ENDORIBONUCLEASE-MEDIATED mRNA DECAY INVOLVES THE SELECTIVE TARGETING OF PMR1 TO POLYSOME-BOUND SUBSTRATE

DISSERTATION

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ABSTRACT

Endonuclease-mediated mRNA decay is one important pathway of regulating mRNA turnover rate, and PMR1 is the best characterized vertebrate mRNA endonuclease. It was originally identified as an estrogen-induced ribonuclease activity that catalyzed the degradation of Xenopus albumin mRNA. Although the mechanism involved in targeting substrate mRNA to degradation is unknown, previous evidence suggested that the functional unit of endonuclease-mediated mRNA decay is a ~680 kDa polysome-bound complex. In this study, I have demonstrated that endonuclease-mediated mRNA decay catalyzed by PMR1 is distinctly different from the general exonuclease-mediated mRNA decay pathways, and PMR1 selectively targets and degrades its translating substrate mRNA by formation of a ~680 kDa polysome-bound complex that contains both PMR1 and its substrate mRNA. I have identified two domains involved in targeting PMR1 to polysomes, an N-terminal domain that lies between residues 100 and 150, and a C-terminal domain that lies within the last 100 residues. Loss of either domain inactivated PMR1 targeting to polysomes and stabilized albumin mRNA. A phosphorylated tyrosine residue within the C-terminal polysome targeting domain is required for PMR1-mediated mRNA decay. Changing this tyrosine to phenylalanine inactivated the targeting of PMR1 to
polysomes, blocked binding of PMR1 to the functional complex containing its
substrate mRNA, prevented the targeting of a GFP fusion protein to this complex,
and stabilized albumin mRNA to degradation by PMR1 in vivo. These results
indicate that endonuclease-mediated mRNA decay occurs on a polysome-bound
complex containing PMR1 and its substrate mRNA. Furthermore, the
requirement for tyrosine phosphorylation of PMR1 directly links this effector
endonuclease to key signal transduction pathways.
Dedicated to all of the people who supported me through this work.
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ABBREVIATIONS

\(\alpha\)-PMR1 – anti-PMR1 antibody
ABP – ARE binding protein
Ab 4 – anti-PMR1 antibodies 4
Apo II – apolipoprotein II
ARE – AU-rich element
ATP – adenosine triphosphate
AUF1 – AU-rich element binding factor 1
AU-rich – adenosine-uracil rich
AYUGA – A pyrimidine UGA
\(\beta\)-AR - \(\beta\)-adrenergic receptor
\(\beta\)-ME - \(\beta\)-mercaptoethanol
bp – base pair
cat – chloramphenicol acetyl transferase
cDNA – complementary DNA
CRD – coding region determinant
CRD-BP – coding region determinant binding protein
CRE – cytosine rich element
CTP – cytosine triphosphate
dCTP – deoxycytosine triphosphate
DAN – deadenylating nuclease
Dcp – decapping protein
DEPC – diethyl pyrocarbonate
DNase - deoxyribonuclease
DMSO – dimethyl sulfoxide
DNA – deoxyribonucleic acid
DSE – downstream element
DTT – dithiothreitol
eIF4E/G/F – eukaryotic initiation factors 4 E/G/F
E. coli – Escherichia coli
EDTA – ethylenediaminetetraacetic acid
EGTA – ethylene glycol-bis(\(\beta\)-aminoethyl Ether) N,N,N’,N’-tetraacetic acid
EJC – exon-junction complex
ELAV – embryonic lethal abnormal visual
EMSA – electrophoretic mobility shift assay
ER – estrogen receptor
eRF1/eRF3 – eukaryotic release factor 1/3
E-RmRNAsF – estrogen-regulated mRNA stabilizing factor
ErEN – Erythroid-enriched endonuclease
FBS – fetal bovine serum
G3BP – RasGAP Src homology 3 binding protein
GC-rich – guanosine-cytosine rich
GTP – guanosine triphosphate
3’-OH – 3’-hydroxyl group
hMPO – human myeloperoxidase
IgG - immunoglobulin
IL-2/IL-3/IL-6 – interleukin 2/3/6
IRE – iron response element
IRE-BP – iron response element binding protein
IRP – iron regulatory protein
K_D – dissociation constant
KH domain – K homology domain
7mG-cap – 7-methyl guanosine cap
7mGDP – 7-methyl guanosine diphosphate
7mGMP – 7-methyl guanosine monophosphate
GM-CSF – granulocyte-macrophage colony stimulating factor
hsp70 – heat shock protein 70
LPS – lipopolysaccharide
Lsmp – Sm-like protein
LSM-I – Lsm complex
MAP kinase – mitogen activated kinase
MBU – molecular biology unit
MIE – MATα1 instability element
MONAP – monocyte-derived neutrophil-activating peptide
mRNA – messenger ribonucleic acid
mRNase – messenger ribonuclease
mRNP – messenger ribonucleoprotein particle
NMD – nonsense mediated decay
NSD – non-stop decay
NTF2 – nuclear transporter factor 2
NP-40 – Nonidet P-40 (detergent)
nt – nucleotide
oligo-dT – thymine oligonucleotide
ORN – oligoribonuclease
PABP – poly(A) binding protein (vertebrates)
Pabp – poly(A) binding protein (yeast)
PAN – poly(A) nuclease
PAP – poly(A) polymerase
PARN – poly(A) ribonuclease
PBS – phosphate buffered saline
PCR – polymerase chain reaction
PGK1 – phosphoglycerate kinase 1
pl – isoelectric point
PMR1 – polysomal ribonuclease 1
PMSF – Phenylmethylsulfonyl fluoride
PNPase – polynucleotide phosphorylase
POL III – RNA polymerase III
Ppk – polyphosphate kinase
PTC – premature termination codon
PVDF – Polyvinylidene fluoride
RasGAP – GTPase activating protein (for ras)
REP – repeated extragenic palindrome
rRNA – ribosomal RNA
RNA – ribonucleic acid
RNAi – RNA interference
RNase(s) – ribonuclease(s)
RNP(s) - ribonucleoprotein
RPM – revolutions per minute
RRM – RNA recognition motif
S100 – 100, 000 x g supernatant
S. cerevisiae – Saccharomyces cerevisiae
SDS – sodium dodecysulfate
SDS-PAGE – sodium dodecylsulfate polyacrylamide gel electrophoresis
SGs – stress granules
siRNA – small interference RNA
SKI – super killer
snRNA – small nuclear RNA
snoRNA(s) – small nucleolar ribonucleic acid(s)
SSC – sodium chloride/sodium citrate
SV40 – simian virus 40
TAP – tandem affinity purification
TBS – Tris-buffered-saline
TBST – Tris-buffered-saline Tween-20
TfR – transferrin receptor
TNF-α - tumor necrosis factor α
tRNA – transfer RNA
uORF – upstream open reading frame
UTP – uracil triphosphate
UV – ultraviolet
5'ETS – 5' external transcribed spacer
5'UTR – 5' untranslated region
3'UTR – 3' untranslated region
VEGF – vascular endothelial growth factor
v/v – volume per unit volume
vhs – viral host shutoff
w/v – weight per unit volume
Xa160 – Xenopus albumin RNA 160
Xa470 – *Xenopus* albumin RNA 470
XLN1 – *Xenopus laevis* nuclease 1

**Units**
- µg/ml – microgram per millilitre
- µg – microgram
- µM – micromolar
- µCi – microcurie
- µl – microliter
- µJ/cm² – microjoules per square centimeter
- cm - centimeter
- fmol - femtomole
- g – gram
- g/L – grams per liter
- g/ml – grams per millilitre
- kb – kilobase
- kDa – kilodalton
- mA - milliampere
- ml – milliliter
- ml/g – milliliters per gram
- mg – milligram
- M – molar
- mM – milimolar
- mmol – milimole
- nm - nanometer
- pmol – picomole
- V – volt
- V/cm – volts per centimeter
CHAPTER 1

INTRODUCTION

1.1 Role of mRNA decay in gene expression

Messenger RNA (mRNA) degradation plays an important role in gene expression regulation, quality control of mRNA biogenesis, and antiviral defenses. Post-transcription regulation is especially important when a rapid change in protein level is required or when coordinated expression is required in cells. Many post-transcriptional regulation events are directly related to RNA, and may occur in every step during mRNA biogenesis.

The biogenesis of mRNA involves a complex network of enzymatic processes including transcription, splicing, polyadenylation, editing, export, cytoplasmic localization, translation and degradation. The level of mRNA in the cytoplasm, therefore, reflects a sum of all previous steps, especially a balance between the rate of mRNA synthesis and rate of mRNA degradation. The primary role of mRNA is as a template for protein synthesis, and the amount of protein that can be translated from each molecule of mRNA is determined by the half-life of that molecule. The half-lives of mRNAs can differ as much as 100-fold within
a cell. In *Saccharomyces cerevisiae* transcripts usually have half-lives between 1 to 30 minutes, whereas in mammalian cells, they can vary from 5 minutes to hundreds of hours (1).

Transcripts are thought to possess many sequence and structural features acting in *cis* that participate in modulating mRNA stability. These *cis* acting stability determinants exist in the untranslated region (UTR) or mRNA body. *Trans* acting factors such as RNA binding proteins and ribonucleases are also involved, and act through the *cis* elements to mediate changes in the half-life of the mRNA. Thus, mRNA stability is determined by the interaction between these *cis* and *trans* elements, which imparts a characteristic half-life on a particular mRNA in a cell specific manner and under defined growth and environmental conditions.

Many mRNAs are degraded through a general degradation pathway, while other mRNAs are believed to be associated with a specific pathway. Each of these pathways will be discussed in detail later. The mechanism involved in these pathways adds an additional level of regulation of protein production. A transcript that is stabilized in one cell may be destabilized in other cells or destabilized when growth conditions are changed. Regulation of mRNA turnover is important in both normal physiological conditions and clinical events, including development and differentiation, reproduction, immune responses, and viral infections. Changes in mRNA decay rates occur during the cell cycle and in response to changes in cell environment.
1.2 Degradation of mRNA in E. coli

E. coli has been one of the most studied model systems of mRNA decay. E. coli contains over a dozen ribonucleases that can be classified into two groups: exoribonucleases, which digest mRNA one nucleotide at a time from the mRNA 5’ or 3’ ends, and endoribonucleases, which cleave internal phosphodiester bonds within mRNA. The current model of mRNA decay in E. coli involves a series of endo- and exonucleolytic events: decay is initiated by the endoribonuclease RNase E; the resulting fragment is then degraded by the 3’-5’ exonucleases RNase II and polynucleotide phosphorylase (PNPase). However, this model is oversimplified as many prokaryotic transcripts are polycistronic and the individual cistrons can have significantly different decay rates. Furthermore, the susceptibility of mRNA to ribonucleases is determined by many factors, especially features on the RNAs themselves, including the presence of specific RNase susceptible sequences, RNA structural motifs and translation. Consequently, mRNAs display a wide range of half-lives in E. coli, from a fraction of a minute to over an hour (1).

1.2.1 Endoribonucleases

There are at least four endoribonucleases in E. coli: RNases E, G, III and I/M. Although RNase III and I/M have been hypothesized to participate in mRNA decay, lack of significant change in decay of either pulse-labeled or specific mRNA in deletion mutants suggested that these RNases do not play a major role in mRNA decay.
The degradation of mRNA in Escherichia coli is initiated principally through RNase E (2). RNase E is an endonuclease that plays a central role in both mRNA processing and degradation. RNase E was first identified in a temperature-sensitive mutant strain, where a single amino acid change in the RNase E gene significantly reduced the degradation rate of pulse-labeled mRNA. RNase E plays an important role in decay of a number of specific transcripts as well. Therefore, RNase E is involved in general mRNA decay as well as decay of specific transcripts.

RNase E is a 1061-amino acid large protein. It contains three distinct domains, a N-terminal domain that is responsible for catalytic activity, a middle region rich in arginine residues that binds RNA, and a C-terminal region that is involved in interactions with other E. coli proteins and assembly of a multi-protein complex called the degradosome (3). The C-terminal region is important to its function, as E. coli carrying mutations in the C-terminal region of RNase E shows significant deficit in mRNA decay. RNase E is also involved in processing of a large number of non-translated E. coli transcripts. It is responsible for the maturation of the 5S ribosomal RNA from a 9S precursor. In addition, it is also involved in the processing of the 5' end of 16S rRNA, the maturation of the RNA subunit of RNase P, the degradation of anti-sense inhibitor (RNA I) of plasmid colE1 DNA replication, and the processing of tRNA.

RNase G, encoded by E. coli rng gene (previously called cafA), is another endoribonuclease that plays an important role in mRNA decay. In contrast to the large size of RNase E, RNase G is comprised of only 489 amino acid residues.
RNase G shares 49.5% sequence similarity and 34.1% sequence identity to the amino half of RNase E but lacks an arginine-rich region and a scaffold domain. Like RNase E, RNase G is involved in both RNA processing and mRNA degradation. For example, RNase G initiates the degradation of RNA I and *ompA* mRNA *in vitro* (4). RNase G is also involved in mRNA degradation *in vivo*. The half-lives of *adhE* and *eno* mRNAs were significantly longer in the rng mutant than in the wild-type (5), indicating that both *adhE* and *eno* mRNAs are substrates of RNase G in *vivo*. Although there may be limited functional overlap between RNase G and RNase E, over-expression of RNase G was not sufficient to restore growth to E. coli cells that lack RNase E, indicating that RNase E is essential and RNase G only plays a minor role in mRNA decay in E. coli (6).

