992; Wilkinson and Pringle, 1974). The MAPK signaling cascade leads to phosphorylation of the mating gene transcription factor, Ste12 (Elion et al., 1993). Once phosphorylated, Ste12 translocates into the nucleus and activates transcription by binding to pheromone response elements (PREs) within proximal promoters of responsive genes (Sengupta and Cochran, 1990). The mating response is attenuated in part by Ste12-mediated transcription of the gene encoding the Bar1 periplasmic protease which degrades the α-factor peptide (Manney, 1983), thus preventing continued activation of the GPCR (Ballensiefen and Schmitt, 1997; MacKay et al., 1988).

In order to identify the genes regulated by the α-factor signal transduction cascade, Roberts *et al.* stimulated *S. cerevisiae* cells using α-factor and monitored the steady-state level of over 6000 yeast transcripts over time using cDNA microarrays, (Roberts et al., 2000). The temporal induction profile for many *S. cerevisiae* mating genes, especially those displaying a very rapid and robust immediate-early response, is remarkably similar to that observed for the mammalian serum-inducible genes (Iyer et al., 1999), raising the question of whether gene expression during the mating response in *S. cerevisiae* is regulated by coordinated transcriptional and post-transcriptional programs, similar to GPCR / Growth Factor Receptor immediate-early gene (IEG) regulation in

higher eukaryotes (Dibrov et al., 2006; Lasko, 2003; Misquitta et al., 2006). The study by Roberts *et al.* provided evidence that the yeast MAP kinase pathway does not increase the base-line transcript levels of mating genes when the transcription factor, Ste12, is deleted, and on the basis of this data, they proposed that increases are regulated solely at the level of transcription (Roberts et al., 2000). However, given that some of the transcripts were not synthesized under these conditions, especially those genes whose transcription depends exclusively upon Ste12, post-transcriptional regulation, including possible modulation of mRNA stability, could not be conclusively ruled out. It remained formally possible that mRNA stability regulation could occur in synergy with Ste12-mediated transcriptional activation.

We therefore directly and rigorously tested the hypothesis that the *S. cerevisiae* α-factor stimulated MAPK cascade, like similar signal transduction pathways in higher eukaryotes (Dibrov et al., 2006; Farhat et al., 2008; Skinner et al., 2008), regulates gene expression through regulating both transcription and mRNA stability. Our data did not uncover any change in the rate of mRNA decay for any of the α–factor responsive transcripts examined. This finding suggests that the *S. cerevisiae* mating response signal transduction pathway does not exploit bimodal transcriptional/post-transcriptional immediate early gene response mechanisms that are observed in mammalian cells.

Results

Mating genes are differentially induced in wildtype vs. *deltaBar1* cells

In order to evaluate the mRNA expression levels of *S. cerevisiae* genes most likely to exhibit signal-mediated coordinated control of transcription and mRNA stability, we selected several genes that either contained (*ECM18*, *FIG2*, *FUS1*, *FUS2*, and *PRM6*) or lacked (*FIG1* and *PRM2*) canonical Pheromone Response Element (PRE) in their 5' promoter region (Table 3-2). On the basis of previously reported mating gene induction profiles (Roberts et al., 2000), we postulated that PRE-regulated genes might respond to yeast GPCR activation as do the higher eukaryotic immediate early genes that are coordinately transcriptionally and post-transcriptionally regulated (Iyer et al., 1999). The PRE-lacking genes were included based upon the reasoning that robust induction of genes lacking an obvious PRE might be dependent upon regulated post-transcriptional mechanisms. Table 3-2 summarizes the protected band sizes for the gene riboprobe set and indicates the presence or absence of well-defined PRE elements in the proximal promoters of the genes selected for analysis.

To assess the mating factor-induced increases in the steady state mRNA levels for the selected genes, wildtype cells, and *bar1delta* cells that lack the protease that degrades mating pheromone (Brachmann et al., 1998; Wach et al., 1994), were grown to mid-log phase and then stimulated with 50 nM α-factor. Cells pellets were harvested and total RNA was extracted over a time course from 0 to 50 minutes. The levels of the target mRNA transcripts were measured using a gene-specific multi-probe RNase protection assay (RPA). Since the Bar1 protease assists in mating response signal attenuation by

degrading periplasmic α-factor (Ballensiefen and Schmitt, 1997), the advantage of using *bar1delta* cells is that the GPCR pathway is active longer.

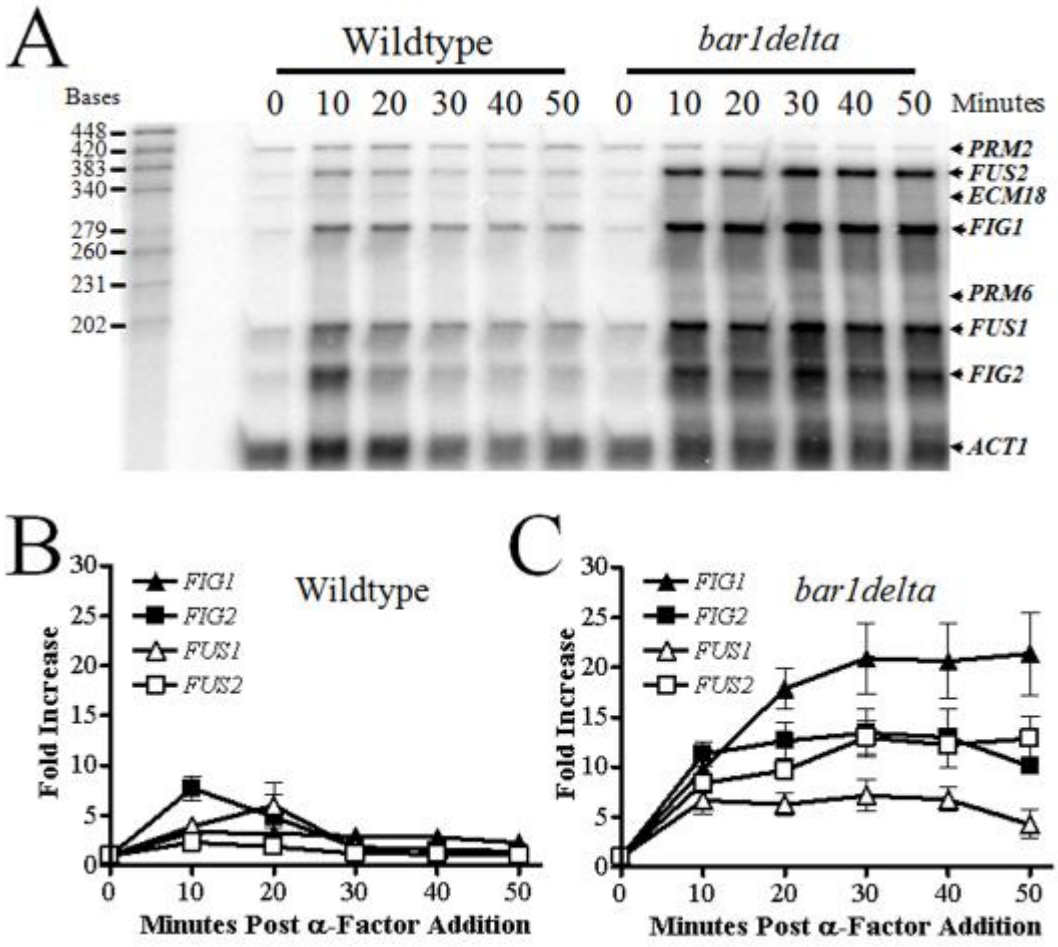
As shown in Figure 3-1A, α-factor stimulation robustly induces several of the genes chosen for analysis, including *FIG1*, *FIG2*, *FUS1*, and *FUS2*. As predicted, among the more significantly induced transcripts, *FUS1*, *FUS2*, and *FIG2*, possess PREs (Refer to Table 3-2) in their promoters. Interestingly, the *FIG1* gene was the most robustly induced among the set but this gene lacks a canonical PRE. Compared to the induction detected in wildtype cells, the mating genes are induced more strongly and for a longer duration in the *bar1delta* mutant cells.

To quantitate the results of this experiment, the mRNA intensity for each gene was divided by the corresponding *ACT1* mRNA intensity in each sample. These ratios were then divided by the baseline ratio at time zero to produce the "fold increase" value. These fold increase values are presented graphically for wildtype (Figure 3-1B) and *bar1delta* mutant cells (Figure 3-1C). For subsequent experiments, we focused on *FUS1*, *FUS2*, *FIG1* and *FIG2* as candidate post-transcriptionally regulated genes due to their relatively low baseline expression levels, their low stability in wildtype cells, and their robust stimulus-regulated induction. Mammalian immediate early genes that are regulated at the level of mRNA stability frequently share these characteristics (Bolognani and Perrone-Bizzozero, 2008).

Table 3-1. Genes selected for RNase Protection Assay (RPA).

Gene	RPA Protected Band Size (bases)	Canonical Pheromone Response Element (PRE)
PRM2	448	No
FUS2	390	Yes
ECM18	353	Yes
FIG1	310	No
PRM6	249	Yes
FUS1	230	Yes
FIG2	201	Yes
ACT1	172	No

Figure 3-1. Both wildtype and bar1delta cells show rapid induction of mating pathway genes following stimulation with α-factor. Either wildtype (ACY402) or *bar1delta* (ACY667) cells were grown to mid-log phase, 50 nM α-factor was added, aliquots were removed at the indicated time points, and total RNA was prepared as described in Materials and Methods. (A) RPA was performed using the gene-specific riboprobes described in Table 3-2. The specific transcripts corresponding to the protected fragments are indicated on the right. Quantitative results for wildtype (B) and *bar1delta* (C) cells were obtained by densitometric scanning of appropriate bands as described in Materials and Methods. Data are presented as fold increase over basal expression levels in unstimulated cells. Results are representative of three independent experiments and standard deviations in the data are indicated by the error bars.


A
Wildtype
bar1delta
Bases
0 10 20 30 40 50 0 10 20 30 40 50 Minutes
448
420
383
340
279
260
231
202
PRM2
FUS2
ECM18
FIG1
PRM6
FUS1
FIG2
ACT1
B
Wildtype
FIG1
FIG2
FUS1
FUS2
Fold Increase
Minutes Post α-Factor Addition
C
bar1delta
FIG1
FIG2
FUS1
FUS2
Fold Increase
Minutes Post α-Factor Addition

Mating gene induction is impaired in cells defective for Ste12 nuclear import

In response to pheromone stimulation, the Ste12 transcription factor enters the nucleus and induces transcription of responsive genes (Blackwell et al., 2007; Leslie et al., 2002). Previously, Roberts *et al*. used *ste12delta* cells in order to test whether mating gene induction depends exclusively upon Ste12 transcriptional activation, but since *ste12delta* cells produce little to no α-mating factor GPCR (Ste2), a constitutively active Ste2 had to be transformed into the cells in order to activate the mating MAPK cascade (Roberts et al., 2000). No mating genes were induced by α-factor in the *ste12delta* cells, leading to the conclusion that induction of mating genes is due to exclusively to transcriptional activation by Ste12. However, the study did not directly examine mRNA stability changes in response to mating factor. If these stability changes were indirectly dependent upon Ste12, or if these changes were dependent upon some minimal mating gene mRNA level, these changes might not be detected in *ste12delta* cells.

As an alternate method to test whether mating genes are induced by mechanisms other than Ste12-dependent transcriptional activation we used the temperature sensitive yeast mutant *pse1-1* that is defective in Ste12 nuclear translocation at the nonpermissive temperature of 37°C (Seedorf and Silver, 1997). This approach allows for the conditional exclusion of Ste12 from the nucleus in the absence of other cellular effects, such as the loss of Ste2 expression.

As shown in Figure 3-2A, mating genes are induced in the *pse1-1* cells at the permissive temperature with a profile that is similar to that seen for wildtype cells (compare to Figure 3-1A). In contrast, *pse1-1* cells shifted to the nonpermissive temperature show significant delays or decreases in the induction of all mating genes

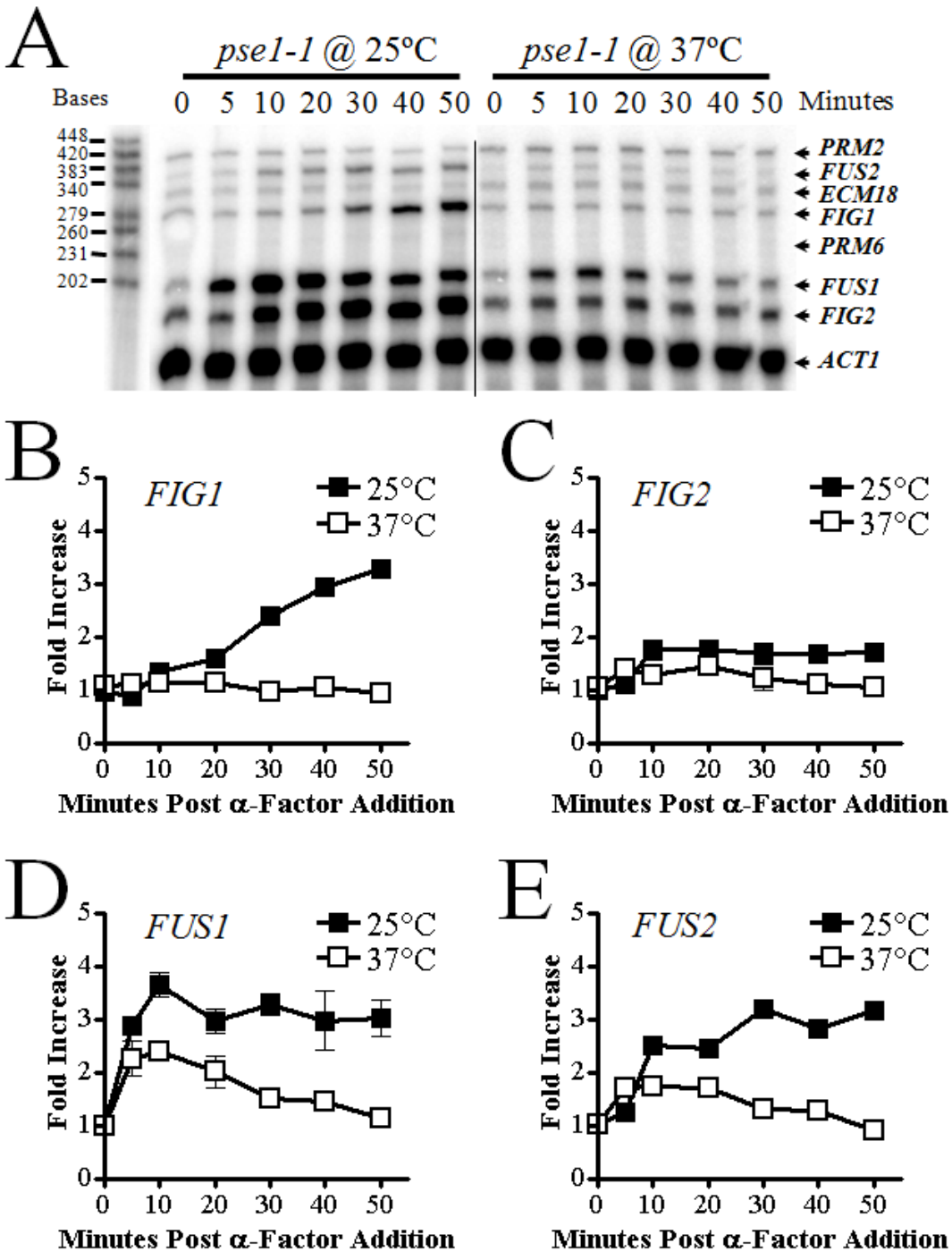
examined. Notably, *FIG1* is not induced detectably at the nonpermissive temperature. In contrast, *FUS1* and *FUS2* are induced modestly in *pse1-1* cells where the Ste12 nuclear translocation is deficient. Results of this experiment implicate transcriptional regulation but cannot dissect coupled transcription from control of transcript stability.

Transcription shut-off experiments using the RNA polymerase II mutant rpb1-1

To directly examine whether pheromone signaling could cause signal-induced stabilization of target transcripts, we employed a system where transcription could be turned off, and the *in vivo* decay of specific transcripts over time could be monitored. We exploited the conventionally used temperature sensitive mutant allele *rpb1-1* because it displays a preferential lack of RNA Pol II-dependent transcription at the nonpermissive temperature of 37 °C (Nonet et al., 1987).

To determine whether α-factor stimulation can lead to stabilization of target transcripts, *rpb1-1* cells were simultaneously stimulated with α-factor and shifted to the nonpermissive temperature. As expected, levels of the PRE-independent *FIG1* mRNA begin to decline upon switch to the non permissive temperature, but no change in the rate of decay was detected with concurrent α-factor stimulation, as would be expected if the signaling pathway stabilizes the mRNA (Figure 3-3A). The mRNA transcripts for each of the PRE-containing genes, *FIG2* (Figure 3-3B), *FUS1* (Figure 3-3C) and *FUS2* (Figure 3-3D) begin to decay upon the temperature switch in the absence of α-factor, but are modestly induced (2- to 3-fold) with α-factor signaling.

Figure 3-2. *pse1-1* cells stimulated with α-factor show a decreased induction of mating factor responsive relative to wildtype cells. *pse1-1* mutant cells (ACY230) were grown to mid-log phase, 50 nM α-factor was added and cells were either maintained at the permissive temperature (25 °C) or shifted to the nonpermissive temperature (37 °C) for one hour. Aliquots were removed at the indicated time points and total RNA was prepared as described in Materials and Methods. (A) RPA was performed using gene-specific riboprobes described in Table 3-2. The sizes (in bases) of the undigested riboprobes are indicated on the left. To quantitate results for expression of *FIG1* (B), *FIG2* (C), *FUS1* (D), and *FUS2* (E) at 25 °C and 37 °C, the appropriate bands from several RPA autoradiograms were scanned by densitometry using the Typhoon Imaging software package. Results are graphed as fold increase over levels of each transcript measured in basal unstimulated cells. Results are representative of three independent experiments.


A
pse1-1 @ 25°C
pse1-1 @ 37°C
Bases
0 5 10 20 30 40 50 0 5 10 20 30 40 50 Minutes
448
420
383
340
279
260
231
202
PRM2
FUS2
ECM18
FIG1
PRM6
FUS1
FIG2
ACT1
B
FIG1
25°C
37°C
Fold Increase
Minutes Post α-Factor Addition
C
FIG2
25°C
37°C
Fold Increase
Minutes Post α-Factor Addition
D
FUS1
25°C
37°C
Fold Increase
Minutes Post α-Factor Addition
E
FUS2
25°C
37°C
Fold Increase
Minutes Post α-Factor Addition

Although this response is consistent with mRNA stabilization, it could also reflect incomplete penetration of the RNA polymerase II defect in conditional *rpb1-1* cells and thus residual PRE-dependent transcriptional induction.

Tet-regulatable mating minigenes are not stabilized by α-factor

Although results obtained with the *rpb1-1* mutant cells could suggest that control of mRNA stability contributes to the regulation of mating gene expression, it was critical to confirm that the α-factor stimulated increase was not due to a slight delay in transcription shut-off in the *rpb1-1* mutant cells. To address this point, and to examine the possibility of α-factor-mediated control of mRNA stability without globally shutting down RNA Pol II-dependent transcription, we exploited a tetracycline regulated expression system that allows for production of full-length mating gene mRNA transcripts whose transcription can be rapidly and selectively repressed using anhydrotetracycline (AnTet). For this experiment, the full-length cDNAs (i.e. from 5' transcription start site to just past the 3' upstream poly adenylation signal) for *FUS1*, *FUS2* and *FIG2* were cloned into an episomal expression vector behind a tetracycline-regulatable promoter (Belli G, 1998; Gari et al., 1997). Gene-specific RPA riboprobes were designed to allow simultaneous measurement of these AnTet-suppressible synthetic mRNA transcripts as well as the endogenous mRNA transcripts.

The data in Figure 3-4A shows that AnTet has no effect on basal or α-factor stimulated levels of endogenous *FIG2* mRNA expression, indicating the drug does not affect the regulated expression of the endogenous mRNA transcript. AnTet also did not affect the expression of *FUS1* or *FUS2* endogenous mRNA transcripts (data not shown). In contrast, the addition of AnTet causes a rapid, time-dependent reduction of the

synthetic forms of the *FIG2* (Figure 3-4B), *FUS1* (Figure 3-4C), and *FUS2* (Figure 3-4D) mRNA transcripts. Based on these results, α-factor signaling neither affects the rate of decay nor does it induce basal levels of these synthetic mRNA transcripts, as might be expected if signaling stabilized transcripts. These experiments rule out a role for post-transcriptional stabilization of these mRNA transcripts by signal transduction pathways involved in the *S. cerevisiae* mating pathway.

Figure 3-3. *rpb1-1* mutant cells stimulated with α-factor show some stabilization of mating factor stimulated transcripts. Either *rpb1-1* or *rpb1-1 bar1delta* cells were grown to mid-log (OD_{600} ~ 0.5) then switched to the nonpermissive temperature to turn off RNA Polymerase II-driven transcription. At this time, either water or α-factor was added, and total RNA was isolated from aliquots removed at the indicated time points. The RPA was performed using gene-specific riboprobes and densitometry was performed to quantitate the level of each transcript. Each symbol represents the percent of mating gene expression remaining (percent remaining) at the indicated time points after transcription shut-off with the level at time zero set to 100%. A, *FIG1*; B, *FIG2*; C, *FUS1*; and D, *FUS2* . Data are results of three independent experiments.

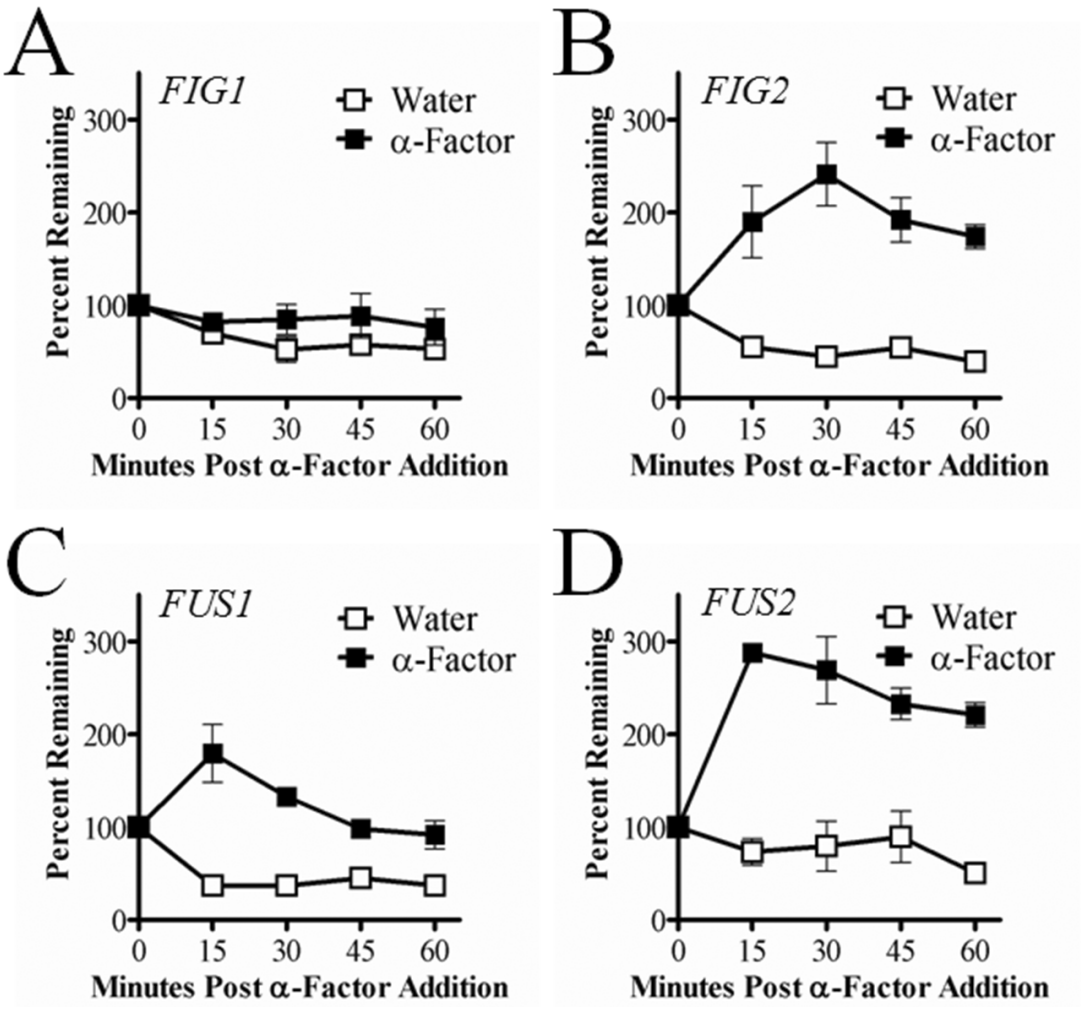
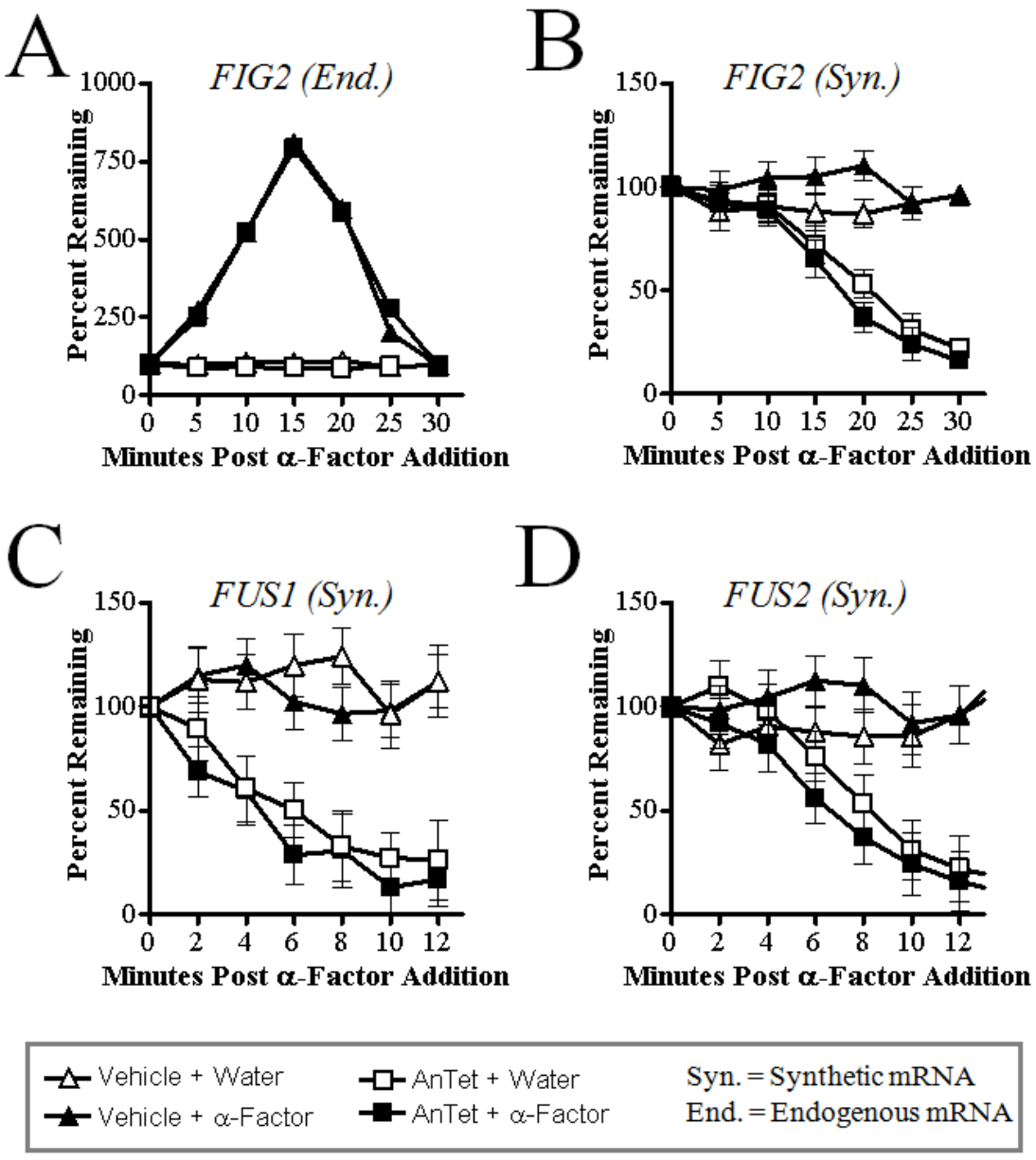

A
FIG1
Water
α-Factor
Percent Remaining
300
200
100
0
0
15
30
45
60
Minutes Post α-Factor Addition
B
FIG2
Water
α-Factor
Percent Remaining
300
200
100
0
0
15
30
45
60
Minutes Post α-Factor Addition
C
FUS1
Water
α-Factor
Percent Remaining
300
200
100
0
0
15
30
45
60
Minutes Post α-Factor Addition
D
FUS2
Water
α-Factor
Percent Remaining
300
200
100
0
0
15
30
45
60
Minutes Post α-Factor Addition

Figure 3-4. Synthetic AnTet-regulated *FIG2*, *FUS1*, and *FUS2* minigenes reveal no change in transcript stability upon α factor stimulation. *Bar1delta cells* (ACY667) harboring tetracycline suppressible *FIG2*, *FUS1*, or *FUS2* synthetic constructs were grown to mid-log phase. A zero time point sample was collected from each sample before the addition of Antet (1 μg/mL), α-factor (50 nM), or their vehicles as indicated. Total RNA was isolated from aliquots removed at the indicated time points following these additions. RPA was performed and results were quantitated by densitometry as described in Materials and Methods. Each symbol represents the percent of mating gene expression remaining (percent remaining) at the indicated time points after transcription shut-off with the level at time zero set to 100%. A, endogenous *FIG2* mRNA expression level; B, synthetic *FIG2* expression level; C, synthetic *FUS1* expression level; and D, synthetic *FUS2* expression level. Data are the results of three independent experiments.