Both RNase E and RNase G are 5' end dependent endoribonucleases with a preference for cleaving mRNAs that bear a monophosphate, rather than a triphosphate or hydroxyl, at the 5' end. RNase E preferentially cleaves single stranded RNA at an AU-rich region. The first endonucleolytic cleavage is the rate-determining step in mRNA degradation by RNase E. The endonucleolytic cleavage product containing 5'-monophosphate residues can be rapidly cut at other RNase E sensitive sites and then further processed by other enzymes. It was originally hypothesized that the 5'-terminal phosphorylation can influence distant cleavage events because these RNases contain domains that bind to 5' end of mRNAs, which contributes to the efficiency of cleavage. However, kinetic analysis of substrate cleavage by RNase E and RNase G has revealed that 5' monophosphorylation accelerates the reaction not by improving substrate binding,
but rather by enhancing the catalytic potency of these ribonucleases (7).

Furthermore, the presence of a 5' monophosphate can increase the specificity of cleavage site selection within mRNA.

1.2.2 Exoribonucleases

The mRNA fragments generated by endonucleolytic cleavage serve as substrates of exoribonucleases for further degradation. In E. coli, there are no known 5' to 3' exoribonucleases. In contrast, E. coli contains at least eight 3' to 5' exonucleases (8). Genetic evidence has indicated that only polynucleotide phosphorylase (PNPase), RNase II and oligoribonuclease are directly involved in mRNA decay (9,10).

Although both PNPase and RNase II are 3'-5' exonucleases, several lines of evidences suggest that there are significant differences between them. RNase II uses water as a nucleophile, liberating nucleotide monophosphates (11), whereas PNPase uses phosphate ion as a nucleophile to generate nucleotide diphosphates (12). Although nonviability is only observed in the absence of both enzymes, PNPase and RNase II are not functionally redundant. Biochemical data suggests that the majority of mRNA degradation in E. coli occurs by a hydrolytic mechanism and RNase II represents 90% of the exonucleolytic activity in E. coli cell extracts (13). Of course, as mRNAs constitute only a small percentage of the total RNA synthesized in exponentially growing cells, it is not clear whether the large amount of RNase II present in the cell is primarily used in the processing of rRNAs and tRNAs or in mRNA degradation. The most important observation is
that PNPase is part of a multi-protein complex, called the degradosome (14) (discussed in detail in section 1.2.3). PNPase is less sensitive to secondary structure than RNase II and thus can more completely degrade highly structured RNA molecules. On the other hand, RNase II does not appear to be associated with any other protein(s), therefore, it may not work as efficiently as PNPase on certain RNAs with secondary structures (15). Inactivation of PNPase led to an increase in the steady-state level of most mRNAs, while a large number of E. coli mRNAs, including ribosomal protein genes, were destabilized in the absence of RNase II (17). This suggests that RNase II might function to protect specific mRNAs from the activity of other ribonucleases. In addition, PNPase also functions in residual polyadenylation of mRNA fragments in the absence of poly(A) polymerase I (PAP I) and the incorporation of non-A residues into poly(A) tails in wild-type E. coli (16), providing additional support for a significant role of this enzyme in mRNA metabolism.

Small oligonucleotide fragments generated by mRNA degrading enzymes such as RNase II and PNPase require additional enzyme activities to further process to mononucleotides. PNPase and RNase II dissociate from RNA when the size of the RNA fragment is less than ten nucleotides. The rest of the fragment becomes the target of another exonuclease - oligoribonuclease (ORN). ORN is a processive enzyme that initiates attack at a free 3' hydroxyl group on single-stranded RNA, releasing 5'-mononucleotides in a sequential manner (17). The function of ORN is required for converting RNA oligonucleotides into mononucleotides and ORN is essential for cell viability. Experiments using a
temperature-sensitive ORN construct and pulse-chase experiments with radiolabeled mRNA observed an accumulation of small RNA oligonucleotides at the non-permissive temperature, and suggested a role for ORN in the final steps of mRNA degradation (10).

In E. coli, there are different mechanisms to protect nascent transcripts from degradation by exonucleases. RNase II and PNPase both require free single-stranded 3' ends for binding to their substrates. The 3' ends of nascent transcripts are sequestered in a ternary elongation complex containing the DNA template and RNA polymerase. The protection of nascent messages from the exonucleases also is achieved by the RNA structure formed by the rho-independent transcription termination mechanism. The short oligo (U) stretch at the 3' end of these messages is insufficient for exonuclease binding and the adjacent RNA stem-loop, which is often very GC-rich, locks the 3' end into a stable, double-stranded structure. Another mechanism that E. coli uses against the exonucleases involves repeated extragenic palindrome (REP) sequences. E. coli has nearly six hundred REPs located in intercistronic regions in single copy or more often in multiple copies (18). When transcribed, they fold into stable RNA structures between cistrons within a polycistronic message.

1.2.3 Degradosome

RNase E exists in a complex with several proteins, including PNPase, enolase (glycolytic enzyme), DnaK, RhlB (RNA helicase) and GroEL (3,14,19-21). This complex was designated as the “degradosome”. Various approaches have
revealed protein-protein interactions in the degradosome, where the carboxy-
terminal region of RNase E serves as a scaffold that directly binds PNPase, RhlB, and enolase (3,22). The discovery of endonuclease, exonuclease and helicase in the degradosome suggests that this complex may play an important role in mRNA decay, and that mRNA decay may need these enzymes to work together coordinately. The endonuclease RNase E can initiate degradation; the exonuclease PNPase can degrade the mRNA fragments; and the helicase RhlB can unwind RNA secondary structure that would normally impede the activity of PNPase. The degradosome is required for the degradation of highly structured mRNA (23). Although the exact composition of the degradosome is still unknown, reconstituted complexes from purified components degrade mRNAs efficiently *in vitro*. The functions of all these proteins are necessary for normal mRNA turnover (24).

Several other proteins have been found to co-immunoprecipitate with the degradosome, including polyphosphate kinase (Ppk) and two heat shock proteins GroEL and DnaK (19,25). However GroEL associated with a temperature-sensitive mutant but not wild-type RNase E, suggesting that heat shock proteins may function as chaperones in the assembly or folding of the degradosome (19). Polyphosphate kinase is the enzyme that catalyses the reversible polymerization of the gamma-phosphate of ATP into polyphosphate. Polyphosphate has been found to inhibit mRNA degradation by the degradosome *in vitro*. Thus regulation of polyphosphate levels may control degradosome activity.
Poly(A) polymerase (PAP) is another decay factor that associates with RNase E, although it has not been found in the degradosome. PAP has previously been implicated in mRNA decay (26,27). PAP inactivation prevented polyadenylation and stabilizes the rpsO mRNA if RNase E is inactive (28). PAP may function to help the degradation machinery, in particular PNPase, overcome strong secondary structures by adding a poly(A) tail to facilitate degradosome binding and allow the helicase to unwind these structures (2,23). Both RhlB and PAP were required to facilitate the degradation of REP-stabilized mRNAs; in the absence of RhlB and PAP, mRNA decay fragments containing REP-stabilizers accumulated to high levels (29). REP degradation by RhlB and PNPase requires their association with RNase E as components of the RNA degradosome, providing further evidence that degradosome is involved in the degradation of structured mRNA fragments.

The decay of some E. coli mRNAs in vivo depends on the action of assembled degradosomes, whereas others are acted on by degradosome proteins functionally independent of the complex, implying the existence of different structural features of degradosomes proteins. Electron microscopy studies (30) revealed that degradosomes exist in vivo as multi-component structures that associate with the cytoplasmic membrane via the N-terminal region of RNase E. The steady state levels of individual degradosome proteins differ and degradosome protein components exist both bound and unbound to RNase E in vivo (30). PNPase and enolase are present in large excess relative to RNase E. Helicase RhlB is present in approximately equal amounts to RNase
E at all cell growth stages. RhlB can physically bind to PNPhase both in vitro and in vivo (31). PNPhase, RhlB and enolase may interact physically and functionally with each other independent of RNase E. This raises the prospect that the RNase E-independent complexes of RhlB and PNPhase, which are detected in vivo, may constitute mini-machines that assist in the degradation of duplex RNA in structures physically distinct from multi-component degradosomes.

1.3 Yeast mRNA decay

The Yeast Saccharomyces cerevisiae (S. cerevisiae) has been extensively studied as model system for eukaryotic mRNA decay. Eukaryotic mRNA decay is quite different from prokaryotic decay in that eukaryotic mRNAs experience significant post-transcriptional modifications prior to translation process. These modifications, including splicing, 5’- capping and 3’- polyadenylation, not only bring structural changes to mRNA but also lead to more stable transcripts. Since few yeast transcripts undergo pre-mRNA splicing, 5’ and 3’ end formation play the most important roles in increasing the stability of mRNA and efficiency of translation.

Yeast mRNAs generally have short half-lives, and are degraded by several pathways. The major route for global mRNA degradation is termed the poly(A)-dependent pathway; this pathway is initiated by the shortening of the 3’ poly(A) tail, catalyzed by exonucleolytic activity, followed by removal of the 5’ cap by the decapping enzyme. The remainder of the mRNA can be degraded in a 5’-3’ direction, by the exonuclease Xrn1p, or degraded 3’-5’ by different
exonucleases, termed the exosome. These two pathways are thought to be the primary pathways for general mRNA decay in yeast, because cells that contain mutations affecting both pathways are nonviable.

1.3.1 Deadenylation

In *S. cerevisiae* the major cytoplasmic deadenylase is the Ccr4-Not complex, and Ccr4 is the catalytic subunit within this complex (32,33). The Ccr4-Not complex was initially identified as a general transcriptional regulatory complex. The proteins of this complex are involved in several aspects of mRNA metabolism, including transcription initiation, elongation and mRNA degradation. The second important component of this complex is Pop2 (also known as Caf1), a protein previously identified as a transcription factor. Pop2 is an RNase D family nuclease. *In vitro* studies have demonstrated that yeast Pop2 can display deadenylase activity and is involved in mRNA turnover (34), but *in vivo* its RNase activity may not be required for its function; instead it may function to enhance the activity of Ccr4p (35). Removal of Pop2 did not impair Ccr4 activity *in vitro*, whereas mutations in the Ccr4 putative catalytic residues abolished Ccr4-Not deadenylase function *in vivo* (32,36). Although a Pop2 deletion reduced the rate of *in vivo* poly(A) shortening, over-expression of Ccr4 could complement this defect. In contrast, over-expression of Pop2 could not complement a Ccr4 defect (37). All of these data point toward Ccr4 as comprising the major functional deadenylase activity in yeast. The Ccr4p deadenylase was inhibited *in vitro* by
addition of the poly(A) binding protein (Pab1p), suggesting that dissociation of Pab1p from the poly(A) tail may be rate limiting for deadenylation in vivo (32).

Additional deadenylases including poly(A) binding protein-dependent poly(A) nuclease (referred as to Pan2/Pan3 complex) also function in yeast. The Pan2/Pan3 complex is recruited by Pab1p and is involved particularly in trimming the poly(A) tail (37). Studies carried out using Pan-deficient mutants showed that Pan is responsible for controlling the poly(A) length of newly synthesized mRNA. In addition, accumulation of transcripts at the transcription site was observed in strains lacking subunits of the Pan2/Pan3 complex, indicating that the Pan2/Pan3 complex may also contribute to the release of mRNAs from transcription sites (38). The Pan2/Pan3 complex consists of a catalytic 135-kDa Pan2 subunit with sequence motifs characteristic of RNase D-like 3'-5' exonucleases (39) and a 72-kDa Pan3 subunit of unknown function. The primary function of Pan seems to be to "preset" poly(A) tails to message-specific lengths before or during the nucleocytoplasmic export of mRNAs (40), but it may also contribute to cytoplasmic mRNA turnover as an alternative or complementing pathway to Ccr4-Caf1 (37).

1.3.2 Decapping

In yeast Dcp1p and Dcp2p function together as the decapping complex (41). Dcp2p is the catalytic subunit; Dcp2p alone has demonstrated decapping activity in vitro in the presence of manganese or a high level of magnesium (42). Dcp2p contains a nucleoside diphosphate linked moiety X (NUDX) motif, which
is found in a family of diverse enzymes that catalyze the hydrolysis of nucleoside diphosphate derivatives (43), and appears to be the active site for decapping. Mutations in this motif inactivated the decapping activity of Dcp2p both in vivo and in vitro (44). In addition to the UNDIX domain, Dcp2p also has two other highly conserved domains, termed Box A and B. These two domains have no homology to other functional motifs, but Box B is required for both RNA binding and decapping activity in vitro (45). Box A has been suggested to facilitate the interaction between Dcp1p and Dcp2p.

It is not clear whether Dcp1p also has decapping activity, but the primary function of Dcp1p appears to be to enhance the activity of Dcp2p. Recombinant Dcp1p can clearly enhance the activity of yeast Dcp2p, allowing Dcp2p to decap efficiently under conditions in which it has little activity on its own (44). The crystal structure of Dcp1p reveals two sites that are important for its function: one is required for interacting with Dcp2p in the Dcp1p/Dcp2p complex; another is probably a binding site for other decapping regulatory proteins (46).

The cleavage reaction catalyzed by the Dcp1p/Dcp2p decapping complex converts the substrate of m$^7$GpppX of mRNA to the products of m$^7$GDP and 5'-monophosphate mRNA (47). Production of transcripts with a 5’-monophosphate is very important for the rapid degradation of transcripts by Xrn1p, the 5’-3’ exonuclease, because Xrn1p prefers to degrade transcripts with 5’-monophosphate over those with 5’-triphosphate end and its activity is blocked by cap structure (48).
The specificity of Dcp1p/Dcp2p is mainly contributed by the 7-methyl group of the cap structure. However, addition of the cap analog m$^7$G$_{OH}$ failed to completely inhibit the decapping activity, suggesting that the body of the transcripts might also contribute to the substrate specificity (47). Several additional observations also support the hypothesis that the decapping enzyme might interact with the body of transcripts. First, uncapped mRNAs can effectively inhibit Dcp1p/Dcp2p decapping activity. Second, the decapping complex prefers substrates that are longer than 25 nucleotides. Third, Dcp2p has RNA-binding activity and can bind to RNA (45). These observations suggest that Dcp1p/Dcp2p recognizes mRNA substrate by interactions with both the cap structure and the RNA body.

The second type of decapping enzyme in eukaryotic cells is the scavenger decapping enzyme, Dcs1 in yeast and DcpS in human. DcpS contains a histidine triad (HIT) motif and hydrolyzes the 7-methylated cap structure to generate the products of m$^7$GMP and 5’ diphosphate oligonucleotide. This makes DcpS biochemically different from Dcp1p/Dcp2p. DcpS was first described as an enzyme for decapping of short substrates such as dinucleotide cap structure or capped oligonucleotides (49). DcpS co-immunoprecipitates with the exosome, suggesting that DcpS may function in the 3’-5’ decay pathway to decap the capped oligonucleotide-decay-intermediates produced by exosome mediated 3’-5’ degradation of mRNA (50). DcpS also functions in the 5’-3’ decay pathway where it hydrolyzes m$^7$GDP produced by mRNA decapping to m$^7$GMP and phosphate (51). This prevents the accumulation of m$^7$GDP in cells and the
potential substrate inhibition of Dcp1p/Dcp2p. The breakdown of m\textsuperscript{7}GDP should also prevent mis-incorporation of methylated nucleotides into nucleic acids and generate a unique indicator allowing the cell to monitor mRNA decay (51).

Several proteins that function as regulators of the decapping process in vivo have also been reported. The first class of regulators includes those that can inhibit the decapping process. Translation initiation factors play a significant role in regulating mRNA decapping and decay. The cap-binding protein eIF4E inhibits decapping both in vivo and in vitro, and eIF4E mutants have a more rapid turnover of their RNAs than the wild type (52). Although poly(A) binding protein (Pabp) is necessary for efficient deadenylation, it can inhibit decapping when bound to the poly(A) tail (53). This suggests that deadenylation efficiency is regulated through the interaction between Pabp and deadenylase, and decapping will not proceed until Pabp has been removed from the poly(A) tail. These observations imply that there is a competition between the cap-binding complex and the decapping complex for access to the m\textsuperscript{7}G-cap.

The second class of regulators may not be absolutely required for decapping but their presence enhances decapping efficiency in vivo. These proteins include Lsm complex, Pat1p and Dhh1p. The Lsm complex is a RNA-binding complex which functions not only in splicing and translation initiation but also in decapping by forming distinct complexes, and one of which contains Lsm1 (Lsm1-7p complex) and functions in decapping (54). Another protein, Pat1p/Mrt1p/Spb10p, which is bound to the 40S ribosomal subunit, is also necessary for efficient decapping (55). Interestingly, the Lsm1-7p complex also
affects decapping, suggesting that it may be involved in the competition for cap binding between eIF4E and Dcp1p. Another family of proteins that functions to enhance decapping includes Edc1p, Edc2p and Edc3p (56). Edc1p and Edc2p bind to mRNA and function to recruit Dcp1p/Dcp2p for decapping \textit{in vitro} (57). This suggests that Edc1p and Edc2p may function to nucleate mRNA-specific decapping complexes on specific mRNAs.

Another protein that interacts with Lsm proteins through Pat1p is the major 5’-3’ exonuclease Xrn1p (58). The \textit{Xrn1} gene encodes a 5’-3’ exonuclease that degrades RNA lacking a 5’ cap \textit{in vitro} (59). Xrn1p requires a 5’ monophosphate at the 5’-terminus to degrade single stranded RNA or single stranded DNA and generates mononucleotides (60). Following decapping, the generation of a 5’- monophosphate at the 5’ terminus of the transcript makes the RNA susceptible to Xrn1p activity. Deadenylated and decapped degradation fragments accumulated in yeast strains harboring Xrn1 mutations, indicating that Xrn1p is the major exonuclease activity associated with mRNA decay and the predominant decay pathway in yeast appears to proceed in a 5’-3’ direction (61). Xrn1p shares a high level of homology with the nuclear protein Xrn2p/Rat1p, which is functionally interchangeable with Xrn1p and is required for transcription termination and mRNA processing (62).

1.3.3 \textbf{P bodies and 5’-3’ decay}

In yeast, the predominant decay pathway appears to be 5’-3’ decay.

Although it has been suggested that a stable complex of Dcp1p/Dcp2p, Lsm1-7p,
and Xrn1p represents an mRNA 5'-3' degradation apparatus (63), the precise location for this decay remains unknown. Recent work in Parker’s lab shed light on this issue. Tharun and Parker (64) identified a critical transition in the translating mRNP prior to activation of mRNA decay, in which deadenylated mRNA dissociates from the translating complex of eIF4E, eIF4G and Pab1p, and becomes associated with the decapping complex of Dcp1p and Dcp2p, Lsm1-7p, Pat1, Dhh1p, and Xrn1p. These proteins involved in mRNA decapping and 5'-3' exonucleolytic decay are localized within discrete cytoplasmic foci, termed P bodies (65). GFP-tagged Dcp1p, Dcp2p, Lsm1-7p, Pat1p, Dhh1p, Edc3p and Xrn1p are localized to P bodies (56,65). The size and number of P bodies varies and correlates with the mRNA decapping event, indicating that P bodies are specific sites where mRNAs are decapped and degraded in a 5' to 3' direction. This hypothesis is supported by the observation that mRNA decay intermediates are also found in P bodies (65). The discovery of P bodies as sites of mRNA 5'-3' decay suggests that there might be distinct pools of mRNAs; translating mRNAs are spatially separated from degrading mRNAs. This introduces potential new points of regulation of gene expression. Furthermore, localized mRNA decay in P bodies can prevent premature degradation of translatable mRNAs that are not yet decay substrates. These findings also suggest that the processes of mRNA decapping and 5'-3' degradation involve both biochemical and physical transitions of mRNAs from a polysome-bound translating mRNP complex to complexes that are associated with decay pathways.
1.3.4 Exosome and 3'-5' decay

The exosome was first identified in yeast in 1997 (66). Surprisingly all four components identified at that time are homologous to characterized bacterial 3'-5' exoribonucleases. Rrp44p is homologous to RNase II, while Rrp41p, Rrp42p, and Rrp43p are related to RNase PH. The exosome consists of at least 10 proteins including seven distinct 3'-5' exonucleases that function in both RNA processing and degradation pathways (67). The exosome is an ATP dependent complex that is located in both the cytoplasm and the nucleus. Nuclear and cytoplasmic forms of the complex share 10 common components and differ by the presence of the RNase Rrp6p in the nuclear complex and the putative GTPase Ski7p in the cytoplasmic complex, respectively (68). The 10 common components (Rrp4p, Rrp40p, Rrp41p/Ski6p, Rrp42p, Rrp43p, Rrp44p/Dis3p, Rrp45p, Rrp46p, Mtr3p, Csl4p) are all essential for the activity of the exosome since a defect in one of them can affect the formation of the complex, which is essential for viability (66,68). Six of these proteins are phosphorolytic enzymes that share homology with RNase PH and PNPase, and generate nucleotide diphosphates as products. The other four proteins are hydrolytic enzymes that generate nucleotide monophosphates (69).

The exosome was initially characterized in the pre-rRNA processing pathway where it functions in 3' end trimming of a number of RNAs as well as several small nucleolar RNAs (snoRNAs) and small nuclear RNAs (snRNAs) (68). Mutation of exosome components inhibited the conversion of 7S pre-rRNA to 5.8S rRNA (66). RNA analysis of rrp6? mutants revealed a distinct defect in 7S
pre-rRNA processing, with the accumulation of 5.8S rRNA species that was 3' extended by 30 nucleotides (70). Like most mutations that inhibit the synthesis of 5.8S and 25S rRNA, exosome mutants also showed indirect effects on early pre-rRNA cleavage events that are required for 18S rRNA synthesis (71,72).

In addition to its function in RNA processing in the nucleus, the exosome is involved in 3'-5' mRNA degradation in the cytoplasm. Mutations that affect components of the exosome confer synthetic lethality with mutations in genes involved in the 5'-3' degradation pathway, and conditional double mutants show extremely long mRNA half-lives (73). Interestingly, the two major functions of the exosome in RNA processing and in mRNA degradation can be genetically separated. For example, a point mutation within the RNA binding domain of Ski4p (Csl4p) strongly inhibits mRNA degradation but has no effect on RNA processing, while the csl4-1 allele of the same gene affects RNA processing but not mRNA degradation (74). Rrp47p, as another example, exhibited characteristics of an exosome cofactor required specifically for the 3’ processing of stable RNAs. Rrp47? mutants were defective in the nuclear processing of pre-rRNA, snoRNAs, and snRNAs. In contrast, Rrp47p is not required for the exosome-mediated cytoplasmic 3'-5' mRNA decay pathway or the nuclear degradation of 3'-extended read-through transcripts generated in the rna14-1 mutant (67).

The exosome also functions to degrade RNA in the nucleus where it is required for the degradation of the 5’ external transcribed spacer (5'-ETS) fragment of 5.8S rRNA (66). Mutations in some components of the exosome lead
to accumulation of polyadenylated transcripts in the nucleus, suggesting a role in nuclear RNA degradation (75). The exosome also functions in the degradation of pre-rRNA transcripts that are not properly processed (72). It may also be involved in the deadenylation of the telomerase RNA (76) and degradation of excised and debranched introns (77).

*In vivo* function of the exosome requires the activity of several additional cofactors such as Mtr4p/Dob1p and Ski2p that are not found in exosome preparations or are present only at substoichiometric levels. Since all the functions of the exosome require 3’-5’ exonucleolytic activity, it is likely that the exosome itself is composed of a core set of enzymes whose specific function is modified by the interaction with additional proteins in different processes. Mtr4p and Ski2p are closely related members of the superfamily II RNA helicases. This family of helicases interacts with RNA and utilizes ATP hydrolysis to promote conformational changes in either RNA structure or RNA-protein interactions.

The Mtr4p gene encodes a nuclear RNA helicase that is required for the processing of a variety of RNA species by the exosome. The 3’ end processing of 5.8S rRNA, degradation of the 5’-ETS and normal pre-snoRNA processing require the RNA helicase Mtr4p (78). Defects in nuclear Mtr4p inhibited the processing of 5.8S rRNA, snRNA, and snoRNA, as well as degradation of the 5’-ETS region of the pre-rRNA (68,78). Mtr4p therefore appears to function as a cofactor of the exosome in many of its nuclear functions in pre-rRNA processing and degradation. Mtr4p may function to unwind the RNAs with its helicase
activity, and may also function to target the exosome to these RNAs since many substrates that require helicase do not have any obvious secondary structure.

While all the characterized nuclear functions of the exosome require the RNA helicase Mtr4p/Dob1p, exosome-mediated cytoplasmic mRNA turnover pathways require the superkiller (Ski) complex as a cofactor. The Ski genes were initially identified from mutations that cause over-expression of the killer toxin encoded by an endogenous double-stranded RNA (79). The Ski complex, consisting of the putative RNA helicase Ski2p and the proteins Ski3p and Ski8p (80,81), has been suggested to be an mRNA decay-specific cofactor for the exosome because mutations in the Ski genes inhibited 3'-5' mRNA decay, but had no effect on the functions of exosome in nuclear RNA processing (74,82). The exosome mediated 3'-5' mRNA degradation required Ski2, Ski3, and Ski8 (80,83). The Ski2 gene encodes a cytoplasmic RNA helicase that is required for 3'-5' mRNA degradation. Ski3p is a tetratricopeptide repeat (TPR) protein, and Ski8p is a WD-repeat containing protein. The three Ski proteins form a stable complex with 1:1:1 stoichiometry, and the complex is localized in the cytoplasm (82). A domain in Ski8p involved in mediating interactions with Ski3p and Ski2p was recently identified and found to be required for Ski8p binding to Ski3p and Ski2p in yeast two-hybrid and GST pull-down assays (84). Another required cofactor for the exosome mediated 3'-5' decay pathway is the putative GTPase Ski7p. Ski7p is a cytoplasmic protein whose C-terminal GTPase domain exhibits similarity to the translation factors EF1A and eRF3 (80,85). Ski7p plays an important role in mediating physical interactions between the Ski complex and
the exosome complex. Different regions of the N-terminal domain of Ski7p interact with the exosome and the Ski complex respectively, which recruits both the Ski complex and the exosome to the 3’ end of mRNA for degradation (80).

1.3.5 Specialized pathways

The exosome also functions in several specialized mRNA decay pathways that recognize and degrade aberrant mRNAs. One of these is the nonsense-mediated mRNA-decay (NMD) pathway, where transcripts with premature translation termination codons are degraded either by deadenylation independent decapping (5’-3’ NMD), or by accelerated deadenylation and 3’-5’ exonucleolytic digestion by the exosome (3’-5’ NMD) (81,86,87). The NMD depends on a group of proteins called Upf proteins, and will be discussed in detail in 1.5.