A
FIG2 (End.)
Percent Remaining
1000
750
500
250
0
0 5 10 15 20 25 30
Minutes Post α-Factor Addition
B
FIG2 (Syn.)
Percent Remaining
150
100
50
0
0 5 10 15 20 25 30
Minutes Post α-Factor Addition
C
FUS1 (Syn.)
Percent Remaining
150
100
50
0
0 2 4 6 8 10 12
Minutes Post α-Factor Addition
D
FUS2 (Syn.)
Percent Remaining
150
100
50
0
0 2 4 6 8 10 12
Minutes Post α-Factor Addition
Vehicle + Water
Vehicle + α-Factor
AnTet + Water
AnTet + α-Factor
Syn. = Synthetic mRNA
End. = Endogenous mRNA

Discussion

This study was designed to rigorously test the hypothesis that *S. cerevisiae* coordinates transcriptional control and mRNA stability control mechanisms to regulate the level of mating gene expression induced in response to the GPCR-controlled α-factor MAPK pathway. The rationale for testing this hypothesis came from two compelling observations. First, recent evidence indicates that a related yeast species, *S. pombe*, uses coordinated transcription / mRNA stability control mechanisms to regulate precisely the level of at least one labile mRNA transcript (Sugiura, et al., 2004; Sugiura, et al., 2003). It was reasonable to ask whether *S. cerevisiae* could similarly regulate its unstable mating gene transcripts. Second, many immediate early genes appear to be synergistically induced by both transcriptional and post-transcriptional mechanisms. Although mating gene transcripts are not induced in *ste12delta* cells (Roberts, et al., 2000), an untranscribed mRNA cannot be post-transcriptionally regulated. It therefore remained possible that mRNA stability control could contribute to mating gene transcript accumulation. We tested this hypothesis by using an RNAse protection assay to monitor the steady-state level of a selected set of yeast mating genes over time under conditions where we could control transcription yielding a direct measure of mRNA decay.

We first measured potential α-factor-mediated mRNA stability by using a relatively less specific, more global transcriptional shutdown method, the *rpb1-1* mutant allele, which allows for conditional termination of Pol II-dependent transcription (Nonet et al., 1987). Though this method is used conventionally where transcription shut-off is required, our results suggest that *rpb1-1* transcription termination may be too slow to

measure the potential mRNA stability effects attributable to acute administration of α-factor. The induction of several of the mating genes occurs in a little as 2.5 minutes (data not shown), so transcription shut-off should ideally happen as quickly as experimentally possible. The *rpb1-1* mutant does not appear to provide this needed rapidity.

To address the concern of transcript specificity and speed of transcription termination, we generated tet-regulatable mating minigenes to enable the selective transcriptional control of specific synthetic mRNA transcripts. Taking this approach allowed us to examine simultaneously the effects of α-factor on both the level of the synthetic mRNA constructs, and the level of the endogenous mating genes. The tet-regulatable experiments have the advantage of being unencumbered by potentially confounding effects of global transcription control strategies. Our data with this approach provides strong evidence that the *S. cerevisiae* mating pathway activates downstream genes through control of transcription, but not through control of mRNA stability.

The emergence of approaches based upon tet-regulated promoters, rather than the use of transcriptional poisons, have been crucial to proving that kinase regulated post-transcriptional processes are involved in controlling mammalian mRNA stability (Xu and Murphy, 2000; Xu et al., 2000). Our most compelling data that supports the assertion that similar regulatory systems do not operate in *S. cerevisiae* downstream of the yeast mating factor pathway are those produced using the tet-regulated system. Mating factor signaling affects neither the steady-state levels nor the decay rates of the mRNA transcripts transcribed from these tet-regulated constructs.

Since we have only rigorously tested the mating MAPK signaling pathway, we cannot rule out the possibility that other signal-induced pathways in *S. cerevisiae* utilize coordinated transcription and mRNA stability control to regulate gene expression, similar to how mammalian MAPK pathways regulate the expression of many immediate early genes. For example, the *S. cerevisiae* ELAV homolog, Pub1, stabilizes transcripts containing either the mammalian TNFα AU-rich element (ARE) or the *S. cerevisiae TIF51A* ARE, in glucose media, but *de*-stabilizes these same transcripts in media lacking glucose, or when the Hog1/p38 MAPK pathway is blocked (Vasudevan and Peltz, 2001). In addition, the putative Poly-C Binding Protein (PCBP) homolog, Rnc1, stabilizes the *S. pombe* MAPK phosphatase transcript, pmp1+, in response to Pmk1 MAPK activity (Sugiura et al., 2004; Sugiura et al., 2003).

The absence in *S. cerevisiae* of coordinated transcriptional and post-transcriptional mRNA stabilization mechanisms in response to the α-factor induced MAPK signaling pathway suggest this model system could be used to develop a screening platform for identifying mammalian post-transcription factors that functionally interact with MAPK responsive *cis*-acting mRNA elements. Since *S. cerevisiae* appears to be unencumbered by an endogenous GPCR-controlled post-transcriptional regulatory program, it may be possible to reconstitute signal-regulated mRNA stability, useful to identify or characterize binding partners for signal-responsive mammalian *cis*-acting regulatory elements.

Chapter 4: A novel system to investigate the interactions between functional RNA *cis*-elements and candidate *trans*-factors

Abstract

A screening system is described that can read out the functional consequences of combining various proteins with chimeric mRNA constructs that contain both the firefly luciferase coding sequence, under control of a tetracycline regulatable promoter, and a functional *cis*-acting mRNA regulatory element. This system is ideal for screening tens or hundreds of full-length cDNA expression clones, encoding putative SMART factors. To evaluate the system, stringently testing whether several mammalian mRNA elements were functional in *S. cerevisiae*. When expressed as a chimera with the luciferase cDNA, a 2.5kB full length COX2 mRNA 3'UTR significantly reduced the amount of luciferase reporter activity, relative to control, indicating that the system could read out the functional consequences of placing different mRNA regulatory elements into the expression system. Co-expression of CUGBP2 and PCBP3 significantly reduced luciferase activity only in the context of the 3'UTR, suggesting a specific interaction. *In vitro* binding studies performed using CUGBP2 validated the interaction.

Introduction

Mammalian cells respond to extracellular cues by altering gene expression using transcriptional and post-transcriptional mechanisms (Moore, 2005). This gene regulation was once thought to be driven primarily by changes in the rate of transcription, but it has become apparent that the regulated induction of certain classes of very unstable mRNA transcripts, such as those that code for cytokines, inflammatory mediators, and oncogenes, could not be accounted for by changes in the rate of transcription alone (Ross, 1995; Wilusz et al., 2001). Experimental evidence implicates post-transcriptional mechanisms, such as mRNA stability control, in signal regulated gene expression changes (Garneau et al., 2007; Raghavan and Bohjanen, 2004). Recently, genome wide studies provide evidence that a significant portion of regulated gene expression can be attributed to changes in the rate of mRNA decay, indicating the important role played by mRNA stability control (Gingerich et al., 2004; Wilusz and Wilusz, 2004).

Signal regulated mRNA stability control is now an undisputed biological process, but studying the molecular details of this process has presented significant experimental challenges. The large number of mRNA processing and regulatory steps, which are each potentially subject to override control by signal transduction systems, including splicing, editing, transport, and decay, contribute to this challenge (Alonso, 2005; Bevilacqua A, 2003; Bolognani and Perrone-Bizzozero, 2008; Garneau et al., 2007; Meyer et al., 2004; Newbury, 2006; Shim and Karin, 2002; Shyu et al., 2008). The precise molecular details remain largely unknown, but at least one aspect of post-transcriptional gene regulation is commonly held to be accomplished by what can be termed as **S**ignal **M**odulated m**R**NA **T**urnover (SMART) *trans*-acting factors interacting with *cis*-acting mRNA stability

control elements (Mignone et al., 2002; Rajagopalan and Malter, 1997; Wilusz et al., 2001).

A first step to understand regulated mRNA stability control often begins with the identification of a functional *cis*-acting regulatory element, such as the AU-rich elements (ARE), and other emerging mRNA regulatory *cis*-elements such as those identified using computer-assisted sequence analysis (Khaladkar et al., 2008; Pedersen et al., 2006; Torarinsson et al., 2006; Washietl et al., 2005; Zhao et al., 2008). Typically, a gene of interest is demonstrated to be either stabilized or destabilized in response to a signaling event by using transcription shut-off assays. Deletion analysis of the mRNA can then reveal a necessary and sufficient minimal mRNA regulatory element s that couple extracellular signaling to changes in mRNA stability. Such elements are usually tens to more than one hundred nucleotides in length, such as those present in the gm-CSF (Putland et al., 2002), utrophin A (Chakkalakal et al., 2008), IFN-β (Paste et al., 2003), and uPAR (Shetty, 2005), transcripts.

An experimental challenge is identifying SMART factors and tying them to specific regulatory responses. Several methods have been deployed to identify sequence-specific SMART factor/mRNA *cis*-element interactions. These methods typically depend upon *in vitro* SMART protein/mRNA binding conditions, and have included affinity capture followed by mass spec (Blaxall et al., 2000; Chrestensen et al., 2004; Skalweit A, 2003), mRNA mobility shift assays (Cok SJ, 2003), and yeast three hybrid (Bernstein et al., 2002; Piganeau et al., 2006; Putz et al., 1996; Riley et al., 2006; Seay et al., 2006; SenGupta et al., 1996).

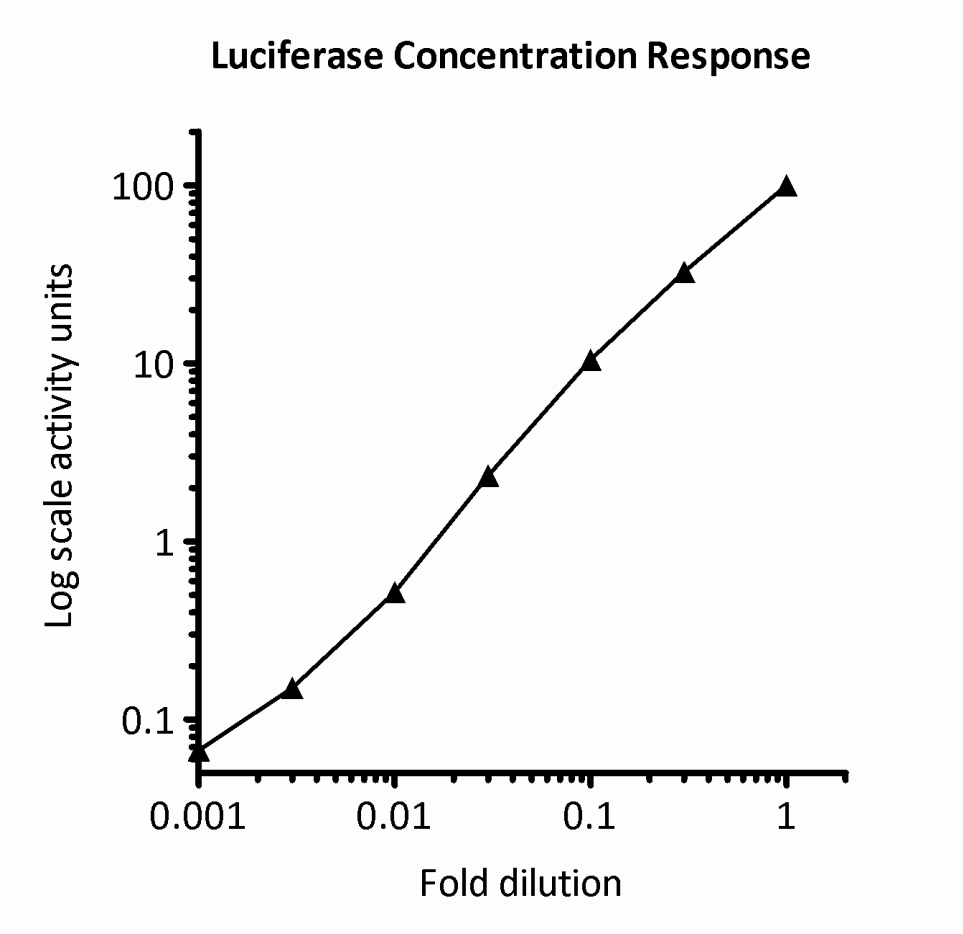
Within the COX2 mRNA 3'UTR, we have identified several elements that together couple COX2 transcript stability control to p38 MAPK, tyrosine kinase, and MEK signaling pathway activation (Xu et al., 2007; Xu et al., 2000). To test the functionality of our screening platform, we have therefore placed the entire COX2 mRNA 3'UTR into one of the luciferase chimeric expression vectors to screen for candidate interacting SMART proteins. In this system the full-length COX2 3'UTR acts as a functional element in *S. cerevisiae*, in that it modulates luciferase expression. Co-expression of the candidate SMART factors PCBP3, hnRNP C, and CUGBP2 modulate luciferase activity further, and demonstrates a functional interaction with the full-length COX2 3'UTR in *S. cerevisiae*. This system can be used to rapidly screen through sub-libraries of RNA interacting proteins suspected of participating in the post-transcriptional regulation of gene expression.