In addition, mRNAs lacking translation termination codons are recognized and rapidly degraded in 3’ to 5’ direction by the cytoplasmic exosome in a process referred to as nonstop decay (NSD) (85,88). In contrast to NMD, nonstop decay does not rely on the Upf proteins. mRNAs lacking termination codons are believed to arise primarily from premature polyadenylation within the open reading frame. Genetic studies in S. cerevisiae have shown that a nonstop mRNA was stabilized in yeast strains lacking exosome subunit proteins but not in an Upf1 mutant strain (88). The exosome-associated protein Ski7p is required for the degradation of nonstop transcripts. It has been proposed that when the ribosome reaches the 3’ end of an mRNA lacking a termination codon, the GTPase domain of Ski7 binds to the empty ribosomal A site and recruits the
cytoplasmic exosome to degrade the transcript from the 3’ end (85,88). Nonstop decay has also been detected in mammals, but very little is known about its mechanism.

Under certain limiting conditions where the process of translation initiation is slowed down due to the effects of mutations in general translation initiation factors such as eIF3, eIF4E and eIF2B, some yeast mRNAs are degraded rapidly through the initiation-mediated mRNA decay pathway. mRNAs from several heat-shock genes, including Hsp70 and Ksp90, are degraded by initiation-mediated decay (89). The two proteins that facilitate nonsense-mediated mRNA decay, Upf1p and Upf2p, are also involved in initiation-mediated decay of these heat-shock mRNAs, although the situations that trigger these two pathways are different. The mechanistic relationship between initiation-mediated decay and nonsense-mediated decay is still unclear. Interestingly, initiation-mediated mRNA decay also requires the Dcp1 and Xrn1 enzymes, and ongoing transcription by RNA polymerase II (90).

1.4 Mammalian mRNA decay

In mammalian cells, cytoplasmic mRNA decay is also normally initiated by deadenylation, followed by either degradation of the mRNA body in a 3’-5’ direction, or removal of the 5’ cap and degradation in a 5’-3’ direction (53). However, in contrast to yeast where the predominant decay pathway appears to be 5’-3’ decay, the relative contributions of 5’-3’ and 3’-5’ mRNA decay in mammals is yet to be definitively elucidated.
1.4.1 Mammalian deadenylases

The major mammalian deadenylase enzyme is poly(A) ribonuclease (PARN) (91). Being absent from yeast, this 3´-5´ exoribonuclease preferentially degrades poly(A), and is a principle component of cell-free extracts capable of efficient poly(A) shortening. Similar to yeast PAN2, PARN is also a member of the DEDD family of RNases (92). However, PARN becomes activated in the presence of the mRNA cap, whereas PAN2 is specifically activated by the poly(A)-binding protein (93). Experiments conducted in *Xenopus* oocytes demonstrated that PARN is a potential deadenylating nuclease *in vivo* and that it is involved in default poly(A) tail removal during *Xenopus* meiotic maturation (94). In somatic cells, PARN also functions in mRNA deadenylation that leads to mRNA decay. Supporting data include the demonstration that the deadenylation activity of HeLa cell S100 extracts was lost after immunodepletion using anti-PARN antibodies (95). PARN is widespread in all species, and is an essential gene required during early development (96).

The second deadenylase enzyme is PABP-dependent poly(A) nuclease (Pan2/Pan3) complex that consists of catalytic Pan2 and regulatory Pan3 subunits. Pan2 has 3' to 5' exoribonuclease activity and requires Mg$^{2+}$ for the enzyme activity. Pan3 interacts with PABP to stimulate Pan2 nuclease activity. Interestingly, the Pan2/Pan3 nuclease complex has higher substrate specificity for poly(A) RNA upon its association with PABP. Consistent with the roles of
Pan2 and Pan3 in mRNA decay, the two subunits exhibit cytoplasmic co-localization (97).

The third deadenylase enzyme Caf1 is an ortholog of the yeast Caf1 protein. The yeast Caf1 is a component of the Ccr4-Not complex, the major cytoplasmic deadenylase of *S. cerevisiae*. Although Caf1 protein belongs to the DEDD family of RNases, Ccr4 appears to be the principle deadenylase of the Ccr4-Not complex. Caf1 is a 3′-5′ exoribonuclease with a preference for poly(A) substrates. In contrast to the two other DEDD family members, Pan2 and PARN, Caf1 is activated by neither poly(A) binding protein nor capped RNA substrates. The rate of deadenylation *in vitro* by Ccr4 and Caf1 are both strongly influenced by secondary structures present in sequences adjacent to the poly(A) tail, suggesting that the ability of both enzymes to deadenylate might be affected by the context of the mRNA 3′-untranslated region sequences (35).

### 1.4.2 Mammalian decapping enzymes and 5′-3′ exonucleases

Several mammalian homologs of yeast decapping proteins have been identified including human Dcp2 (42), and two homologs of Dcp1 (Dcp1A and Dcp1B) (98). The major decapping activity is Dcp2 (99) in mammals. Similar to yeast Dcp2, the decapping activity of mammalian Dcp2 is inhibited by the presence of poly(A) tail; however unlike yeast, competition of cap-binding proteins by cap analog did not influence the efficiency of decapping, and the mammalian Dcp2 contains intrinsic decapping activity (99). This activity is specific to N7-methylated guanosine containing RNA. The hDcp2 enzyme does
not function on the purified cap structure and is not sensitive to competition by cap analog, suggesting that hDcp2 requires the mRNA body for cap recognition. hDcp2 is an RNA-binding protein and its recognition and hydrolysis of the cap substrate is dependent on an initial interaction with the RNA moiety (45). Biochemical characterization of hDcp2 revealed that a 163 amino acid region consisting of two evolutionarily conserved regions, the Nudix fold hydrolase domain and the adjacent Box B region, contains methyl-cap-specific hydrolysis activity. Maximum decapping activity for wild-type as well as truncation mutants of hDcp2 required Mn$^{2+}$ as a divalent cation. Dcp2 is detected in both nuclear and cytoplasmic fractions of mammalian cells, although it is primarily cytoplasmic and co-sediments with polysomes (99).

In mammals, enzymes for decapping and 5’-3’ decay function together in discrete cytoplasmic foci that resemble the P-bodies in yeast. These discrete cytoplasmic foci were originally identified using antibodies specific to a 182-kD protein GW182 and termed GW bodies (100). GW182, characterized by 60 repeats of a glycine (G) and tryptophan (W) motif, contains an RNA recognition motif (RRM) that binds a subset of mRNAs. Further work revealed that GW182 colocalizes with hDcp1 and hLsm4 in GW bodies (101). Co-localization of proteins related to 5’-3’ mRNA decay including hDcp2, hDcp1a, hDcp1b, hCcr4, hLsm1-7, and rck/p54 in specific cytoplasmic structures that are equivalent to yeast P-bodies (42,102,103) suggests that a process of mRNA decay similar to yeast also functions in the degradation of mRNAs in higher eukaryotes. Functional analysis using fluorescence resonance energy transfer confirmed that
hDcp1a and hDcp2 interact in vivo in these cytoplasmic foci. These cytoplasmic foci were dynamic and disappeared after mRNA decay is abolished by cycloheximide and actinomycin D. Inactivation of the Xrn1 exonuclease resulted in poly(A)+ RNA accumulation in these cytoplasmic foci, indicating that they represent active mRNA decay sites (102).

The second mammalian decapping activity is the scavenger decapping enzyme DcpS (104). DcpS preferentially hydrolyzes cap structures from terminal oligo-ribonucleotides generated by the exosome-mediated 3’-5’ decay pathway. Its binding affinity to 5’ cap structures is sensitive to the length of the RNA onto which the cap is attached (105), explaining why intact mRNAs are not substrates for DcpS. DcpS has greater activity on a capped dinucleotide versus a full-length capped mRNA substrate. Moreover, DcpS can efficiently compete with eIF4E for cap binding as demonstrated by its ability to hydrolyze cap structures even in the presence of excess eIF4E, implying DcpS can function to prevent accumulation of complex between cap and eIF4E following mRNA 3’-5’ decay (106). DcpS is also responsible for converting m7GDP produced by Dcp2 cleavage of the cap to m7GMP (51). DcpS functions as an asymmetric dimer with an open non-active conformation and a closed active conformation. This structural feature is important to prevent premature decapping of mRNA because long mRNAs block the conformational changes that are required to form a closed productive active site (105). In HeLa cells, DcpS is predominantly a nuclear protein with low levels of protein detected in the cytoplasm (106), indicating that the catalytic or regulatory role of DcpS is mainly in the nucleus. The presence of cytoplasmic
DcpS suggests that there are likely different decapping pathways that are
catalyzed by Dcp2 and DcpS, and that the two pathways are likely to occur in
different subcellular locations in the cytoplasm.

There is little known about prominent 5’-3’ exonuclease activity in
mammalian cells despite the cloning of a murine homolog to Xrn1p (mXrn1) (107)
and human orthologs to Rat1 (also called Xrn2) (108). Mouse Xrn1 localizes to
the cytoplasm as evidenced by indirect immunofluorescence, and purified Xrn1
functions as a 5’-3’ exoribonuclease, which preferentially digests G4 tetraplex
substrates (107). However, mXrn1 does not have the same dominant activity as
the yeast Xrn1p, and its role in mRNA turnover is unclear.

1.4.3 Mammalian exosome and 3’-5’ exonucleases:

In contrast to the poorly characterized 5’-3’ exonuclease activity,
mammalian homolog to the yeast exosome that contains the major 3’-5’
exonuclease activities has been well studied, and is likely to be the major form of
degradation after deadenylation. Human homologues for 9 of the 11 yeast
exosome proteins have been identified and two of them correspond to
autoantigens produced by patients with polymyositis/scleroderma (PM/Scl)
overlap syndrome (68). These homologues, including Rrp4, Rrp40, Csl4, Rrp41,
PM/Scl75, Rrp46, Dis3p, and PM/Scl100, have been demonstrated to have 3’-5’
exonucleolytic activities (109), suggesting that the function of the exosome in the
3’-5’ decay pathway is conserved in higher eukaryotes.
The mammal exosome complex is present in both the cytoplasm and the
nucleus, and is functionally equivalent to the yeast exosome (94). The exosome
interacts in vitro with the decapping machinery and other regulatory binding
proteins (50). The mammalian exosome recognizes ARE-containing mRNAs
through its interaction with certain ARE binding proteins. This interaction recruits
the exosome to unstable mRNAs, and promotes their rapid degradation. PM-
Scl75 protein was also found to interact specifically with AREs, suggesting that
the interaction between the exosome and AREs plays a key role in regulating the
efficiency of ARE-containing mRNA turnover (110). Immunodepletion of the
human exosome from cell extracts increased the half-life of unstable mRNAs
(111), suggesting that human exosome complex is required for efficient 3'-5'
exonucleolytic decay. Results obtained using mammalian in vitro extracts
suggest that the degradation of ARE-containing mRNAs proceeds predominantly
in the 3'-5' direction, implicating the exosome as a major contributor to ARE-
containing mRNA degradation (110,111). However, the relative contributions of
the exosome and of 5'-3' exonucleases in mRNA decay in vivo have not been
directly tested.

1.4.4 The role of AU-rich elements in mRNA decay

The best-studied cis elements contributing to short half-lives of
mammalian mRNAs are the adenylate, uridylate-rich (AU-rich) instability element
(ARE). AREs are found in the 3' untranslated regions (UTRs) of transiently
expressed genes, including early response genes such as lymphokines,
cytokines, transcription factors, and proto-oncogenes (112). AREs generally consist of repeats of the sequence AUUUA, and function to promote the rapid deadenylation and rapid degradation of the mRNA body (113). Three classes of ARE have been identified based on their sequence features and decay characteristics. Class I AREs contain one to three scattered copies of the pentanucleotide AUUUA embedded within a U-rich region, and are found in c-fos and c-myc mRNAs. Class II AREs have at least two overlapping copies of the nonamer UUAUUU(U/A)(U/A)U in a U-rich environment, and are only found in cytokine mRNAs. Class III AREs, such as the one in c-jun mRNA, lack the hallmark AUUUA but contain U-rich stretches (114). All three ARE classes contribute to mRNA instability in cultured cells through increased rate of mRNA deadenylation.

The diversity of ARE sequences suggests that there may be a number of different trans acting factors that associate with the elements, imparting different regulatory characteristics on transcripts. Indeed, many ARE-binding proteins (Table 1.1), including BRF1, hnRNP D (AUF1), tristetraprolin (TTP), TIAR, TIA-1, KSRP, and the Hu proteins (HuB, HuC, HuD, and HuR), have been identified by various biochemical approaches, such as RNA electrophoretic mobility shift assays (EMSAs) and cross-linking experiments. However, the function and physiological significance of some of those RNA-protein interactions and their roles in ARE-mediated mRNA decay remain unclear. The influence of some ARE binding proteins on mRNA turnover has been extensively studied. BRF1 and TTP function to enhance ARE-mRNA degradation, whereas HuR and NF90 function
to increase ARE-mRNA stability. Both destabilizing and stabilizing effects have been reported for hnRNP D (115).

hnRNP D, the first identified ARE-binding protein, was isolated based on its ability to induce c-myc mRNA decay in vitro (116). hnRNP D is also called AUF1, for ARE/poly-(U)-binding/degradation factor 1. hnRNP D exists as a family of four protein isoforms (p37, p40, p42 and p45) that are derived from an alternatively spliced pre-mRNA, and that differ by their sequences at their N- and/or C-termini (117). Some differences in the RNA binding activity of the various hnRNP D isoforms have been documented with p37 and p42 displaying the highest mRNA binding activities and the most profound effects on mRNA stability. More interestingly, hnRNP D isoforms may have different effects on target mRNA decay: p42 and p45 stabilize but p37 and p40 destabilize ARE-mRNAs (118). The larger isoforms, p42 and p45, are largely nuclear, probably due to the presence of a binding determinant for components of the nuclear scaffold (119). By contrast, p37 and p40 lack this sequence determinant and may be found in both nuclear and cytoplasmic compartments. All four hnRNP D proteins are capable of undergoing rapid nucleocytoplasmic shuttling, and binding of hnRNP D to mRNAs defines the nuclear history of individual mRNAs and can influence their cytoplasmic fate (120).