Results

Determination of optimal luciferase assay reading conditions

In order to determine the optimal luciferase assay conditions, we subjected serially diluted, high luciferase activity lysates to multiple combinations of assay buffer incubation times and luminescence incubation times. Figure 4-1 illustrates the luciferase activity of increasingly concentrated *S. cerevisiae* cell lysates is linear over three orders of magnitude.

Figure 4-1. Optimization of Luciferase Assay Incubation and Integration Read Times. Lysates with high luciferase activity were serially diluted and subjected to a 2 second incubation delay followed by a 5 second integration time.



The mammalian MEK-sensitive element is not functional in *S. cerevisiae*, but the full-length COX2 3'UTR is functional

We produced the tetracycline regulatable luciferase chimeric expression vectors (Figure 4-2), transformed them into *S. cerevisiae*, then performed luciferase activity assays to determine the effect of the 3'UTR sequences on luciferase activity levels. In order to control for the presence of a second protein expression vector, as would be present in subsequent studies, an appropriate expression vector was co-transformed along with the luciferase reporter vectors. For each luciferase construct, the luciferase activity of 20 independent colonies was evaluated, both before and after a 6 hour incubation with 1 μg/mL Anhydrotetracycline (antet) to selectively shut off transcription. The activity was corrected for cell growth differences by normalizing with OD_{600} values. As indicated in Figure 4-3A, treatment with antet reduces luciferase activity from all 3 expression constructs, consistent with transcriptional arrest. The presence of the full length 3'UTR significantly reduced luciferase activity relative to the control, indicating it has a functional property. In contrast the luciferase activity produced from expression of the chimeric luciferase-MEK mRNA is not significantly different from control.

To determine if differences in luciferase activity levels are paralleled by steady-state mRNA levels, total RNA isolated from five independent *S. cerevisiae* cultures, each transformed with either the control luciferase without a 3'UTR, the luciferase with the MEK element 3'UTR, or the luciferase with the full COX2 3'UTR luciferase construct. RNAse protection assay (RPA) was used to determine luciferase mRNA expression levels, which were normalized by *ACT1* mRNA

Figure 4-2. Map of Parent Vector and Insert Sizes for the Tetracycline Regulatable Luciferase mRNA Expression Vectors. The tetracycline regulatable *S. cerevisiae* expression vector pCM190 (A) served as the parent vector for constructing each of the test luciferase chimeric expression vectors. The inserts were cloned into the NotI and PstI sites as indicated, to produce the following constructs: 1) parent, 2) no 3'UTR, 3) MEK element, and 4) full COX2 3'UTR.

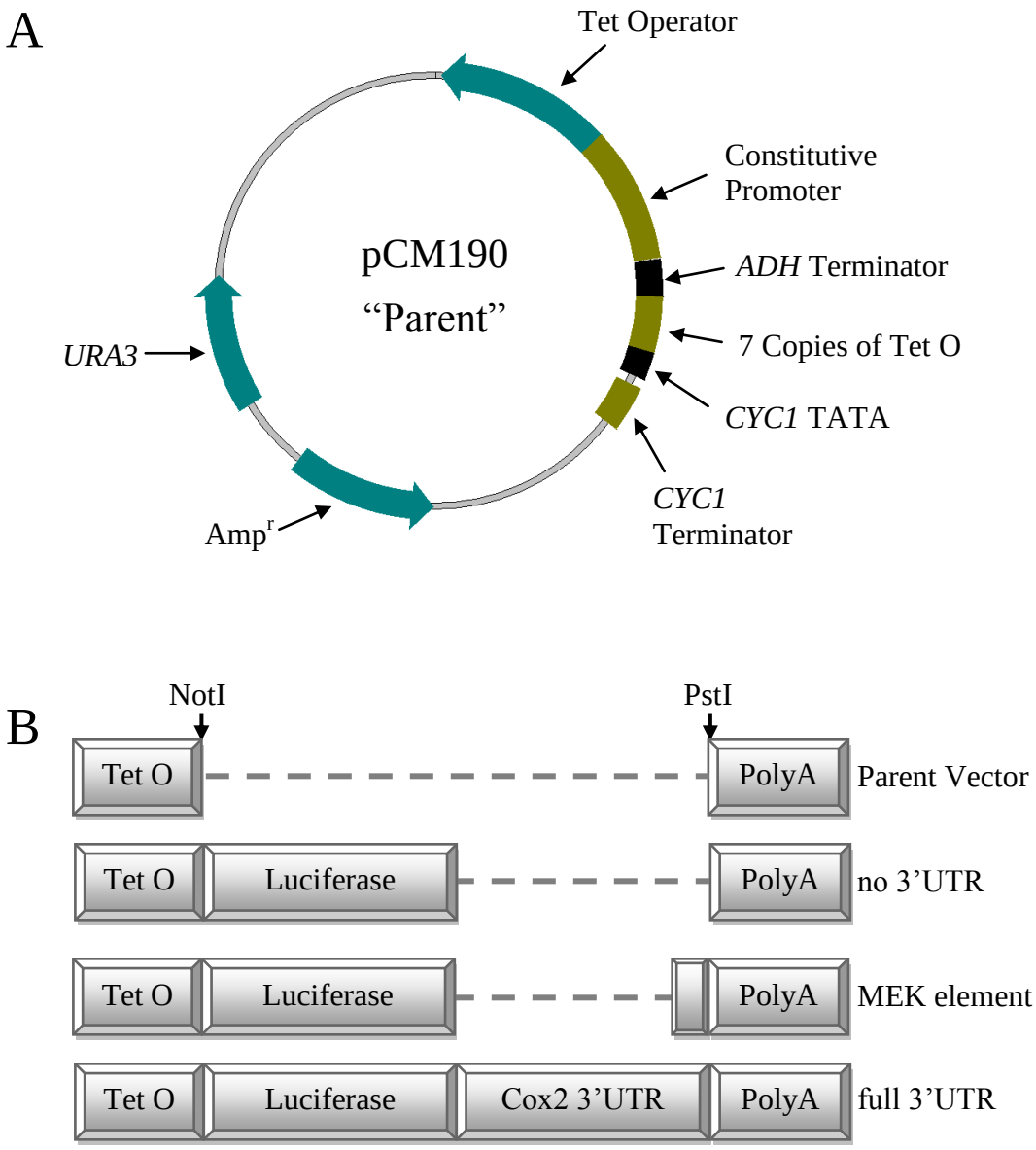
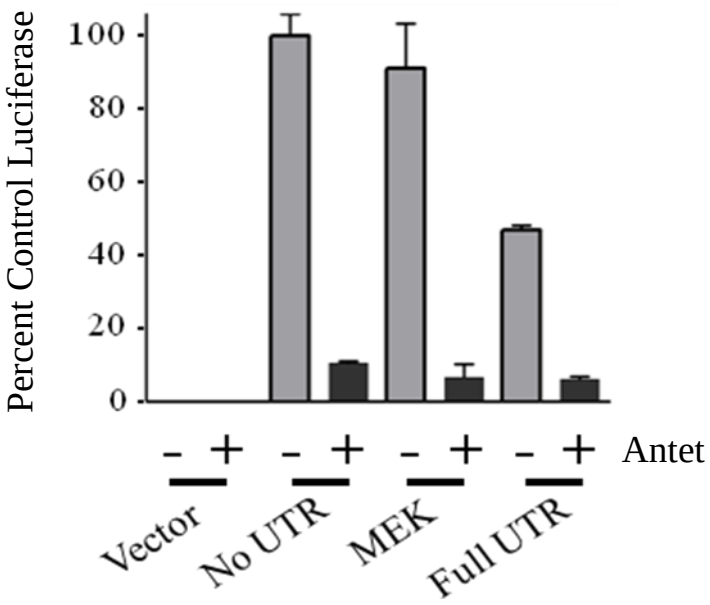
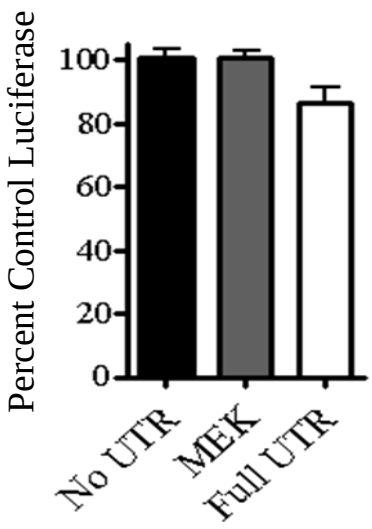


Figure 4-3. Relative luciferase assay values for selected mRNA binding protein luciferase construct combinations. The no UTR, MEK Element, and Full 3'UTR containing luciferase vectors were transformed into *S. cerevisiae* cells. Transformants were allowed to grow for 4 days on selective media, and 12 colonies from each luciferase construct were processed to evaluate steady-state luciferase activity (A). Luciferase activity values were normalized using OD_{600} values for each culture. The no UTR control was set to equal 100% relative luciferase activity, and the results represent the average of 2 independent experiments each evaluating 12 colonies from each protein/mRNA pairing. Total RNA was then isolated from no UTR, MEK element, and FULL UTR transformed cells (5 colonies for each construct) and RPA was performed to measure the level of luciferase and *ACT1* mRNA (B), and the values were graphed as a percent of the no UTR control mRNA level (C).

A


Percent Control Luciferase
100
80
60
40
20
0
- + - + - + - +
Antet
Vector
No UTR
MEK
Full UTR

B


Percent Control Luciferase
100
80
60
40
20
0
No UTR
MEK
Full UTR

expression levels (Figure 4-3B). This indicates that the steady-state level of the chimera luciferase containing the full length 3'UTR mRNA is slightly, but significantly less than that of the control and of the MEK element chimera luciferase mRNA, which are not significantly different than one another. Notably, luciferase activity gives a broader signal to noise ratio than does mRNA expression levels.

Pilot screening of mRNA binding protein mini-library

To test the ability of our system to screen for functional interactions between *cis*-element and SMART factor, a test panel of several full length proteins was created to screen against these constructs. Table 4-1 summarizes the protein identities and the rationale for their inclusion in the screen. In general, proteins were selected that had literature linkages to either COX2 mRNA stability or to MAPK-mediated gene regulation. Several mRNA-binding proteins were chosen that possess putative MAPK-binding or phospho acceptor sites, as predicted by Scansite (Obenauer et al., 2003).

Luciferase activity produced by full COX2 3'UTR luciferase chimeras is altered by PCBP3, CUGBP2, and hnRNP C

The initial pilot screen revealed several proteins that appeared to interact with the full COX2 3'UTR luciferase chimera mRNA. However, the variability of reporter activity produced by independent colonies transformed with the exact same constructs is significantly high. To increase precision in measuring the differences in luciferase activity attributable to the presence of a candidate protein, a high replicate secondary screen was performed. Luciferase assays were performed on cultures co-expressing one of the three luciferase chimera mRNA transcripts along with

Table 4-1. Proteins screened for functional interaction with luciferase mRNA transcripts. The following proteins were co-transformed into *S. cerevisiae* that contained either the control no 3'UTR, the MEK element, or the full COX2 3'UTR luciferase chimeric expression vector.

Gene ID	Screen Inclusion Rationale	Reference
BRUNOL5	Related to CUGBP2, a COX2 mRNA SMART factor	(Barreau et al., 2006)
CUGBP2	COX2 mRNA SMART factor	(Mukhopadhyay et al., 2003a; Murmu et al., 2004; Sureban et al., 2007; Xu et al., 2007)
hnRNP C	mRNA binding protein, MAPK Phosphoprotein	(Esnault and Malter, 2003)
HuR	mRNA binding protein	(Doller et al., 2008; Yang et al., 2004b)
Nucleolin	mRNA binding protein linked to MAPK signaling	(Westmark and Malter, 2001)
PCBP3	mRNA binding protein related to hnRNP E2	(Kim et al., 2000; Wang et al., 2006)
PTBP2	mRNA binding protein, associated with nucleolin	(Singh et al., 2004)
RBM4	Splicing protein, potential MAPK-binding	Scansite (Obenauer et al., 2003)
RBMS2	Interacts with single-stranded RNAs, non-specific control	
RPS16	Ribosomal Protein S16, non-specific control	
GRB10	Non-mRNA binding control	(Nantel et al., 1998)
RhoGDI	Putative CUGBP2 interacting protein, signaling protein	Our Observations
RBM21	Putative CUGBP2 interacting protein	Our Observations
IRBIT	Putative CUGBP2 interacting protein	Our Observations, (Ando et al., 2003)

candidate proteins or several negative controls, including RBM21 as a general RNA-binding protein that does not bind to the COX2 3'UTR. Figure 4-4 presents the luciferase activity produced by *S. cerevisiae* that were transformed with the control no 3'UTR luciferase chimera, the MEK element luciferase chimera, or the full 3'UTR luciferase chimera plus either empty vector, PCBP3, hnRNP C, RBM21, or CUGPB2. Both PCBP3 and CUGBP2 significantly affected the luciferase activity level produced by the full COX2 3'UTR luciferase chimeras, whereas neither protein had a significant effect on the luciferase activity produced by either the control or the MEK-element luciferase chimeras.

PCBP3, CUGBP2, and hnRNP C effect steady-state luciferase COX2 mRNA 3'UTR chimera

To test whether the differences in luciferase activity reflect changes in mRNA levels, the latter were measured in cells co-expressing the chimera luciferase constructs or control luciferase lacking the COX2 mRNA 3'UTR, along with CUGBP2, PCBP3, or hnRNP C. Each of these proteins had no effect on the expression of the control luciferase chimera (Figure 4-5A), but all proteins enhanced expression of the full length COX2 3'UTR luciferase chimera (Figure 4-5B).

PCBP3 and hnRNP C affect mRNA stability

To understand whether the observed increases in steady-state luciferase mRNA levels were due to increased mRNA stability, the time-dependent decay of luciferase mRNA was measured after stopping transcription using antet. As shown in Figure 4-6 neither of the proteins affect stability of the control luciferase mRNA. In

Figure 4-4. Relative luciferase assay values for selected combinations of mRNA binding protein and luciferase mRNAs. The no UTR, MEK Element, and Full 3'UTR containing luciferase vectors were co-transformed with either control, PCBP3, hnRNP C, RMB21, or CUGBP2 into *S. cerevisiae* cells. Luciferase activity was measured and raw values for the MEK-element luciferase chimera and for the full 3'UTR luciferase chimera-transformed cells were normalized to their corresponding control no 3'UTR luciferase chimera.

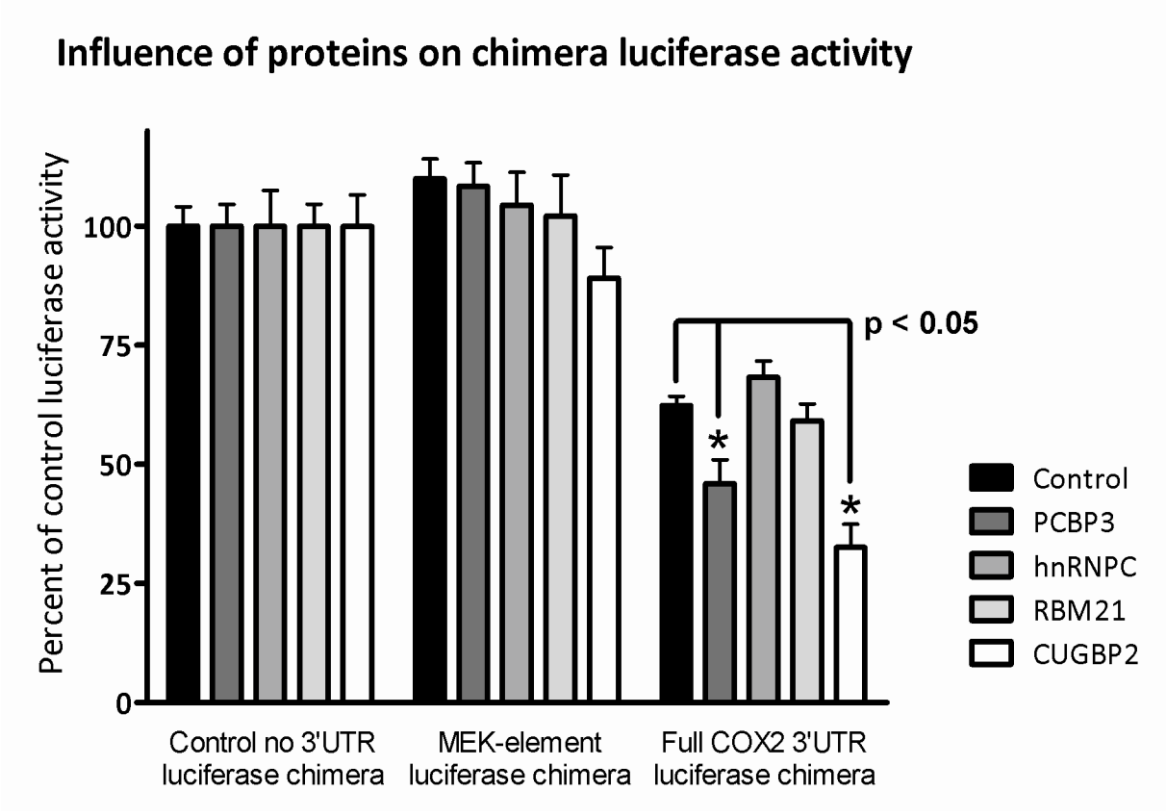


Figure 4-5. Steady-state luciferase mRNA levels for control and full COX2 3'UTR luciferase chimera. The control and full COX2 3'UTR luciferase chimera vectors were co-transformed with either control, PCBP3, hnRNP C, or CUGBP2 into *S. cerevisiae* cells. Total RNA was isolated and RNAse protection assay was performed to measure the levels of the control luciferase mRNA (A) and the full COX2 3'UTR luciferase chimera mRNA (B).

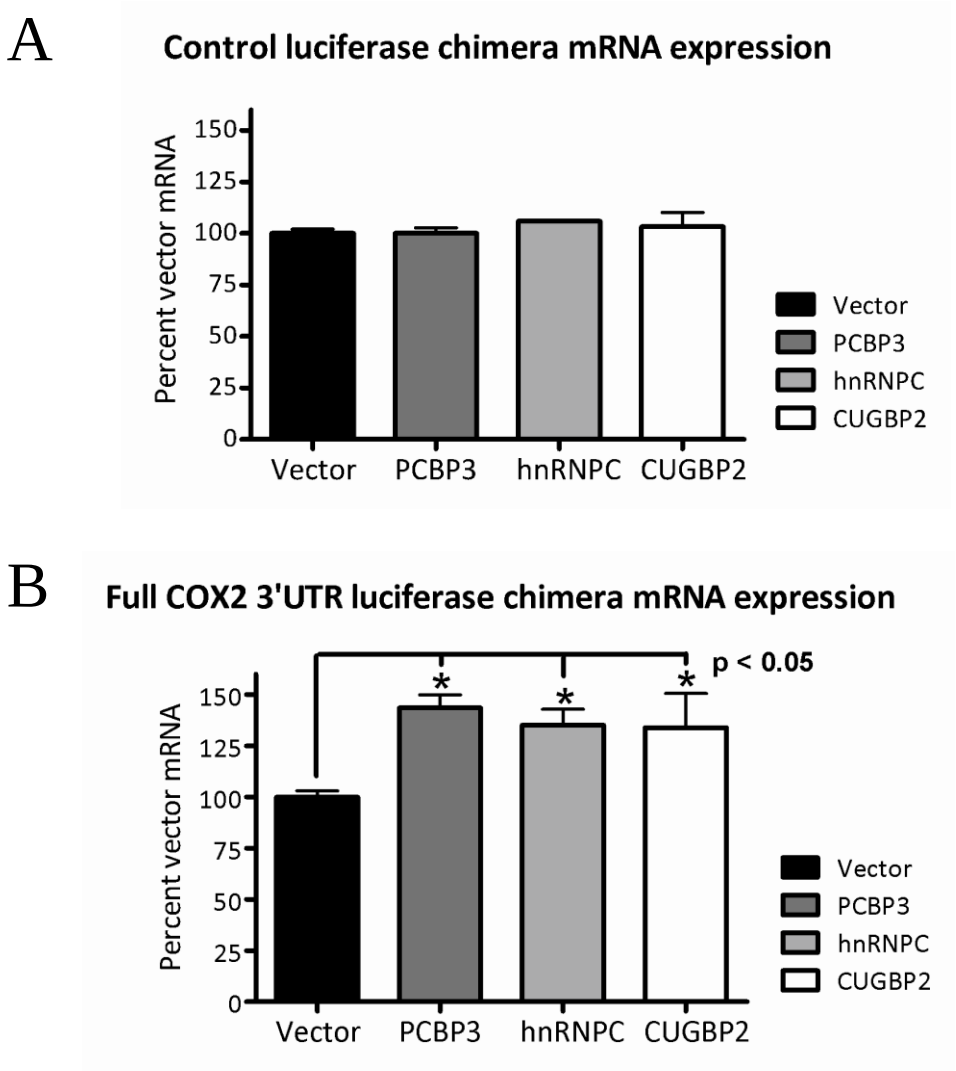
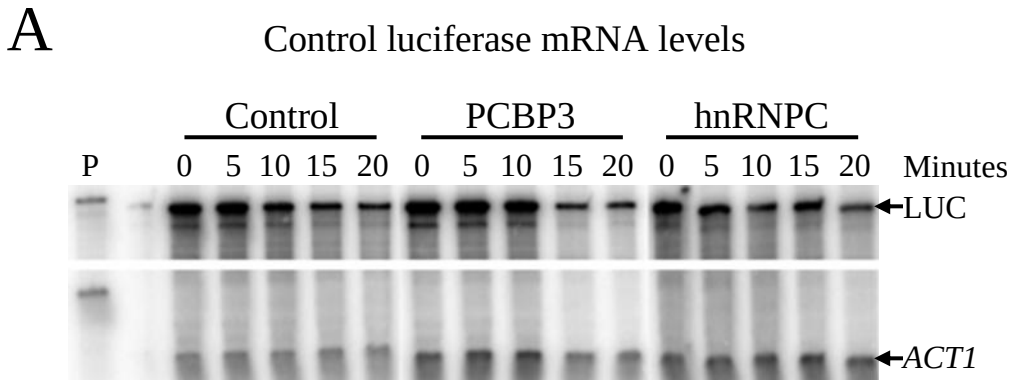


Figure 4-6. Influence of PCBP3 and hnRNP C on the control luciferase mRNA decay rate. *S. cerevisiae* transformed with control luciferase were co-transformed with either control vector, PCBP3, or hnRNP C. Transcription was arrested using 1 mg/mL antet, then RNA was isolated at the indicated time points, and RNAse protection assay was performed (A) using luciferase and *ACT1* gene-specific riboprobes to measure the levels of the control luciferase mRNA (B).



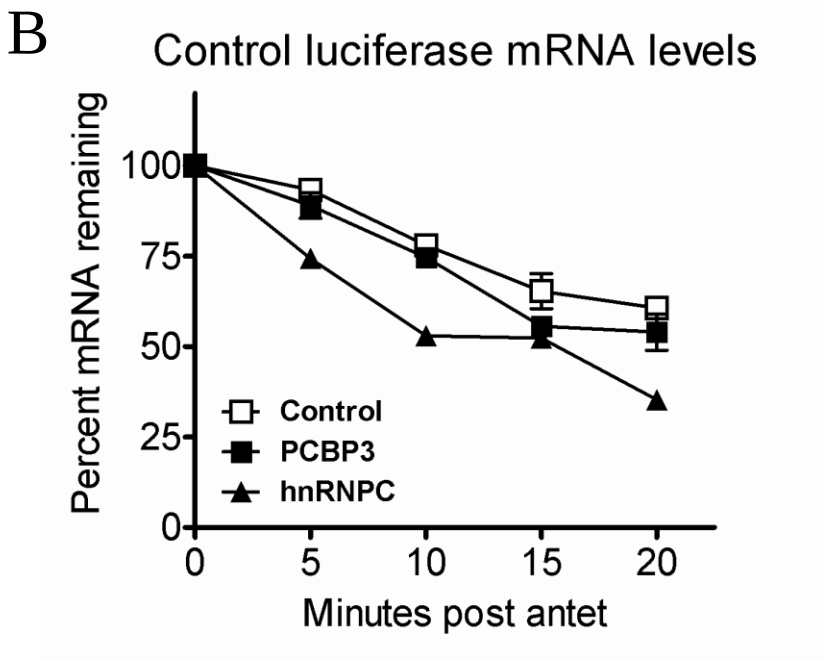
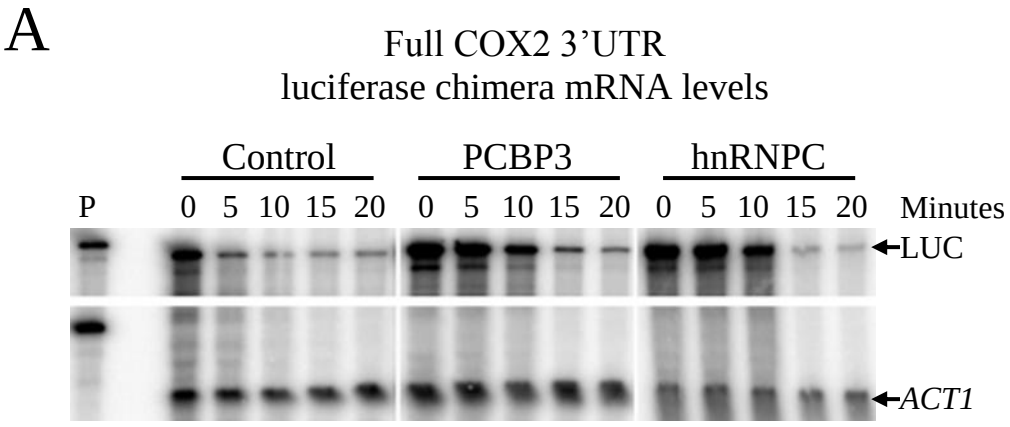
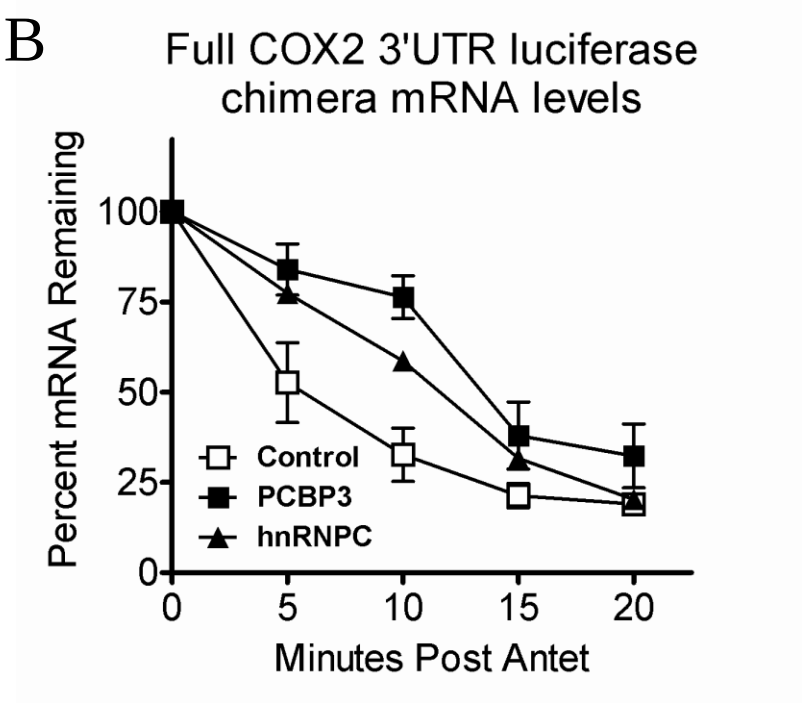


Figure 4-7. Influence of PCBP3 and hnRNP C on the full COX2 3'UTR luciferase chimera mRNA decay rate. *S. cerevisiae* transformed with full COX2 3'UTR luciferase chimera were co-transformed with either control vector, PCBP3, or hnRNP C. Transcription was arrested using 1 mg/mL antet, then RNA was isolated at the indicated time points, and RNAse protection assay was performed (A) to measure the levels of the full COX2 3'UTR luciferase chimera mRNA (B).





contrast, Figure 4-7 shows that the chimera COX2 3'UTR mRNA is stabilized by expression of either protein.