The high affinity binding activity of hnRNP D to class I and II AREs relies on its two central RNA recognition motifs (RRMs) in combination with both the alanine/glycine-rich region near the N-terminus and the short glutamine-rich region near the C-terminus. Multimerization of hnRNP D is another major affinity
determinant for ARE binding. hnRNP D self-associates into dimeric and hexameric complexes. The alanine/glycine-rich region of the N-terminus is required for dimerization of hnRNP D. The hexameric complex binds the c-fos ARE with nanomolar affinity and correlates with accelerated mRNA decay (121). The multimerization of hnRNP D is hypothesized to provide a large interaction surface for interaction with other proteins. hnRNP D formed complex with the heat shock protein hsp70, the translation initiation factor eIF4G and PABP (118).

ARE-mediated rapid mRNA degradation is regulated through the p38 signal transduction pathway. The p38 pathway has an important role in post-transcriptional regulation in addition to its role in transcriptional regulation. The p38 pathway consists of MAPK kinases (MKK)-3 and 6, p38 itself and the downstream kinase MAPK-activated protein kinase (MAPKAPK)-2. Some class II AREs from inflammatory mRNA conferred instability of a ß-globin reporter mRNA when inserted into the 3´UTR of the stable ß-globin mRNA. This was reversed upon the activation of the p38 pathway (122). Treatment of human monocytes with p38 inhibitor blocked stabilization of ARE-mRNA and reduced ARE-binding by hnRNP D (123). hnRNP D is phosphorylated in vivo, making it a possible candidate for mediating mRNA stabilization by p38 (116). The two phosphorylated serine residues of hnRNP D p40 in resting cells are dephosphorylated upon phorbol ester treatment, suggesting that ARE-binding and mRNA destabilization by hnRNP D might be regulated by phosphorylation (124). The p38 pathway may regulate ARE-directed mRNA turnover by reversible phosphorylation of polysome-associated p40. As another example, c-Jun N-
terminal kinase pathways were required for stabilization of ARE-containing mRNAs associated with the tumor cell metastasis (125).

Another well characterized ARE binding protein is HuR. HuR belongs to a family of RNA-binding proteins related to the Drosophila embryonic lethal abnormal visual (ELAV) proteins. Unlike other members of the ELAV-like family (HuB, HuC, and HuD) which are expressed specifically in the brain and neuronal tissues, HuR has a wide tissue distribution and is expressed ubiquitously in spleen, thymus, intestine and reproductive organs (126). ELAV-like proteins contain a conserved arrangement of three RRMs. Two are arranged in tandem near the N-terminus and the third is at the C-terminus. RNA-binding studies confirmed that the two tandemly arranged RRMs are involved in ARE binding, whereas the C-terminal motif associates with the poly(A) tail (127).

HuR acts as an ARE mRNA-stabilizing factor (128,129), probably by competing with other ARE-binding factors and by protecting the ARE-mRNAs from degradation. Over-expression of HuR stabilizes a reporter mRNA carrying the c-fos ARE by blocking the decay of deadenylated mRNA and stabilizing a deadenylated poly(A)-minus intermediate (130). Addition of recombinant HuB (or Hel-N1) protein (which shares high sequence identity with HuR) to cell extracts also stabilized the in vitro decay of an ARE-containing mRNA by inhibiting the decay of the body of the mRNA (113). HuR is not an abundant protein, and HuR levels can be easily perturbed by over-expression, resulting in mRNA stabilization. Over-expression of HuR has been shown to stabilize reporter mRNAs that contain the p38-regulated AREs from GM-CSF (131), TNF- α (132),
IL-3 (133) and COX-2 (134). However, it appears that HuR and p38 operate by different mechanisms because the p38 pathway stabilizes mRNA by blocking deadenylation, while over-expression of HuR in vivo had little effect on the rate of deadenylation (135). Suppression of HuR expression by RNA interference resulted in destabilization of the ARE-containing mRNAs for VEGF (136), cyclins A and B1 (137) and p21 (138), which is in agreement with the over-expression results. Interestingly, binding of HuB to a class III ARE of the neurofilament M mRNA 3’UTR leads to mRNA recruitment into polysomes in human teratocarcinoma cells, without affecting mRNA turnover (139). This observation indicates that ELAV-like proteins are not only stabilizers of ARE-containing mRNAs, but can also act as translational activators in some circumstances.

HuR is predominantly a nuclear protein. The cytoplasmic HuR may be due to an artificial increase in the level of HuR in the cytoplasm resulting from the stabilization following HuR over-expression. There are dramatic changes in the complexes formed on ARE-containing mRNA probes in electromobility shift assay (EMSA) when HuR is over-expressed (140). mRNA stabilization by endogenous HuR has also been linked to an increase in the cytoplasmic level of the protein. HuR translocates to the cytoplasm in RKO colorectal carcinoma cells following UV treatment and this is associated with an increase in stability of the ARE-containing cyclin-dependent kinase inhibitor p21 mRNA (138). Similarly, cytoplasmic levels of HuR increase dramatically at the onset of myogenesis in a myocytic cell line (141). Over-expression of an active mutant of MAPKAPK-2 results in an increase in the level of cytoplasmic HuR (142).
In general, mRNA turnover mediated by AREs consists of rapid 3'-5' shortening of the poly(A) tail, followed by decay of the mRNA body (63). Deadenylation \textit{in vitro} is more processive on substrates that contain ARE elements than on similar substrates lacking an ARE (113), suggesting that ARE binding proteins may act, directly or indirectly, to recruit deadenylase to the mRNA. Surprisingly, ARE-stimulated deadenylation rates are apparently unaffected by either hnRNP D or HuR (113,143). Presumably, other proteins bound to the ARE interact with the deadenylation machinery and stimulate poly(A) tail shortening. hnRNP D can mediate ARE-mRNA degradation by recruiting the exosome (111). hnRNP D was also found as a component of the a-globin mRNA stability complex, which is not involved in ARE recognition (144). This raises the possibility that the function of ARE-binding proteins is conditioned by the complexes into which they are integrated. Increased levels of HuR or other ELAV-like proteins \textit{in vitro} lead to stabilization of deadenylated, ARE-containing mRNAs (113). In contrast, \textit{in vivo} depletion of hnRNP D leads to a strong stabilization of diverse ARE-containing mRNAs, whereas ectopic expression restores destabilization (145). HuR and AUF1 bind to many common AU-rich target mRNAs and compete with each other, exerting opposing influences on target mRNA stability. Both HuR and hnRNP D are predominantly nuclear but shuttle between the nucleus and the cytoplasm (131,146), suggesting that they bind to nascent ARE-containing nuclear pre-mRNAs and are transported to the cytoplasm as part of the mRNP particles. Therefore, functional links between the two RNA binding protein families have been postulated. HuR and hnRNP D may
be bound to target transcripts on either distinct, non-overlapping sites, or on common sites in a competitive fashion (147). In the nucleus, both proteins were found together within stable ribonucleoprotein complexes; in the cytoplasm, HuR and AUF1 were found to bind to target mRNAs individually, as HuR colocalizes with the translational apparatus and hnRNP D colocalizes with the exosome (147). In addition, the composition and fate (stability, translation) of HuR- or hnRNP D-containing ribonucleoprotein complexes depend on the target mRNA of interest, RNA-binding protein abundance, stress condition, and subcellular compartment.

1.5 mRNA decay surveillance pathway

Eukaryotic mRNAs containing a premature termination codon (PTC) are rapidly destroyed in a process termed nonsense-mediated decay (NMD). This translation-dependent surveillance pathway is one of the best-characterized mRNA decay mechanism in both yeast and mammals. PTCs may result from genomic mutation, inaccurate transcription or improper pre-mRNA processing events, such as splicing and polyadenylation (88).

The key protein factors in NMD are the Upf1, Upf2, and Upf3 proteins. Recognition of a PTC causes the translating ribosome to pause, leading to the assembly of a surveillance complex consisting of Upf proteins on the mRNA, which targets mRNA for rapid decay. Humans possess homologues of the Upf1p and Upf2p proteins and two Upf3p proteins: Upf3A and Upf3B (148). Tethering of any of these proteins downstream of a termination codon targets an mRNA for
rapid decay (149), but how the Upf proteins recruit degradation enzymes is unclear. The Upf complex is highly dynamic as Upf proteins accumulate at different cellular locations. hUpf1 is cytoplasmic, hUpf2 is mainly perinuclear, whereas hUpf3 is a predominantly nuclear, nucleocytoplasmic shuttling protein (148). Recent results demonstrate that phosphorylation of Upf1 by SMG1 and dephosphorylation of Upf1 promoted by SMG5, SMG6, and SMG7 play an important role in NMD (150).

Despite the conservation of Upf factors, there are some key differences in the mechanism of identification of substrates for decay between yeast and mammalian cells. In yeast, a cis-acting downstream sequence element (DSE) appears to be required for recognition of a stop codon as premature (151). During cytoplasmic translational termination, the local mRNP environment in the vicinity of the termination codon may be sensed by the surveillance complex. If the context is poor, indicating inappropriate termination, downstream sequences are scanned by the surveillance complex. The identification of a downstream element (DSE), downstream of the stop codon, will dictate whether the mRNA is subjected to NMD or if translation is reinitiated (151). The sequences of the DSE that are important for activation of NMD have been characterized from a number of transcripts and have revealed that they are typically degenerate sequences that may be present in multiple copies in a given mRNA (152).

The yeast protein Hrp1p has been shown to bind to a DSE as well as Upf proteins (153). Mutations in the Hrp1 gene stabilized NMD susceptible mRNAs without affecting the decay of normal transcripts. Hrp1p binds to Upf1p while
bound to the DSE. A mutant Hpr1p protein that stabilizes PTC containing transcripts has lost the ability to bind the DSE or interact with Upf1p. Thus, Hrp1p bound to the DSE may represent the marker that the surveillance complex, via Upf1p, recognizes during scanning to trigger decay of the transcript.

In mammals, the key element for identifying substrates for NMD is the presence of a splice junction downstream of the termination codon. A PTC is recognized by its position relative to the last exon-exon junction. As a general rule, mammalian transcripts that contain a nonsense codon more than 50 nucleotides upstream of the last exon-exon junction will be subjected to NMD (154). The exon-exon junction is marked by the exon-junction complex (EJC) that is deposited 20-24 nucleotides upstream of the exon-exon junction as a result of pre-mRNA splicing (155). The EJC is a highly dynamic structure that consists of at least eight proteins, some of which leave the nucleus with the mRNA (156,157). The EJC also functions in nuclear export of spliced mRNA and mRNA localization.

Studies in yeast and mammals indicate that degradation of PTC containing mRNA can occur from both the 5' end of the message, involving decapping and 5'-3' exonucleolytic digestion by Xrn1, and the 3' end, through accelerated deadenylation and exosome-mediated 3'-5' decay (87). In yeast, NMD features deadenylation-independent decapping, which is accomplished by Dcp1 (158). Decapping of an NMD substrate does not require the Lsm-I complex or Mrt1p involved in default mRNA decay, suggesting that the decapping enzymes are recruited by a distinct mechanism (55). In mammals, decapping is
mediated by Dcp2. Downregulating Dcp2, exosome component PM/Scl100, or PARN increased the abundance and half-lives of steady-state nonsense-containing mRNAs (159). Furthermore, NMD factors Upf1, Upf2, and Upf3X coimmunopurified with decapping enzyme Dcp2, 5'-3' exonuclease Xrn1/Rat1, exosome components PM/Scl100, Rrp4, and Rrp41, and PARN, indicating that in mammalian cells NMD degrades mRNAs from both 5' and 3' ends by recruiting decapping and 5'-3' exonuclease activities as well as deadenylating and 3'-5' exonuclease activities (159).

In yeast a nuclear degradation pathway exists that removes defective pre-mRNAs using the exosome and Rat1p/Xrn2p 5'-3' exonuclease. In vertebrate cells, aberrant pre-mRNAs that have not been spliced properly or are otherwise deficient in mRNP structure are rapidly degraded. However, even though the exosome complex has a homologous complex identified in vertebrate cells and a murine Xrn1p has been identified, it is not known if this pathway is conserved in mammals.

Nonsense mediated decay of certain mRNAs may be initiated by endonuclease cleavage. β0 thalassemia appears to be caused by endonuclease cleavage upstream of the nonsense codon in exon I and II of the β-globin gene. This endonuclease, with preference for cleavage at UG dinucleotides, is involved in not only the degradation of nonsense-containing β-globin mRNA but also nonsense-free human β-globin mRNAs in mouse erythroid cells (160). This endonuclease activity was localized in polysomes of MEL cells and cleaved β-globin and albumin mRNA in vitro and in vivo at many of the same sites as
polysomal ribosomal ribonuclease I (PMR1) (161). These results suggest that the endonuclease activity functions on translating mRNA, and endonucleolytic cleavage of nonsense-containing β-globin mRNA constitutes one important step in the process of β-globin mRNA decay in erythroid cells. As another example, degradation of PTC-containing messages in Drosophila is initiated by endonucleolytic cleavage(s) in the vicinity of the nonsense codon. The resulting 5’ fragment is rapidly degraded by exonucleolytic digestion by the exosome, whereas the 3’ fragment is degraded by Xrn1 (162).

1.6 Endonuclease-mediated mRNA decay

Both the exonuclease and endonuclease-mediated mRNA decay include processes that interrupt the interaction between the 5’ cap and the 3’ poly(A) tail. Endonucleases are key effectors of mRNA degradation, particularly for mRNAs whose turnover rates are regulated by extracellular stimuli. A growing number of mRNAs have been found that are degraded through endonucleolytic cleavage, including mRNAs encoding the transferrin receptor (163), the cytokine gro a (164), avian apo-very low density lipoprotein II (165), insulin-like growth factor II (166), c-myc (167) and a-globin (168). However, with few exceptions (such as PMR1, which will be discussed in 1.8), the endonucleases responsible the cleavage have not been characterized. It has been shown that these endonucleases prefer to cleave mRNA within specific motif such as AREs or sequences with specific secondary structures; however, this specificity is not strictly followed based on the cleavage products that have been mapped.
The best example of structure-directed cleavage is described by transferrin receptor (TfR) mRNA. The 3´ UTR of TfR mRNA contains five stem-loop structures, which constitute an iron responsive element (IRE) that mediates a large iron-dependent effect on TfR mRNA levels (163,169). Under conditions of low intracellular iron, these stem-loop structures are bound by the iron regulatory protein (IRP), allowing stabilization of TfR mRNA. In contrast, conditions that favor the increase of iron salts or hemin lower the binding of IRP to the stem-loop structures, with the consequent destabilization of TfR mRNA (169,170).

Degradation of the TfR appears to be initiated by an endonuclease whose identity is presently unknown. The endonuclease cleavage site was mapped to a portion of the TfR 3´ UTR containing three IREs, and its mutation was shown to cause TfR mRNA stabilization (163). The endonucleolytic cleavage does not require prior shortening of the poly(A) tail. Given the location of the cleavage site in the vicinity of IRE, it was suggested that binding of IRP may either sterically occlude access to the endonuclease or, alternatively, alter the RNA structure in such a way that prevents endonuclease cleavage.

A similar mechanism was proposed to explain the turnover of a-globin mRNA during erythroid differentiation. The a-globin mRNA contains a C-rich region (CRE) in its 3´ UTR that interacts with a ribonucleoprotein complex, called a-complex. The binding of this complex protects target sequences from a sequence-specific erythroid cell-enriched endoribonuclease (ErEN) activity involved in the turnover of the stable a-globin mRNA (168,171). Interestingly, binding of the a-complex is enhanced by PABP, indicating that the poly(A) tail
may have a negative effect on α-globin mRNA degradation (172,173). β-globin mRNA is also rapidly degraded during erythrocyte differentiation. An endonuclease activity that localized with β-globin mRNA containing polysomes and mRNP complexes is responsible for the rapid degradation (161).

AREs can also serve as endonuclease cleavage sites. One example is the mRNA for monocyte-derived neutrophil-activating peptide (MONAP). This mRNA is cleaved at AUUUA sites following induction of HL-60 cells with phorbol ester, indicating that these sequences may serve as loci for specific endonucleolytic events (174). Several endonucleases have been described to have the ability to cleave AREs in vitro, including a 60-70 kDa endonuclease that degrades the interleukin 2 (IL 2) mRNA in an Mg^{2+}-dependent manner but does not seem to require the cap structure or the poly(A) tail (175). A 65 kDa human site-specific single-strand endonuclease that functionally resembles E. coli RNase E was also shown to target ARE sequences. The 65 kDa protein-mediated cleavages occurred at several positions that resemble RNase E cleavage sites with no preference for a single sequence motif (176).

Some mRNAs, such as c-myc, are regulated by multiple pathways that utilize different endonucleases. Endonuclease G3BP cleaves the ARE of mouse c-myc mRNA in vitro (177). However, the 3´ UTR is not the only determinant of the half-life of c-myc mRNA. The coding region contains a 180–320-nucleotide purine-rich segment, which interacts with a protein that shields it from endonucleolytic cleavage (178,179). The endonuclease that attacks this region is
also tightly bound to polysomes, but unlike G3BP, it is magnesium dependent and appears to degrade polyadenylated c-myc mRNA (167).

G3BP is Ras-GTPase-activating protein (GAP)-binding protein with molecular mass of 68 kDa. It comprises a carboxyl terminal RNA-binding domain (180), the RRM-type domain, an amino terminal domain homologous to nuclear transporter factor 2 (NTF2), and a central domain rich in acidic residues. G3BP binds specific sequences through its C-terminal RRM domain and exclusively cleaves between CA dinucleotides (181). The c-myc mRNA contains a G3BP high-affinity binding site at its 3´ UTR, and decays more rapidly in control fibroblasts compared to fibroblasts deficient in p120 RasGAP. The phosphorylation at Serine 149 was demonstrated to control G3BP subcellular localization (181). Remarkably, G3BP is recruited to stress granules (SGs) in cells exposed to arsenite, suggesting that G3BP may function in regulating mRNAs during cellular stress (182). SGs assembly can be either dominantly induced by G3BP over-expression, or inhibited by expressing a central domain of G3BP. This central domain contains the binding site for RasGAP. Dephosphorylation of serine 149 in this domain after arsenite treatment appears to be important for assembly of SGs (182), suggesting that Ras signaling may contribute to SGs assembly by regulating G3BP dephosphorylation.

1.7 Estrogen regulation of mRNA stability

Estrogens are the best characterized hormones for their roles in regulating the stabilities of specific mRNAs in different species such as frog, chicken and
mammals, implying that this molecular mechanism may be conserved through evolution.

In chicken liver, estrogen specifically stabilizes apolipoprotein II (apoII). ApoII mRNA has a half life of 13 hours in the presence of estrogen; withdrawal of estrogen for 14 days of treatment reduces the half life to 1.5 hours (183). ApoII mRNA does not undergo deadenylatation or decapping. Instead, degradation appears to occur via endonucleolytic cleavage at 5´-AAU-3´/5´-UAA-3´ elements in single stranded loop domains of the 3´UTR of apoII mRNA (165). The cleavages occur in two larger domains of secondary structure of the 3´UTR that bind specifically and independently to two mRNP proteins (184). The protein factor involved in this process is designated as estrogen-regulated mRNA stabilizing factor (E-RmRNASF). E-RmRNASF is responsible for estrogen-mediated stabilization of apoII mRNA. It protects apoII mRNA from targeted endonucleolytic degradation through direct binding of stem loop structure in the upstream region of the 3´UTR of apoII mRNA (185). E-RmRNASF is expressed in the liver in response to estrogen. The expression of E-RmRNASF is also modulated by certain estrogen-mimicking non-steroidal environmental xenobiotics (186) and some therapeutic dietary phytochemicals (187).

In frog, estrogen specifically stabilizes vitellogenin mRNA and destabilizes albumin mRNA (188). The stabilization of vitellogenin mRNA is mediated by vigilin, a 155 kDa estrogen induced hnRNP K homology-domain protein that binds the 3´UTR of vitellogenin mRNA in an unstructured single stranded region (189). The 14 K homology (KH) domains of vigilin are hypothesized to wrap the
RNA around the protein and stabilize the mRNA by preventing PMR1 endonuclease cleavage within this region. Vigilin binds to the vitellogenin mRNA with 30 times more affinity than it exhibits for the albumin mRNA, explaining why vitellogenin mRNA is stabilized while albumin mRNA is actively degraded by PMR1, whose activity is greatly induced by estrogen (190). The competition between vigilin and PMR1 appears to be responsible for the regulation of albumin and vitellogenin mRNA turnover as a result of the endonuclease decay pathway.

Interestingly, estrogen receptor (ER) itself is regulated by estrogen through both transcriptional and post-transcriptional events. The effects of estrogen on regulating the ER mRNA are complex and tissue specific. ER mRNA levels in endometrium were increased after treatment with a physiological dose of estradiol (E2) (191). The 3'-UTR of ER mRNA contains discrete sequences required for E2-enhanced stability. These sequences include a uridine-rich sequence that is predicted to form a loop structure that resembles vitellogenin mRNA in frog liver, suggesting that a protein stabilizing factor is involved in this process. In MCF-7 cells, a breast cancer cell line used widely for ER studies, E2 down-regulates ER mRNA levels by mRNA destabilization. Treatment of MCF-7 cells with E2 resulted in a six-fold decrease in estrogen receptor mRNA half-life, from 4 hours to 40 minutes (192). The destabilization appears to be polysome-dependent. Pactamycin and puromycin, which prevent ribosome association with mRNA, inhibited the effect of E2 on receptor mRNA stability, whereas cycloheximide, which has no effect on ribosome association with mRNA, had no
effect on E2 regulation of estrogen receptor mRNA stability (192). In an *in vitro* degradation assay, polysomes isolated from E2-treated cells degraded ER mRNA faster than polysomes isolated from control cells, suggesting that an estrogen-regulated nuclease activity associated with ribosomes alters the stability of estrogen receptor mRNA (192).

### 1.8 Characterization of PMR1

Polysomal ribosomal ribonuclease I (PMR1) is the first mRNA endonuclease linked to mRNA turnover that has been purified to homogeneity. In *Xenopus* liver, estrogen induces a change of the translational pattern for secreted proteins, resulting in the destabilization of serum protein such as albumin mRNA and transcriptional induction and stabilization of vitellogenin mRNA (193). 95% of the albumin mRNA disappears after 4 days of estrogen stimulation, while transcriptional levels remain unchanged, suggesting that a post-transcriptional event leads to these changes in albumin levels (194). In addition to albumin, other serum protein mRNAs including ?-fibrinogen, transferrin, inter-a-trypsin inhibitor, and serum protein 12B are also destabilized by estrogen in a ER-dependent manner (195). The degradation of all these mRNAs occurs in the cytosol and is associated with polysomes, suggesting that a ribonuclease is involved in this process. Subcellular fractionation showed that 75% of the decay activity was associated with the polysomes while only 10% was associated with the mRNP fraction. Using polysome extract and an *in vitro* degradation assay with radiolabeled RNAs, albumin mRNA was shown to be preferentially degraded.
by the estrogen-stimulated ribonuclease activity over the intracellular protein mRNA, ferritin. This observed activity generated discrete degradation fragments and appeared to be an endonuclease (196).

The endonuclease was then purified from polysomes of estrogen-treated frogs (197) and was named PMR1. The 2512 bp cDNA of PMR1 encodes for an approximately 80 kDa polypeptide that appears to be processed to the amino-terminally truncated 62/64 kDa forms (198). The enzyme bears no sequence or structural similarity to the known superfamily of vertebrate endonucleases, yet shares a 57% sequence identity with human myeloperoxidase (hMPO). Unlike hMPO however, PMR-1 does not possess a heme group, does not have peroxidase activity and is not N-linked glycosylated (198). Nuclease activity was divalent cation independent, resistant to placental ribonuclease inhibitor and did not require an RNA cofactor (197). In vitro mapping using primer extension showed that PMR1 preferred to cleave the sequence AYUGA, but was not limited to this motif. AYUGA is present in unstable serum protein mRNAs (199). However, the sequence is not found in the intercellular ferritin protein mRNA, which is not specifically degraded by PMR1.

PMR1 interacts with its target albumin mRNA and exists in a latent form within albumin mRNA-containing polysomes and messenger ribonucleoprotein particles (mRNPs), yet little activity is detected in the absence of estrogen-stimulation (200). This suggests that PMR1 forms a complex with its target mRNA before being activated by estrogen and initiating mRNA decay. Localization of PMR1 activity onto polysomes correlates with the estrogen-
mediated destabilization of albumin mRNA. A > 670 kDa large mRNP complex can be released from polysomes after EDTA treatment, suggesting that this may be the functional unit of PMR1. This stable complex contains both PMR1 and its substrate albumin mRNA (200), and other protein components. Upon estrogen stimulation, the unit activity of PMR1 on polysomes increased 22-fold while the unit activity of PMR1 associated with the mRNPs remained unchanged. PMR1 co-sediments with vitellogenin-translating polysomes after cytoplasmic albumin mRNA disappears (200).

Finally, isoelectric focusing showed that the purified PMR1 polypeptides were very basic and separated into a triplet with pI’s of 9.6, 9.7 and 9.9 (197). Its mobility was increased after alkaline phosphatase treatment. This suggests that PMR1 is a phosphorylated protein. The protein sequence of the mature 60 kDa polypeptide has one conserved tyrosine phosphorylation site, seven consensus casein kinase II sites, two protein kinase C sites and two JNK/MAP kinase sites. Using several different antibodies to tyrosine phosphorylation, PMR1 was shown to be subject to tyrosine phosphorylation, but there is no discernable difference in the level of tyrosine phosphorylation upon estrogen treatment (unpublished data). Although there is an example in the literature for phosphorylation regulating a specific ribonuclease activity (177), the role of phosphorylation of PMR1 has yet to be fully elucidated.
1.9 Purpose of this study

This dissertation sought to address three issues:

1. Develop a cell culture system to express PMR1 that reconstitutes its properties in *Xenopus* liver.
2. Identify domains of PMR1 involved in targeting to its functional form -- >670 kDa mRNP complex.
3. Characterize the tyrosine phosphorylation of PMR1 involved in endonuclease-mediated mRNA decay.