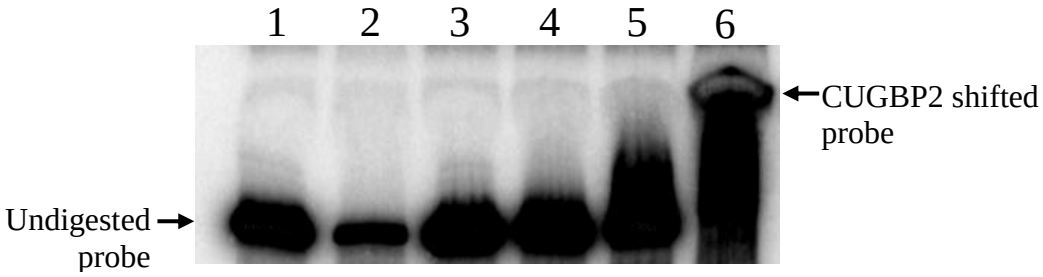
CUGBP2 interacts with the first 250 bases of the 3'UTR of the Cox2 mRNA

To test whether CUGBP2 binds directly to the minimal 250 base COX2 mRNA c-Src responsive element identified above, binding studies were performed. A probe corresponding to this sequence was synthesized, bound with a GST-CUGBP2 protein expressed and purified from bacteria, and binding was assessed using RNA electro mobility shift assay (REMSA). As shown in Figure 4-8, the mobility of this probe is specifically retained by CUGBP2 in a dose dependent manner.

CUGBP2 interacts with the first 50 bases of the Cox2 mRNA

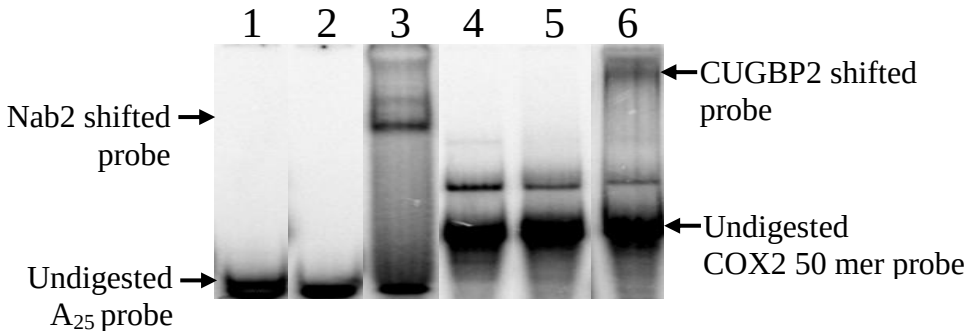
To further refine the sequence that binds to the CUGBP2, a probe from the first 50 nucleotide was transcribed and binding studies were performed as above. As shown in figure 4-9, the probe corresponding to the first 50 nucleotides of the COX2 mRNA 3'UTR is specifically retained by CUGBP2.

Figure 4-8. REMSA using 250 nucleotide ^{32}P-labeled COX2 riboprobe and GST-CUGBP2. Each binding reaction was assembled according to the table below and resolved on a native acrylamide gel.



Lane	Components
1	1 µL ^{32}P UTP Riboprobe (~100ng/2E5cpm)
2	Probe + 200 nM GST
3	Probe + 0.10 µg CUGBP2
4	Probe + 0.33 µg CUGBP2
5	Probe + 1.00 µg CUGBP2

Figure 4-9. REMSA using ^{32}P-labeled riboprobes, GST-CUGBP2, and GST-Human Nab2. Each binding reaction was assembled according to the table and resolved on a native acrylamide gel.

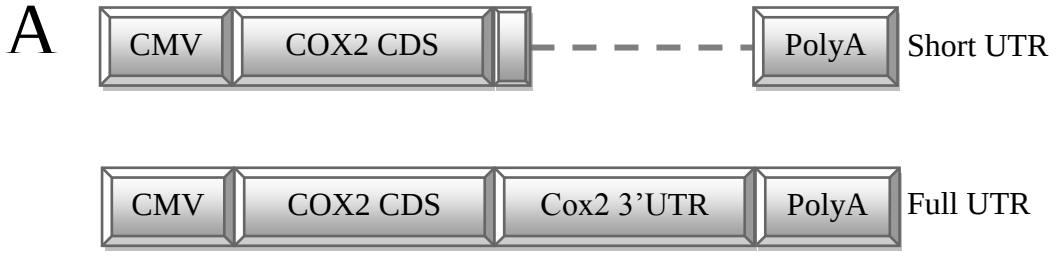


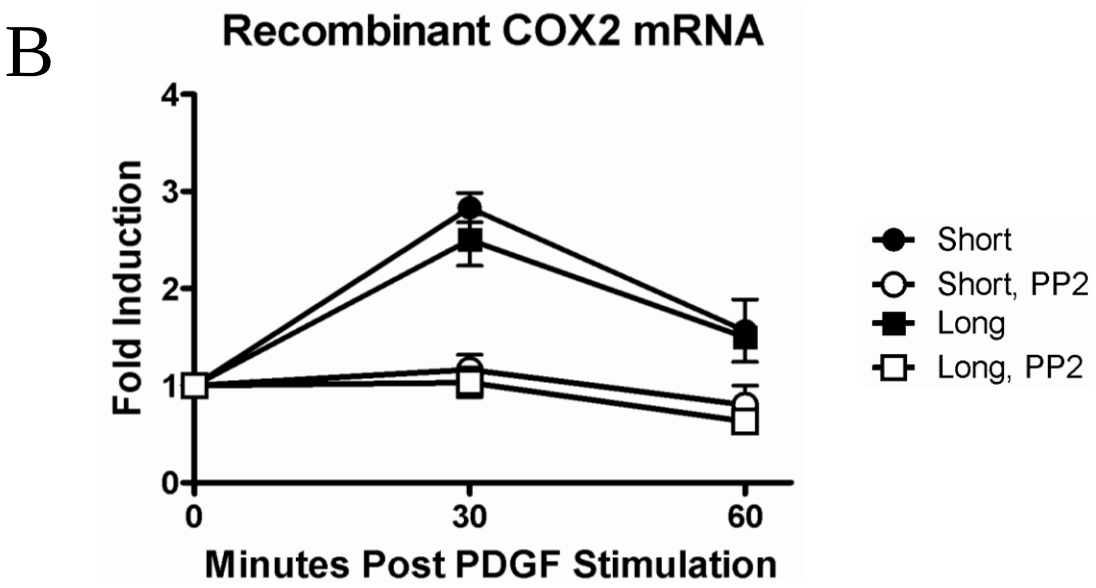
Lane	Components
1	A_{25} probe
2	A_{25} probe + 10µg GST
3	A_{25} probe + 1µg human Nab2
4	COX2 50 mer probe
5	COX2 50 mer probe + 10µg GST
6	COX2 50 mer probe + 1µg CUGBP2

The first 250 nucleotides of the COX2 mRNA 3'UTR is responsive to c-Src signaling

In mammalian cells, CUGBP2 is phosphorylated by c-Src kinase (Xu et al., 2007) and has been shown to interact with the proximal portion of the COX2 mRNA 3'UTR in response to radiation induced signaling (Mukhopadhyay et al., 2003b). To determine whether COX2 mRNA stabilization-mediated by the proximal 250 bases of the 3'UTR is regulated by tyrosine kinase signaling, and therefore possibly by CUGBP2, we produced rat aortic smooth muscle (RASM) cell lines that stably express recombinant COX2 mRNAs consisting of CDS and either the first 250 bases of its 3'UTR, or the full 3'UTR (Figure 4-10A). Each are driven by a CMV promoter and previous studies have shown that any induction of these mRNAs is due to stabilization (Xu et al., 2007). Treatment with PDGF induces these mRNAs 3-fold over basal and the induction of both are inhibited by the c-Src inhibitor PP2, indicating the 250 bases is necessary and sufficient to confer c-Src mediated PDGF stimulated COX2 mRNA stabilization, putatively via CUGBP2.

Figure 4-10. RPA using COX2 and CYP gene specific riboprobes. RASM cells stably expressing the indicated constructs (A) were serum starved for 24 hours and then pre-treated for 1 hour with either the Src-family kinase inhibitor PP2, or control. Cells were then treated with either PDGF for the indicated times, and total RNA was harvested. The RPA band intensity is graphed according to B.





Discussion

This study was conducted to evaluate whether *S. cerevisiae* could be used as a discovery platform for identifying SMART factors for *cis*-acting mRNA stability control elements. This is premised on the notion that if a SMART factor interacts with a specific mRNA sequence coupled to the luciferase coding region, such an interaction may alter luciferase activity. This system does not discriminate based upon the mechanism by which a candidate SMART factor affects changes in luciferase activity levels. For example, luciferase activity could be altered due regulation of mRNA processing, nuclear export, translation, or decay. Conceptually, this system is without prejudice to mechanism; all that is necessary is the co-dependence for both the mRNA sequence and the candidate SMART factor. The system does not require that an mRNA element bind to a candidate protein. Instead, the output of luciferase activity, when controlled properly, is shown to be dependent upon both the mRNA element and the candidate SMART protein. This dependence can result from direct binding of the mRNA element to the candidate protein, or it can result from indirect actions such as SMART factor mediated regulation of endogenous yeast proteins or processes that specifically influence transcripts containing the *cis*-element under investigation.

In the current study, luciferase activity produced by the full length COX2 3'UTR chimera was altered by the presence of PCBP3 or CUGBP2. In concept, it would be possible to determine the specificity of these interactions down to a small mRNA-element level. For example, a luciferase chimera containing only the first 250 nucleotides of the

COX2 3'UTR would likely exhibit altered luciferase activity dependent upon the presence of CUGBP2.

A key advantage of our system is that it can exploit available full-length yeast 2 hybrid libraries to identify proteins that interact with target mRNA sequences. Some SMART factors rely upon a signaling event to cause their subcellular relocalization to the site of interaction with mRNA *cis*-elements (Kitchen et al., *in preparation*). As one approach to ensure that the candidate SMART factors would have an opportunity to interact with the luciferase chimera mRNA transcripts, the screen was performed using nuclear localization signal (NLS) fusion proteins. The NLS is a necessary feature of yeast 2 hybrid libraries, so any such library consisting of full-length proteins would be suitable for use in this system. The nuclear localization of candidate proteins could explain the paradox of higher mRNA levels and lower luciferase activity levels. It is formally possible that the full COX2 3'UTR luciferase chimeras are retained in the nucleus, due to interaction with nuclear localized PCBP3 and CUGBP2. The nuclear retention of mRNA transcripts could produce the observed paradoxical effect of decreased luciferase activity levels (Figure 4-4) and increased mRNA stability (Figure 4-5B).

Though these results are very encouraging overall, there are several areas where the speed and sensitivity of the system could be improved. For instance, there is significant variability between both the luciferase activity and mRNA levels produced by cells that are transformed with the same mRNA and protein expression constructs, thus necessitating large numbers of replicates. This is certainly due in part to the variation of copy number, from colony to colony, of the tet-regulatable luciferase reporter vectors. To reduce mRNA expression level variability, the *cis*-element-containing luciferase

chimeras could be incorporated into the genomes of the *S. cerevisiae* cells. Taking this approach should allow for increased sensitivity and for higher system throughput volumes and speeds.

Finally, the system has contributed new insight into the role of CUGBP2 in the regulation of COX2 mRNA, and has revealed PCBP3 as a new candidate regulatory factor in the post-transcriptional control of COX2 gene expression. CUGBP2 has been implicated in tyrosine kinase mediated post-transcriptional regulation of the COX2 mRNA. Such signaling has been shown to induce the association of COX2 mRNA with CUGBP2, but the region on the mRNA was unknown. This study provides complementary verification of this interaction, not just in the yeast luciferase assay method, but also in the REMSA experiments, which narrowed the site of interaction to within the first 250 nucleotides of the COX2 3'UTR.

Chapter 5: Future Directions and Conclusions

Summary

The goal of my research was to increase our understanding of how cells couple extracellular signaling to changes in mRNA stability. I began by evaluating the ability of existing methods to identify a *trans*-acting factor for the MEK-regulated COX2 mRNA stability control element. Concluding that all methods presented serious selectivity limitations, I chose to take a more functional approach to the problem. I first tested whether the *S. cerevisiae* GPCR-controlled mating pathway (homologous to the higher eukaryotic MEK signaling pathway) adjusted steady-state levels of mating gene transcripts in part by regulating mRNA stability. After providing definitive evidence that *S. cerevisiae* do not regulate mRNA stability downstream of mating pathway activation, I developed a novel yeast-based system to detect interactions between functional RNA elements and candidate *trans*-factors. As part of my efforts to validate the results of the screen, I produced evidence that PDGF receptor activation stabilizes COX2 mRNA transcripts dependent upon c-Src kinase, the putative SMART factor CUGBP2, and the first 250 nucleotides of the COX2 mRNA 3'UTR. Using this system, I also identified PCBP3 as a novel candidate interacting protein for the COX2 3'UTR. Figure 5-1 summarizes my experimental findings.