1.9.1 Expression of PMR-1 in mammalian cells

Much of the work in this study will use recombinant PMR1. I wished to use two basic expression systems: pcDNA3, which drives constitutive expression from the CMV promoter, and pTRE, in which expression from a minimal CMV promoter is regulated by 7 upstream Tet operator elements. Because *Xenopus* hepatocytes are difficult to culture and transfect, I wished to develop mammalian cell lines with the ability to recapitulate the process of PMR1 targeting to polysomes. I wished to express PMR1 in these cells with transient transfection. Polysome association will be assessed by fractionating cell lysates into polysomes (pellet), mRNP (interface) or free protein (upper portion) using sucrose discontinuous (step) gradients or continuous linear gradients as described previously (201). A catalytically inactive form of PMR1 (PMR\(^0\)), prepared by replacing the opposing histidine residues in the presumptive active site with alanines, will be used in order to avoid possible artifacts resulting from
mRNA cleavage events. The selected cell line has to meet two criteria. First, to minimize interference with polysome targeting by exogenous PMR1 or its mutant forms and to restrict changes in albumin mRNA to the activity of the expressed endonuclease, these cells should not express the 67 kDa PMR1-like endonuclease activity that was identified previously in murine erythroleukemia (MEL) cells. Second, transfected cells should display a similar distribution of PMR1 and albumin mRNA on sucrose density gradients as Xenopus hepatocytes (200).

Ultimately I wished to develop a cell line that stably expresses PMR1 either constitutively or in a tet-responsive regulated expression system. This will help to avoid non-physiological conditions caused by over-expression of PMR1 with transient transfection. I also wish to develop a cell line expressing human estrogen receptor a (hERa) to use in reconstituting estrogen regulation of mRNA decay in mammalian cells in order to dissect the signal transduction pathway that leads to activation of PMR1.

1.9.2 Identification of targeting domains in PMR1

A major finding in a previous study was that PMR1 is present in a relatively latent form in a >670 kDa polysome-bound mRNP complex (200). The focus of this study was to determine the molecular interactions that direct PMR1 to this mRNP complex. The first step in this was to identify the portion(s) of PMR1 that is responsible for its binding to the mRNP complex. This is highly significant for the overall field of mRNA turnover because sequences identified
here may be conserved in other mRNA endonucleases, and understanding the nature of these sequences will help determine how these enzymes integrate into their respective mRNP complexes.

I wished to map domains of PMR1 by transiently transfecting cell line with plasmid vectors expressing a series of myc-tagged N- or C-terminal truncated catalytically-inactive form of PMR1 (PMR\(^{\circ}\)). I chose to use catalytically-inactive form of PMR1 because an active RNase is capable of degrading polysome-bound mRNAs and artificially release PMR1 from the mRNP complex. Subcellular targeting of truncated proteins were assessed by sucrose discontinuous or continuous gradients, and the presence of PMR\(^{\circ}\) in each fraction will be determined by Western blot with a monoclonal antibody to the myc epitope. I expected that deleting a domain important in targeting PMR\(^{\circ}\) to polysome-bound mRNP complexes would be apparent by a change in the gradient distribution of the fusion protein. One would also expect that albumin mRNA might no longer co-sediment with the mutated PMR1. The polysome identity would be confirmed by Western blot using an antibody against ribosomal protein and the association of PMR\(^{\circ}\) with polysomes will be confirmed by dissociation of polysomes with EDTA or puromycin.

Should these experiments successfully identify a targeting domain(s) of PMR1, as confirmation of the function of these domains, these putative targeting domains would be fused to GFP, and the association of fusion proteins with polysomes will be determined as previously described and compared to controls lacking the targeting portion of PMR1. If appropriate targeting of GFP fusions to
polysomse is observed, it will indicate that I have correctly identified the polysome-targeting domain. However, it is possible that N- or C-terminal deletions could indirectly alter protein-protein interaction through improper protein folding. If this is the case the targeting domains identified by deletion mutagenesis might not function when fused to GFP, and require adding back portions of PMR1 until targeting is restored.

1.9.3 Characterization of tyrosine phosphorylation in PMR1 mediated mRNA decay

A key issue of endonuclease-mediated mRNA decay is how an mRNA endonuclease finds its target mRNA for degradation. Results from both mammalian and yeast systems indicate mRNA turnover is a complex, dynamic process involving interactions between mRNAs and numerous protein factors, such as large mRNP complexes, translation initiation factors, ribosomes, and nuclease effectors. Since PMR1 was previously identified to associate with its substrate mRNA in the context of the translating mRNP complex, interaction between PMR1 and other proteins in the >670 kDa mRNP complex are likely to play a key role in targeting the mRNA endonuclease to its substrate.

Phosphorylation plays an important role in regulating protein-protein or protein-RNA interactions, and changes in the phosphorylation of RNA-binding proteins modulate the turnover of numerous unstable mRNAs (202,203). It has been reported that hyperphosphorylation of G3BP endonuclease was responsible for activation of the endonuclease activity (177). Given that PMR1 is
activated by estrogen in a transcription independent manner, but the state of tyrosine phosphorylation of PMR1 is not altered in response to estrogen, it is likely that tyrosine phosphorylation may not function directly in activating PMR-1 on membrane-bound polysomes following estrogen-treatment. However, tyrosine phosphorylation may function in regulating protein-protein interactions, and therefore is directly involved in the formation of >670 kDa mRNP functional complex. In addition, tyrosine phosphorylation of PMR1 may be involved in PMR1 subcellular localization and/or directing PMR1 to its target mRNA as well.

I wished to examine the role of tyrosine phosphorylation in PMR1 mediated mRNA decay. Should the tyrosine phosphorylation of PMR1 be confirmed in previously established mammalian expression system, site(s) of tyrosine phosphorylation will be mapped by using site-directed mutagenesis. The potential functions of tyrosine phosphorylation in PMR1-mediated mRNA decay, including protein-protein interactions, PMR1 localization and association of PMR1 with its target mRNA and polysomes, will be studied.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular mass (kDa)</th>
<th>RNA-binding motif</th>
<th>Expression site</th>
<th>ARE</th>
<th>Function</th>
<th>References</th>
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<tr>
<td>hnRNP D</td>
<td>37, 40, 42, 45</td>
<td>RRM</td>
<td>Ubiquitous</td>
<td>c-myc, c-fos, GM-CSF</td>
<td>mRNA destabilization</td>
<td>(116)</td>
</tr>
<tr>
<td>AUBF</td>
<td>ND</td>
<td>ND</td>
<td>T cells</td>
<td>c-fos, interferon, interleukin-3, v-myc, GM-CSF, (AUUUA)_n</td>
<td>ARE-binding correlated with mRNA stabilization</td>
<td>(204)</td>
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<tr>
<td>AU-A</td>
<td>34</td>
<td>ND</td>
<td>T cells</td>
<td>TNF, GM-CSF, c-myc</td>
<td>ND</td>
<td>(205)</td>
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<tr>
<td>AU-B</td>
<td>30</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AU-C</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>hnRNP A1</td>
<td>36</td>
<td>RRM</td>
<td>Human PBMCs</td>
<td>GM-CSF, interleukin-2, c-myc</td>
<td>ND</td>
<td>(206)</td>
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<tr>
<td>hnRNP C</td>
<td>43</td>
<td></td>
<td></td>
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<tr>
<td>ELAV-like</td>
<td>36–40</td>
<td>RRM</td>
<td>Ubiquitous, nervous system</td>
<td>c-myc, c-fos, TNF-a, GM-CSF</td>
<td>mRNA stabilization</td>
<td>(127)</td>
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<td>TIAR</td>
<td>40, 42</td>
<td>RRM</td>
<td>Brain, spleen, lung, liver, testis</td>
<td>TNF, GM-CSF</td>
<td>Translational suppression</td>
<td>(207)</td>
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<td>TIA-1</td>
<td></td>
<td></td>
<td>Brain, spleen, testis</td>
<td></td>
<td></td>
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<tr>
<td>TTP</td>
<td>44</td>
<td>Cys-Cys-His, zinc finger</td>
<td>Fibroblasts, macrophages</td>
<td>TNF, interleukin-3 GM-CSF</td>
<td>mRNA destabilization</td>
<td>(208, 209)</td>
</tr>
<tr>
<td>KSRP</td>
<td>78</td>
<td>KH</td>
<td>Neural cells and other cell types</td>
<td>c-fos</td>
<td>Possibly mRNA destabilization</td>
<td>(111)</td>
</tr>
</tbody>
</table>

Table 1.1 ARE-binding proteins.
CHAPTER 2

MATERIALS AND METHODS

2.1 Plasmid constructions

2.1.1 Cloning of PMR and TAP tagged PMR

The construction of a plasmid for expressing catalytically-active PMR1 with an N-terminal myc epitope tag (myc-PMR60) was described previously (161). Briefly, the 60 kDa portion of Xenopus PMR1 corresponding to the processed, catalytically active form extracted from liver was amplified from a full-length cDNA clone using primers 5'-TGACAAGCTTCCGCCATTACAGGACAGTGC and 5'-GATCGCGGCCGCTTAAGCCACTTTCCAAGGAT (KC42). This was digested with HindIII and NotI, and ligated into the corresponding sites in pTRE-myc (BD Biosciences). The insert bearing the myc epitope tag was PCR amplified using 5'-ACTGGAATTCACCATGGCATCAATGC and KC42. The product was digested with EcoRI and NotI, and ligated into the corresponding sites in pcDNA3. A catalytically inactive form of the protein (myc-PMR60°) was generated by site-directed mutagenesis of the histidines at positions 393 and 479 to alanine using the QuikChange mutagenesis kit (Stratagene). Sense primer 5’-GGTCTTACTGCTTTGCACCACACTTTTTGTGC and antisense primer 5’-
CGCACAAAAAGTGTGGCGAAAGCAGTAAGACC were used to generate the H393A mutation; sense primer 5’-
GTCTTTCCGGATGGGAGCCACTTTAATACAGCC and antisense primer 5’-
GGCTCTATTAAAGTGGCTCCCATCCGGAAGA C were used to generate H479A mutation.

2.1.2 Cloning of PMR deletions

Deletions of myc-PMR60º lacking 50, 100 or 150 amino acids from either the N- or C-terminus were prepared by PCR amplification using the primers listed in Table 2.1. These were denoted as myc-PMRº to distinguish them from myc-PMR60º since they lack portions of the intact protein. PCR amplifications were done in a 100 µl of reaction containing 1 × PCR reaction buffer, 2 mM MgCl₂, 0.2 mM dNTPs mix, 10 ng template DNA, 200 ng each upstream and downstream primers, and 2 U Pfu DNA polymerase (Stratagene). The PCR reactions were cycled as follows:

\[
94°C \times 2 \text{ min} — 1 \text{ cycle}
\]
\[
94°C \times 1 \text{ min} \rightarrow 53°C \times 1 \text{ min} \rightarrow 72°C \times 1 \text{ min} — 30 \text{ cycles}
\]
\[
72°C \times 5 \text{ min} — 1 \text{ cycle}
\]

The PCR products were purified with the QIAquick® PCR Purification Kit from Qiagen. The amplified products were cleaved with the restriction enzymes indicated in the Table 2.1 and cloned into either pcDNA3 or pTRE vectors to generate products with an N-terminal myc tag. The TAP (tandem affinity purification) tag was PCR amplified from pBS1479 with sense primer 5’-
AGTCGGGCCGCGAAGAGAAGATGGAAAAAGAATTTC and antisense primer 5’-TGCAGATATCTCAGGTTAACTTCCCCGC. The amplified product was cleaved with Not1 and EcoRV and inserted downstream of PMR60° in pcDNA3 or pTRE cleaved with the corresponding restriction enzymes.

2.1.3 Cloning of GFP and fusion proteins

Green fluorescent protein (GFP) was amplified from pTracer-SV40 (Invitrogen) by PCR amplification using primers indicated in Table 2.1 to generate GFP or GFP lacking a stop codon (GFPnostop). Both genes were then cloned into pcDNA3. The portion of the gene encoding the N-terminal 100 or 150 amino acids of PMR60° was PCR amplified with the indicated primers in Table 2.1 and inserted into the N-terminus of GFP in pcDNA3 (100N-GFP, 150N-GFP). Similarly, the portion of the gene encoding the C-terminal 50, 100 and 150 amino acids of PMR60° was PCR amplified and inserted at the C-terminus of GFPnostop in pcDNA3 (GFP-50C, 100C, 150C).

2.1.4 Cloning of stem-loop albumin minigene

The preparation of the albumin minigene plasmid was described previously (210). A 24 bp stem loop removed by Hind III digestion of pT7-SL24-luc-A50 (211), was inserted into a Hind III site in the 5’-UTR of the albumin minigene. The stem loop was produced by the following 52 bp palindromic oligonucleotide 5’-AAGCTTGGGCCAGATCTACGCGTACGTACGC GTAGAT
CTGGGCCCAAG CTT containing HindIII-ApaI-BglII-MluI-SnaBI-MluI-BglII-ApaI-
HindIII.

2.1.5 Cloning of Y649F, Y650F, YYFF and Y650D mutants

Y649F, Y650F, YYFF were generated by site-directed mutagenesis of the

Y649F, Y650F, YYFF were generated by site-directed mutagenesis of the
tyrosine at position 649 or 650 or at both positions to phenylalanines using the

Y649F, Y650F, YYFF were generated by site-directed mutagenesis of the
GeneEditor mutagenesis kit (Promega). Primer 5’-

GeneEditor mutagenesis kit (Promega). Primer 5’-

Y649F; primer 5’-

Y649F; primer 5’-

CGGTTTTACTTTGAGCAGCCT was used for Y650F; primer 5’-

CGGTTTTACTTTGAGCAGCCT was used for Y650F; primer 5’-

YYFF. Primer 5’-

YYFF. Primer 5’-

GGAGACCGGTGTTTTCTTTGAGCAGCCTTCAG was used for YYFF.

GGAGACCGGTGTTTTCTTTGAGCAGCCTTCAG was used for YYFF.