Chapter 2 – Directions and Conclusions

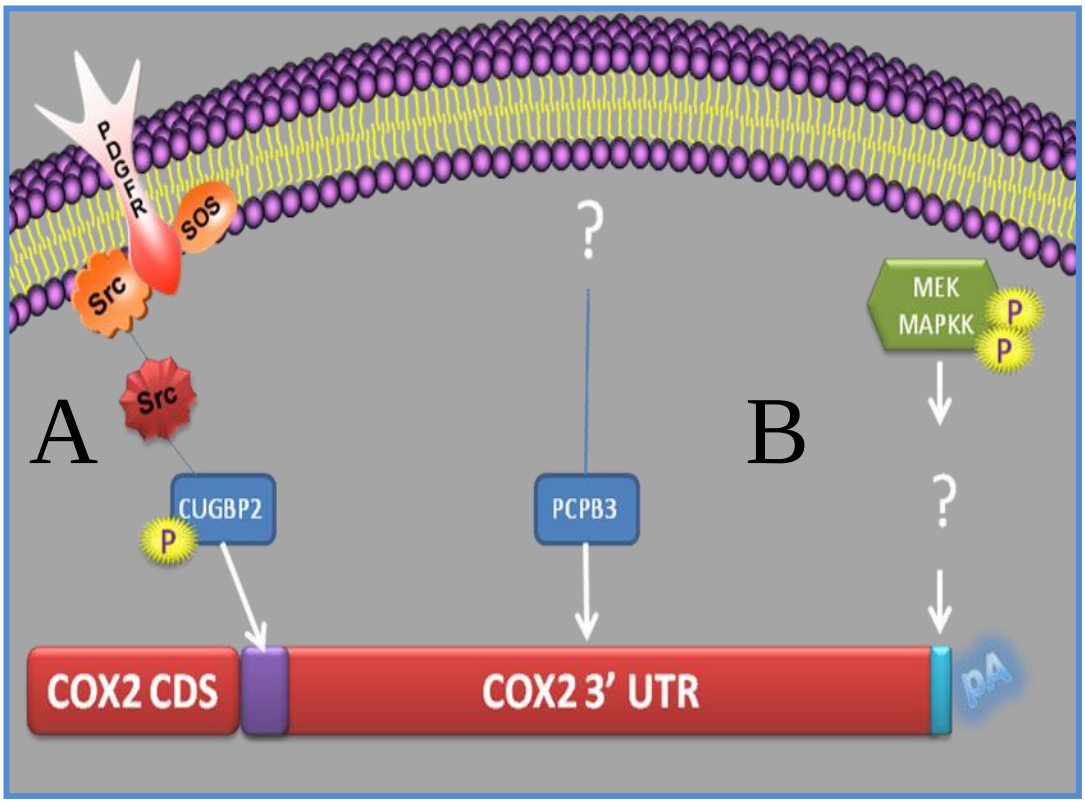
Chapter 2 describes the use of the yeast three hybrid screen to identify MEK-element interacting SMART factors (Hook et al., 2005). Several pieces of evidence suggested that this approach would have a reasonable chance of success. First, the rat MEK-element encoding DNA sequence is highly conserved across mammalian species.

Second, the MEK-element primary sequence is capable of forming secondary structures, as predicted by MFold sequence alignment (Amarzguioui et al., 2000). These secondary structures are similar in organization to those potentially formed by the far distal 3'UTR sequences of several other immediate early genes. Finally, a labeled MEK element mRNA crosslinks to multiple species, as determined by SDS-PAGE. This may indicate that the MEK-element is capable of binding to one or several proteins. Though we discovered no specific SMART factor for the orphan MEK mRNA stability control element, we did develop a rigorous specificity evaluation assay for use in future mRNA/protein interaction studies.

In the future, smaller pieces or mutant versions of the MEK element could be used to perform additional 3-hybrid screens. The rationale for this approach is that because the MEK element contains AU-rich stretches, it may be constitutively bound (in *S. cerevisiae*) by ARE-binding proteins (Duttagupta et al., 2005; Vasudevan and Peltz, 2001). This constitutive binding could cause large complexes of endogenous RNAs and proteins to confound our identification of a specific SMART factor for the MEK stability control element.

Interestingly, according to Figure 2-2 and 2-3, the MEK element appears to be highly conserved in vertebrate lineages only. Recent evidence suggests that certain transcription factors have evolved in response to the increasing developmental demands of multicellular organisms, such as the need for complex nervous and vascular systems (Graef et al., 2001). An example transcription factor is the Nuclear Factor of Regulated T-cells (NFAT), whose appearance on the evolutionary scene coincides with the arrival of vertebrates (Graef et al., 2001; Wu et al., 2007).

Figure 5-1. Summary of dissertation experimental findings. As indicated by the model below, PDGF receptor activation is coupled to COX2 mRNA stability control by c-Src kinase, the putative SMART factor CUGBP2, and a *cis*-acting mRNA stability control element present within the first 250 nucleotides of the COX2 3'UTR (A). The novel interaction system has yet to identify a *trans*-factor that mediates MEK-regulated mRNA stability via the discrete distal regulatory element, but it has successfully identified PCBP3 as a novel candidate SMART factor for the COX2 mRNA (B).



Analogously, certain RNA binding protein *trans*-factors are preferentially expressed in specific cells, such as neurons, vascular smooth muscle cells, or cardiac myocytes (Barreau et al., 2006; Ladd et al., 2001; Ladd et al., 2005; McKee et al., 2005). Taken together, the body of scientific evidence indicates that multicellular organisms regulate gene expression by exploiting both transcriptional regulation and mRNA stability regulation in response to extracellular cues, such as the presence of autocrine, paracrine, or endocrine signaling molecules (Kitchen et al, in preparation). Cross-species sequence conservation analysis provides some evidence that the COX2 mRNA MEK stability control element may be an invention of vertebrates.

Chapter 3 – Directions and Conclusions

Chapter 3 describes a functional approach to the problem defined in Chapter 1. Therein we describe the use of the *S. cerevisiae* α factor mating pathway as a model for MEK-mediated mRNA stability control in a genetically tractable organism. I chose the *S. cerevisiae* mating pathway for two main reasons. First, the induction profile of several mating gene mRNA transcripts mirrors that observed for higher eukaryotic mRNA transcripts that are coordinately induced by transcription and mRNA stability control (Iyer et al., 1999; Roberts et al., 2000). Second, a related yeast species (*S. pombe*) appears to regulate mRNA stability using SMART *trans*-factors and *cis*-acting mRNA regulatory elements downstream of stress pathway activation (Sugiura et al., 2004; Sugiura et al., 2003). We provide strong evidence that mating gene transcript levels are not significantly increased by regulated mRNA stability control (Kitchen et al., 2009). The future direction

for this project rests with the SMART factor / mRNA functional interaction screening system described in Chapter 4.

Chapter 4 – Directions and Conclusions

Chapter 4 describes the development and use of a novel *S. cerevisiae*-based system for identifying mRNA binding proteins that functionally interact with test mRNA transcripts. The screening method involves multiple tet-regulatable yeast expression vectors, each of which contain the entire Luciferase coding sequence (CDS) with either no additional 3'UTR, the MEK element, or the full length COX2 3'UTR. We screened multiple full length mRNA binding proteins for their ability to functionally interact with each of the luciferase constructs as measured by luciferase activity levels. If the presence of a protein is associated with altered luciferase activity, relative to the no protein control, the protein likely regulates the mRNA post-transcriptionally. However, the luciferase assay data alone cannot reveal the type of regulation, such as translational or mRNA stability control. For this reason, we incorporated into the system the ability to precisely measure changes in mRNA decay rates. Overall, the system correctly identified an established mRNA/protein interaction and it may have led to the identification of a novel COX2 mRNA-binding protein.

One of the important strengths of this system is that it can exploit existing yeast 2 hybrid libraries to screen for proteins that interact with functional RNA elements. One caveat to using existing libraries is that these expression constructs necessarily include a nuclear localization signal (NLS). Forcing candidate *trans*-factors to remain in the nucleus may limit the types of interactions that can be detected by this screen. For example, if an interaction between a functional *cis*-element and its corresponding *trans*-

factor occurs exclusively in the cytoplasm, it is possible that luciferase activity will be unchanged, relative to control, and this type of interaction may go undetected. In the future, the episomal tet-regulatable constructs will be integrated into the *S. cerevisiae* genome, to reduce variability due to plasmid copy number. This should significantly improve the utility of this system. We may also explore the possibility of reconstituting mammalian signaling components, for instance by adding in regulatable Src kinase, to determine if the system can read out the influence of post-translational modification of SMART factors on mRNA interaction potential.

Discussion

Careful evaluation of existing methods that are used to study interactions between proteins and mRNA transcripts revealed serious limitations. For all of the methods tested, identifying proteins that interact selectively with specific mRNA transcripts, versus control mRNA transcripts, presents a significant experimental challenge. Taken together, my research suggests that a more functional approach to this problem will be required.

Chapter 6: Materials and Methods

General Methods

Isolation of RNA from *S. cerevisiae*

Flash frozen *S. cerevisiae* cell pellets were resuspended in 150 µL LET (100 mM LiCl, 20 mM EDTA, pH 8.0, 25 mM Tris-HCl, pH 8.0), 150 µL Phenol/LET (LET-saturated Phenol, pH 6.6) and $_{600}$ µL acid-washed glass beads (Sigma, Cat# G8772) was then added. The tubes were alternately vortexed and cooled on ice, each for one minute, for a total of five cycles. After the last vortex/ice cycle, 250 µL DEPC-treated water and 250 µL phenol-chloroform-isoamyl alcohol (50:49:1) was added. The tubes were briefly vortexed and spun down at 16,000 X g for 2 minutes. The aqueous phase (~400 µL) was transferred to clean microcentrifuge tubes, and the remainder of the RNA isolation was completed using chloroform-isoamyl alcohol extraction followed by isopropanol precipitation (Chomczynski and Sacchi, 2006). The pellets were rinsed using 1 mL 70% ethanol, dried for 15 minutes at room temperature, and resuspended in 20 µL DEPC-treated water. The total RNA concentration was quantified using a UV spectrophotometer (Biorad, SmartSpec3000).

RNAse Protection Assay (RPA)

An RNase Protection Assay (RPA) followed by phosphorimage analysis was used to quantitatively determine mRNA levels. To generate riboprobes, 50 ng each of linearized plasmid DNA templates was added to a single reaction tube, and in vitro transcription in the presence of radiolabeled ^{32}P UTP was carried out per the manufacturer's protocol (BD Pharmingen, Multi-Probe Transcription Kit). The probe set was purified using phenol- chloroform extraction followed by ammonium acetate / ethanol precipitation. The probe pellet was rinsed using 1 mL ice cold 70% ethanol, dried

for 5 minutes at room temperature, then resuspended in RPA hybridization buffer (80% formamide, 40 mM PIPES (piperazine-N,N′-bis(2-ethanesulfonic acid), pH 6.7, 400 mM NaCl, 1 mM EDTA, pH 8.0).

To perform the RPA, ~10^5 cpm of the newly transcribed riboprobes was added to 2 µg total yeast RNA (30 µl final reaction volume). The mixture was heat denatured for 5 minutes at 90ºC then immediately transferred to a 42°C incubator for at least 16 hours. Following overnight hybridization, 300 µL RNAse digestion buffer (300 mM NaCl, 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, pH 8.0, and enough RNAse A/T1 cocktail (Ambion, Cat# AM2286) to produce a final dilution of 1:1000) was added. Samples were gently vortexed, spun down for 10 seconds at 10,000 X g, and incubated at 37ºC for 30 minutes. To these digested samples was added 10 µL 20% SDS, 2 µL Proteinase K (20 mg/mL, Promega Cat# 9PIV302), and 1 µL of 10 mg/mL yeast tRNA (Ambion, Cat# AM7119). The samples were gently vortexed, spun down for 10 seconds at 10,000 X g, and incubated at 37ºC for 30 minutes. After incubation, 300 µL 50:49:1 phenol:chloroform:isoamyl alcohol was added and the samples were vortexed for 10 seconds at the highest vortex speed setting. The samples were then centrifuged at 10,000 X g for 5 minutes, and the aqueous phase transferred to clean microfuge tube. The aqueous layer, containing the protected riboprobes, was then ethanol precipitated as previously described (Chomczynski and Sacchi, 1987; Chomczynski and Sacchi, 2006). The radiolabeled protected probes were resuspended in loading buffer (Ambion, Cat# AM8547), heat denatured for 5 minutes at 95ºC. The samples were resolved at room temperature on a 4% vertical urea sequencing gel (Gibco Life Sciences Model SL2) for 1

hour at 65 watts in 0.5 X Tris-Borate-EDTA buffer (45 mM Tris Base, pH 11, 1 mM EDTA, pH 8.0, 45 mM Boric Acid).

The gels were transferred to Whatman® paper and exposed to Typhoon phosphor imaging cassettes for at least 12 hours and the band intensity was measured using the Imagequant Software application. All densitometry summary statistics and graphs were prepared using GraphPad Prism® software.

Chapter 2 Methods

Chemicals, Yeast Strains, and Plasmids

All work with *S. cerevisiae* was performed using standard protocols (Adams et al., 1997; Sambrook and Russell, 2001), and all chemicals were purchased from US Biologicals or Sigma-Aldrich, unless otherwise specified. Summary information for all plasmids and *S. cerevisiae* strains used in Chapter 2 is presented in Table 6-1. The enhanced yeast 3 hybrid plasmids and *S. cerevisiae* cells were a generous gift of Dr. Marv Wickens (Hook et al., 2005).

Cross-Species Conservation Analysis of the MEK-Sensitive mRNA Stability Element

The University of California at Santa Cruz (UCSC) genome browser was used to locate the Mouse COX2 gene (http://genome.ucsc.edu/). We zoomed in to display the proximal promoter sequence, the 5'UTR, the entire coding sequence, the introns, and the relatively large 3'UTR of the COX2 gene. The consensus sequences depicted in Figure 3-3 were produced using WebLogo software (Crooks et al., 2004).

Construction of the mRNA bait vectors

The MEK stability control element (Xu et al., 2000) was blunt cloned into the SMAI site of the pIII-A-MS2-2 yeast 3 hybrid bait vector. DNA sequencing identified the forward (sense) and reverse (antisense) orientation clones, and these were named pCMK70 & pCMK71, respectively. A third "RNA Null" vector, pCMK72, was produced by excising the MS2 stem loop sequence from PIII-A-MS2-2.

RNAse Protection Assays

Performed as described in General Methods (above) except that the gene specific riboprobes used for these experiments were designed to detect the [Bait / MS2 Stem Loop] hybrid mRNA transcripts. Densitometry was used to compare the levels of the control [IRE / MS2 Stem Loop] hybrid mRNA to the levels of the [MEK stability control element / MS2 Stem Loop] hybrid mRNA.

Pre-Transformed Library Screening

The forward MEK stability control element bait vector, pCMK70, was transformed into the yeast 3 hybrid reporter strain YBZ1. Transformed cells were grown in selective media and mated to the pre-transformed murine E11d fibroblast library according to the manufacturers protocol (Clontech, Matchmaker cDNA libraries). A 100 μL aliquot of cells was plated onto double selection media (-Ura / -Leu) to titer the library, and the remaining 10 mL of mated cells was plated onto high stringency quadruple selection media (-Ura / -Leu / -His / -Ade) to identify potential MEK element interacting SMART factors. Colonies were allowed to grow for six days @ 30 °C. Only white colonies were picked for further analysis because the reddish colonies (deficient in Adenine production) very likely represented RNA-independent, false positive

interactions. Though not definitely indicative of RNA-dependent MEK element interacting library clones, the white colonies at least had the potential to be expressing the RNA bait.

5FOA Curing Cells of RNA Bait Plasmids

White colonies from the library screening were scraped and transferred to 96-well assay plates (previously filled with 150 μL 50% glycerol per well). Cells were replica-plated (frogged) onto multiple types of selectable marker plates. One set of plates contained 5FOA / -Leu. Cells would only grow on these plates if 1) they still contained the library plasmid and 2) they had lost the MEK element RNA bait plasmid. The cells growing on the 5FOA / -Leu plates were restreaked onto -Ura / -Leu plates to confirm the absence of the MEK element RNA bait plasmid. The level of reporter gene expression for these "cured" cells was then measured using the Beta-Glo® assay (Promega, described below). Library clones that could activate reporter gene expression in the absence of the MEK element bait plasmid were considered RNA-independent false positives.

Beta-Glo® Assays

Promega's Beta-Glo® assay is a homogeneous bioluminescent assay that couples betagalactosidase activity to a Firefly Luciferase reaction. For all Beta-Glo® assays, cells were grown in double selection media (or single, for the 5FOA-cured cells) in 96-well plate format overnight at 30 °C. The thick cell cultures were diluted 1 to 10, the OD_{600} value was measured for subsequent normalization, and 50 μL of cells from each well was transferred to a 96-well assay dish. An equal volume of Beta-Glo® lysis/assay solution was added to the cells and the mixture was rocked at room temperature for 1 hour. The

luminescence values were then read by a SpectraMax plate reader. The OD_{600} values were used to normalize the raw data to obtain relative Beta-Glo® units.

RNA Dependence and Binding Specificity Assay

MEK element RNA dependent library clones were further subjected to a stringent specificity battery assay. YBZ1 cells were first transformed with either the Forward MEK element (pCK70), the reverse MEK element (pCK71), the Iron Response Element (pIII-A-IRE), or an RNA null version of pIII-A-MS2-2 (pCK72). Each candidate MEK element interacting library clone was then transformed into the battery of RNA bait-transformed YBZ1 cells. Double transformants were subjected to Beta-Glo® assay to assess the influence of each clone on the level of reporter gene expression across the panel of RNA bait-transformed YBZ1 cells.

Mating Pheromone Stimulation of YBZ1 Cells

The battery of RNA bait-transformed YBZ1 cells were transformed with pAD-IRP control or the selected library clones. The cells were grown in 96-well format to ~0.3 to ~0.5 OD_{600}, and then treated with either 50 nM alpha mating factor or an equal volume of water. Cells were harvested at zero, one hour, or three hours after stimulation.

Chapter 3 Methods

Chemicals, Yeast Strains, and Plasmids

All work with *S. cerevisiae* was performed using standard protocols (Adams et al., 1997; Sambrook and Russell, 2001), and all chemicals were purchased from US Biologicals or Sigma-Aldrich, unless otherwise specified. Summary information for all plasmids and *S. cerevisiae* strains used in Chapter 4 is presented in Table 6-1.

PCR Cloning to Produce AnTet-regulatable cDNA

Gene specific primers were designed to amplify the genes *FIG1*, *FIG2*, *FUS1*, and *FUS2*. The forward primers are homologous to sequences that immediately follow the TATA promoter sequence for each gene (White and Jackson, 1992). The reverse primers are homologous to sequences that contain at least 30 nucleotides downstream of the upstream poly A signal (AAUAAA). The PCR fragments were then ligated into BamHI / XbaI linearized tetracycline-regulatable yeast expression vector, pCM190 (Euroscarf, Frankfurt Germany), (Belli G, 1998).

Yeast Cell Growth and α-Factor Stimulation Conditions

S. cerevisiae cultures were grown at 30°C, to mid-log phase, except for temperature sensitive mutant cells. For all experiments, cells were stimulated using 50 nM α-factor at the zero time point. For experiments with the *rpb1-1* mutant, cells were grown at 25°C, to mid-log phase, then switched to the nonpermissive temperature by adding an equal volume of growth media that had been pre-warmed to 49°C (final temperature ~37°C). For experiments with the *pse1-1* mutant, cells were grown to early-log phase, then equilibrated to the indicated temperatures (25°C, 30°C or 37°C) for four hours prior to stimulating with 50 nM α-factor.

Chapter 4 Methods

Chemicals, Yeast Strains, and Plasmids

All work with *S. cerevisiae* was performed using standard protocols (Adams et al., 1997; Sambrook and Russell, 2001), and all chemicals were purchased from US Biologicals or Sigma-Aldrich, unless otherwise specified. Summary information for all plasmids and *S. cerevisiae* strains used in Chapter 4 is presented in Table 6-3 below. Full length RNA-binding protein clones were generously provided by Marc Vidal (Rual et al., 2005).

Construction of Antet-regulatable Luciferase/*cis*-Element Chimeric Plasmids

We previously published a study that made use of luciferase/*cis*-element chimeric expression vectors to identify a minimal MEK-sensitive mRNA control element within the distal COX2 3'UTR (Xu et al., 2000). To shuttle these chimeric constructs into *S. cerevisiae* expression vectors, we cut XF40-Luc (no COX2 element), KX67-Luc (COX2 MEK element), and KX51-Luc (full COX2 3'UTR) just proximal to the Luciferase coding sequence (HindIII-blunt) and immediately distal to the stop codon (PstI). These DNA fragments were purified and ligated into [NotI-blunt / PstI]-linearized tetracycline-regulatable yeast expression vector, pCM190 or Parent Vector (Euroscarf, Germany) (Belli G, 1998).

Table 6-1. *S. cerevisiae* strains and plasmids used in Chapter 2.

Strain / Plasmid	Description	Origin
YBZ1	3-hybrid reporter strain	Marv Wickens Lab
AH109	Library host strain	Clontech
pACT2	Empty library clone vector	Marv Wickens Lab
pIII-A-MS2-2	Empty MS2 stem loop vector	Marv Wickens Lab
pIII-A-MS2-2-IRE	IRE cloned into the pIII-A-MS2-2 vector	Marv Wickens Lab
pAD-IRP	IRP cloned into the pACT2 vector	Marv Wickens Lab
pCMK70	Forward MEK stability control element bait vector. 134nt MEK element inserted into SMAI site of pIII-A-MS2-2	This Study

Table 6-2. *S. cerevisiae* strains and plasmids used in Chapter 3.

Strain / Plasmid	Description	Origin
Wild type (ACY402)	*MATa ura3Δ 0, leu2Δ 0, his3Δ 1, met15Δ 0*	(Brachmann et al., 1998)
bar1delta (ACY667)	*MATa, ura3Δ 0, bar1delta:KAN MX, leu2Δ 0, his3Δ 1, met15Δ 0*	(Brachmann et al., 1998)
ACY203	*MATα, rpb1-1, ura3-52, his4-539*	(Nonet et al., 1987)
ACY1854	*MATa, rpb1-1, ura3-52, his4-539*	This study
ACY1855	*MATa, rpb1-1, ura3-52, his4-539, bar1delta, KAN^R*	This study
pCM190 (pAC2625)	Yeplac195 episomal vector, URA3, AMP^R	(Belli G, 1998)
pAC2626 (pCMK140)	pCM190 + *FUS1* Full-length cDNA insert	This study
pAC2627 (pCMK141)	pCM190 + *FUS2* Full-length cDNA insert	This study
pAC2628 (pCMK142)	pCM190 + *FIG1* Full-length cDNA insert	This study
pAC2629 (pCMK143)	pCM190 + *FIG2* Full-length cDNA insert	This study
pAC2630 (pCMK144)	Riboprobe template for ACY2626	This study
pAC2631 (pCMK145)	Riboprobe template for ACY2627	This study
pAC2632 (pCMK146)	Riboprobe template for ACY2628	This study
pAC2633 (pCMK147)	Riboprobe template for ACY2629	This study
pAC2634 (pUM1)	Riboprobe template for *PRM2*	This study
pAC2635 (pUM2)	Riboprobe template for *FUS2*	This study
pAC2636 (pUM3)	Riboprobe template for *ECM18*	This study
pAC2637 (pUM4)	Riboprobe template for *FIG1*	This study
pAC2638 (pUM5)	Riboprobe template for *PRM6*	This study
pAC2639 (pUM6)	Riboprobe template for *FUS1*	This study
pAC2640 (pUM7)	Riboprobe template for *FIG2*	This study
pAC2641 (pUM8)	Riboprobe template for *ACT1*	This study

RNA-Binding Protein Mini-Library Screening

In order to identify a likely binding partner for the MEK-sensitive mRNA stability control element, a select panel of mRNA-binding proteins was transformed into *S. cerevisiae* harboring either the No 3'UTR, MEK-element, or Full 3'UTR luciferase expression vector. Double transformants were selected for using –Ura/–Trp/+2% Glucose agar plates. After growing for 4 days at 30°C, multiple colonies were picked from each plate, inoculated into 1mL selective media, and rotated at 30°C overnight. For each culture, 50 µL was transferred to 1mL of fresh selection media, and the dilute cultures were grown at 30°C for 4 hours. After incubation, 50µL from each tube was transferred to all-white 96-well luciferase assay plates. Next, 50µL of 2X Luciferase assay buffer was added to all wells, using a multi-channel pipettor, and the assay plates were gently rocked for 1 hour at room temperature. The lysates were then assayed according to the luciferase assay procedure that follows. While the cells were lysing, the OD_{600} value of the dilute cultures was read for later luciferase value normalization.

Determination of Optimal Luciferase Assay Plate Reading Conditions

In order to find the proper luciferase assay reading conditions, we subjected serial dilutions of the highest activity *S. cerevisiae* lysates (i.e. control luciferase transformed cells) to various combinations of luciferase assay buffer incubation times and luminescence integration times. The goal was to find conditions that provided the largest linear detection range surrounding the luciferase values we were likely to observe.

Luciferase Assays

To assess the level of luciferase reporter that was produced in transformed *S. cerevisiae* cells, a 50 µL aliquot from each culture was added to a 96-well assay plate. To each well, Luciferase lysis buffer (25 mM Tris HCl/Phosphate, 4 mM EGTA (E3889), 1% Triton X-100 (Sigma T6878), 10% Glycerol, 2 mM DTT) was added. The assay plate was then rocked at room temperature for 1 hour to lyse the cells. Next, 50 µL Luciferase assay buffer (25 mM Tris/phosphate, 20 mM $MgSO_4$, 4 mM EGTA, 2mM ATP, 1mM DTT, 1mM D-luciferin (Molecular Probes, L2911) was added to the wells, and the luminescence intensity was then read on a luminescence plate reader (SpectraMax).

Antet Transcription Shut-off Assays

S. cerevisiae transformed with one mRNA-binding protein vector and one Luciferase chimera vector were grown to 0.7-1.0 OD then treated with 1 µg/mL anhydrotetracycline (antet). Cells were kept at 30°C and 1 mL aliquots were removed, spun down for 15 seconds, and flash frozen in crushed dry ice at 5 minute intervals. Cell pellets were stored at -80°C until RNA isolation.

Mating Pheromone Stimulation Experiments

To determine whether α mating factor altered the steady-state level of mRNA transcripts containing the MEK element, transformed *S. cerevisiae* were grown to early log phase (OD_{600} ~ 0.3-0.6) in SD media –Ura/–Trp/+2% Glucose at 30°C, then stimulated with 50 nM α factor or water. Aliquots were taken at specific time intervals, then total RNA was isolated as described below. Luciferase and *ACT1* mRNA levels were measured using RNAse Protection Assay (RPA), also described below.

RNA Mobility Shift Assay (REMSA)

All binding reactions were incubated at 20 °C for 30 minutes unless otherwise specified. Samples were either subjected to 10 minutes of UV crosslinking (Stratalinker), or set aside at RT for an equal amount of time. For REMSA using RNAse digestion, 2 μL of 1 to 100,000 diluted RNAse T1 (Ambion, 500 Units / μL) was added (or not) and the resulting mixture was incubated for 30 minutes at 20 °C. All reactions were resolved using a vertical 4% native acrylamide gel run at 4 °C at 30 mA / cm for 45 minutes. The gels were transferred to filter paper then vacuum dried at 80 °C for 30 minutes. The gels were exposed to phosphor image screens for 4 to 8 hours and band intensities were quantified using Image Quant software.

Table 6-3. *S. cerevisiae* strains and plasmids used in Chapter 4.

Strain or Plasmid	Description	Reference
ACY192	*MAT***a** *ura3 leu2 his3 trp1*	(Winston et al., 1995)
pCM190	AnTet regulatable *S. cerevisiae* expression vector, Amp^r, Ura+	(Gari et al., 1997)
pCMK177	pCM190 with Luciferase CDS insert	This Study
pCMK178	pCMK177 with MEK element insert	This Study
pCMK180	pCMK180 with Full COX2 3'UTR insert	This Study
BRUNOL5	Vidal Lab full length human ORF, Amp^r, Trp+	(Rual et al., 2005)
CUGBP2	Vidal Lab full length human ORF, Amp^r, Trp+	(Rual et al., 2005)
hnRNP C	Vidal Lab full length human ORF, Amp^r, Trp+	(Rual et al., 2005)
HuR	Vidal Lab full length human ORF, Amp^r, Trp+	(Rual et al., 2005)
Nucleolin	Vidal Lab full length human ORF, Amp^r, Trp+	(Rual et al., 2005)
PCBP3	Vidal Lab full length human ORF, Amp^r, Trp+	(Rual et al., 2005)
PTBP2	Vidal Lab full length human ORF, Amp^r, Trp+	(Rual et al., 2005)
RBM4	Vidal Lab full length human ORF, Amp^r, Trp+	(Rual et al., 2005)
RBMS2	Vidal Lab full length human ORF, Amp^r, Trp+	(Rual et al., 2005)
RPS16	Vidal Lab full length human ORF, Amp^r, Trp+	(Rual et al., 2005)
GRB10	Vidal Lab full length human ORF, Amp^r, Trp+	(Rual et al., 2005)
RhoGDI	Vidal Lab full length human ORF, Amp^r, Trp+	(Rual et al., 2005)
RBM21	Vidal Lab full length human ORF, Amp^r, Trp+	(Rual et al., 2005)
IRBIT	Vidal Lab full length human ORF, Amp^r, Trp+	(Rual et al., 2005)

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