The Y650D mutation was generated by site-directed mutagenesis using

The Y650D mutation was generated by site-directed mutagenesis using

the QuikChange site-directed mutagenesis kit (Stratagene) using the forward

the QuikChange site-directed mutagenesis kit (Stratagene) using the forward

primer 5’-GACCGGTTTTACGATGAGCAGCC and the reverse primer 5’-

primer 5’-GACCGGTTTTACGATGAGCAGCC and the reverse primer 5’-

GGCTGCTCATCGTAAAACCGGTC. To prepare a tetracycline-regulated

GGCTGCTCATCGTAAAACCGGTC. To prepare a tetracycline-regulated

plasmid expressing PMR60 the sequence encoding the full-length protein was

plasmid expressing PMR60 the sequence encoding the full-length protein was

PCR amplified from the pcDNA3-containing plasmid using the primers 5’-

PCR amplified from the pcDNA3-containing plasmid using the primers 5’-

TGACAAGCTTCCGCCATTACAGGACAGTGC and 5’-

TGACAAGCTTCCGCCATTACAGGACAGTGC and 5’-

GATCGCGGCGGCTTAAGCCACTTTCCAAGGAT, which contain restriction sites

GATCGCGGCGGCTTAAGCCACTTTCCAAGGAT, which contain restriction sites

for NotI and HindIII, respectively. The amplified product was then digested with

for NotI and HindIII, respectively. The amplified product was then digested with

HindIII and NotI and ligated into the corresponding sites in pTRE-myc (BD

HindIII and NotI and ligated into the corresponding sites in pTRE-myc (BD

Biosciences). The sequences of all plasmids were confirmed before use in

Biosciences). The sequences of all plasmids were confirmed before use in

transfection experiments.
2.2 Site directed mutagenesis

2.2.1 Site directed mutagenesis using GeneEditor

Phosphorylation of oligonucleotides was performed by mixing 100 pmol mutagenic oligonucleotide, 2.5 µl kinase 10 X buffer, 2.5 µl ATP (10 mM), 5 U T4 polynucleotide kinase (PNK) and water to a final volume of 25 µl. The above reaction was incubated at 37 °C for 30 min. At the end of reaction, the T4 PNK was inactivated by incubating at 70 °C for 10 min. The double-stranded DNA template was alkaline denatured prior to use by mixing 0.5 pmol of DNA template with 2 µl 2 M NaOH containing 2 mM EDTA and water to a final volume of 20 µl. The mixture was incubated at room temperature for 5 min, followed by addition of 2 µl of 2 M ammonium acetate (pH 4.6) and 75 µl of 100% ethanol. The denatured DNA was incubated at -70 °C for 30 min to precipitation, then centrifugated at 14,000 x g for 15 min at 4 °C. The DNA pellet was washed with 200 µl of 70% ethanol and dried under vacuum. The DNA was suspended in 100 µl of TE buffer (pH 8.0). 0.05 pmol of denatured DNA was then mixed with 0.25 pmol of mutagenic oligonucleotides, 1.25 pmol of selection oligonucleotide complementary to the same DNA strand, 2 µl of 10 X annealing buffer and water to a final volume of 20 µl. The mixture was heated to 75 °C for 5 min and cooled slowly to 37 °C. Five µl of water, 3 µl of 10 X synthesis buffer, 1 µl of T4 DNA polymerase and 1 µl of T4 DNA ligase was added to the mixture. The reaction was incubated at 37 °C for 90 min to perform mutant strand synthesis and ligation. The heteroduplexed DNA was then transformed into the repair minus *E. coli* strain BMH 71-18 *mutS*, and the cells were grown in selection medium to
select for clones containing the mutant plasmid. Plasmids resistant to the novel GeneEditor antibiotic selection mix are then isolated and transformed into the final host stain, JM109, using the same selection condition.

2.2.2 Site directed mutagenesis using QuikChange

A pair of complementary mutagenic primers was designed with the desired mutation located in the middle of the primer and flanked by unmodified nucleotide sequence. These primers were purified by HPLC or PAGE prior to use in the following steps. The PCR reaction was performed with mutagenic primers and *Pfu* DNA polymerase under the cycling conditions:

\[
\begin{align*}
95^\circ C \times 30 \text{ sec} & \quad \text{— 1 cycle} \\
95^\circ C \times 30 \text{ sec} \rightarrow 55^\circ C \times 1 \text{ min} \rightarrow 68^\circ C \times 2 \text{ min/kb of plasmid length} & \quad \text{— 12 cycles} \\
72^\circ C \times 5 \text{ min} & \quad \text{— 1 cycle}
\end{align*}
\]

Following the PCR amplification, the parental non-mutated supercoiled dsDNA was removed by addition of 2.5 U of *Dpn I* restriction enzyme to the mixture and incubation for 1 hr at 37°C. One µl of the *Dpn I* treated DNA was then used for transformation into E.coli DH5a strain according to the standard bacterial transformation protocol. All mutagenic plasmids were sequenced and confirmed before use in transfection experiments.
2.3 Cell culture and transient transfection

Cos-1 cells were cultured in Dulbecco’s Minimal Essential Medium (DMEM) medium plus 10% fetal bovine serum (FBS) and 2 mM glutamine at 37°C in a CO₂ humidified incubator. Passaging of cells was performed when cell confluency was approximately 80% of the cell culture flask surface area. 2.5x10⁶ cells in log phase growth were seeded on a 10 cm dish one day before transfection. Ten µg (total) of plasmid DNA was diluted into 600 µl of serum free DMEM in a sterile tube. To this tube, 36 µl of PLUS Reagent (Invitrogen) was added and the mixture was incubated for 15 min at room temperature. In a second tube, 24 µl of Lipofectamine Reagent (Invitrogen) was diluted into 600 µl of serum free DMEM. The above two solutions were combined and mixed gently and incubated at room temperature for 15 min. Meanwhile, cells were washed once with 10 ml serum-free DMEM. For each transfection, 2.5 ml serum free DMEM was added to each tube containing the Lipofectamine PLUS Reagent – DNA complexes. The complexes were overlaid onto cells and mixed gently. The cells were incubated for 3 hrs at 37°C in a CO₂ humidified incubator. The DNA– containing medium was removed and replaced with 10 ml of DMEM supplemented with 10% fetal calf serum (FCS). Cells were incubated at 37°C in a CO₂ humidified incubator for an additional 40 hrs before harvest. In experiments using puromycin to disrupt ribosomes 200 µg/ml of puromycin (Sigma) was added to the medium for 30 min before harvest.
2.4 Preparation of cell extracts

Cells were washed twice with ice-cold phosphate buffered saline (PBS) and incubated in PBS with 100 µg/ml cycloheximide for 10 min, then were scraped off the dish with a disposable cell lifter (Fisher Scientific). The cells were pelleted by centrifugation at 1000 x g for 5 min, then suspended in cell lysis buffer (10 mM HEPES-KOH, pH 7.5, 10 mM KCl, 5 mM MgCl₂, 50 mM NaF, 0.5% NP-40 (v/v), 2 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 25 µl/ml of protease inhibitor cocktail (Sigma), 10 µl/ml of phosphatase inhibitor I and II (Sigma), and 10 µl/ml of RNaseOUT (Invitrogen)). After incubation for 15 min on ice, the cells were homogenized with 30 strokes of a Dounce homogenizer (A pestle). Nuclei and debris were removed by microfuge centrifugation at 1000 x g for 10 min, and the post-nuclear supernatants were then centrifuged at 15,000 x g for 20 min. The post-mitochondrial supernatants were transferred to a new microcentrifuge tube and protein concentration was quantified by BCA or Bradford assay.

2.5 Quantification of protein concentration

2.5.1 BCA assays

The Micro BCA protein assay kit (Pierce) was used for BCA assays. Fresh working reagent for BCA assay was prepared by mixing 25 parts of reagent MA and 24 parts reagent MB with 1 part of reagent MC. 0.5 ml of each standard or unknown sample was mixed with an equal volume of working reagent and incubated at 60°C in a water bath for 1 hr. The absorbance at 562 nm was
measured using a Beckman DU 640 spectrophotometer to determine the protein concentration.

2.5.2 Bradford assays

The Bradford reagent (Bio-Rad) was diluted 1:4 with water and filtered through a Whatman number 1 filter. Reactions were carried out in a 96 well dish with 200 µl of diluted reagent added to each well. Protein diluted with water to 10 µl was added and incubated at room temperature for 10 min before the absorbance at 595 nm was determined using a Beckman DU 640 spectrophotometer. Either IgG or BSA of a known concentration was diluted in the sample buffer and used to derive the standard curve for each assay.

2.6 Western blotting and antibodies

Protein samples were prepared in an equal volume in 1X SDS sample buffer (50 mM Tris-HCl, pH 6.8, 50 mM β-ME, 2% SDS, 0.1% (w/v) bromophenol blue, 10% glycerol) then heated to 100 °C for 5 min. Samples were then electrophoresed on a 10% polyacrylamide gel (37.5:1 acrylimide:bis-acrylamide) at 150 V until the bromophenol blue dye had migrated to the bottom of the gel. This was then electroblotted to a PVDF membrane (Millipore) that had been presoaked first in 100% methanol then in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol (v/v)). Protein transfer was performed in transfer buffer at 4 °C and 300 mA for 3 hrs or 16 hrs at 15 mA. The PVDF membrane was then
blocked in a solution of TBST (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Tween-20) with 5% (w/v) non-fat dried milk for 2-16 hrs at 4 °C. The membrane was then probed with a primary antibody for 1 hr at room temperature using dilutions ranging from 1:1000 to 1:10,000 (v/v) in TBST plus 5% milk. The blot was then washed 3 times, 10 min each, with 50 ml of TBST. The secondary antibody was then added and incubated with the membrane for 1 hr at dilutions between 1:10,000 to 1:30,000 in TBST plus 5% dry milk. Again the blot was washed 3 times, 10 min each, with 50 ml of TBST. After a final wash with 1 X TBS, the protein was visualized using chemiluminescent substrate (Pierce). The PVDF membrane was stripped with stripping buffer (0.05 M Tris-HCl, pH 6.8, 2% SDS, 0.7% 2-mercaptoethanol) at 60 °C for 30 min, and then re-blotted with a different antibody if necessary.

Antibodies to the myc epitope tag (9E10), GFP (B2) and ribosomal protein S6 (E13) were purchased from Santa Cruz Biotechnology. The antibody to PMR1 was described previously (197). HRP-coupled rabbit anti-mouse IgG and mouse anti-rabbit IgG were purchased from Santa Cruz Biotechnology.

2.7 In vitro endonuclease activity assay

Cells were transfected with TAP fusions of PMR60 or GFP as control and harvested 30 min after puromycin was added to the cells to dissociate polysome-bound complexes. Fusion proteins were partially affinity purified by IgG-sepharose and recovered by Tev protease cleavage. Catalytic activity of purified proteins were determined by incubating with 250 fmol of a 5′-[32P]-labeled 160 nt
transcript derived from the portion of albumin mRNA bearing the mapped PMR1 cleavage sites (corresponding to nt 1690-2002 of the albumin mRNA). The probe was incubated with affinity purified protein in a buffer containing 30 mM Tris-HCl, pH 7.5, 1 mM DTT, 2 mM MgCl₂ and 75 mM KCl, at 37°C and the reaction was terminated at 0, 5, 10, 15 and 30 min by adding an equal volume of stop solution (98% formamide (v/v), 0.1% (v/v) bromophenol blue and 0.1% xylene cyanole) and heating at 95 °C for 3 min. The reaction mixtures were separated on a denaturing 6% polyacrylamide-urea gel and analyzed by phosphorimager.

2.8 Establishment of stable cell lines

2.8.1 Establishment of PMR1 stable LM (TK⁻) cell lines

Plasmid expressing catalytically active myc-PMR60-TAP driven by a tetracycline-regulated promoter was transfected into LM (TK⁻) cells stably expressing the tetracycline repressor. The Zeocin™ (Invitrogen) selection marker on the pTracer vector was used to select stably transfected cells. The optimal plating density and minimal concentration of Zeocin™ required to kill the untransfected parental LM (TK⁻) cells were determined prior to transfection. Cells were selected in Zeocin™-containing medium for 10 days after transfection. Colonies were then transferred first to 24-well then to 6-well plates and selection was continued until cells reached confluency. Cells were screened by Western blot with antibody to the myc tag.
2.8.2 Establishment of tetracycline-regulated Cos-1 cell lines

Plasmid pCMV-tetR expressing tetracycline-controlled transactivators containing modified VP16 activation domains and a hygromycin resistance gene was used to construct a tet-off Cos-1 cell line. The optimal plating density and minimal concentration of 100 µg/ml of hygromycin required to kill the untransfected parental cell line were determined before transfection. Cells were selected in hygromycin-containing medium for 10 days after transfection. Individual colonies were then transferred first to 24-well then to 6-well plates and selection was continued until cells reached confluency. Tet-responsive cells were screened by co-transfection with a plasmid expressing firefly luciferase in a tet-responsive vector and a control plasmid expressing Renilla luciferase. After incubating in medium containing either no doxycycline or 1 µg/ml of doxycycline for 40hrs, cells were harvested and cytoplasmic extracts were assayed with a dual-luciferase reporter assay report system (Promega). Cells exhibiting over 20-fold induction were selected for further experiments.

2.9 Polysome profile analysis with discontinuous sucrose gradients (step gradients)

Previously our lab had established a rapid method to fractionate mRNP and polysome complexes from post-mitochondrial extract (201). Polysomes and mRNP complexes were separated on discontinuous sucrose gradients containing 0.6 ml each of 10% and 35% (w/v) sucrose in the buffers containing 10 mM HEPES-KOH, pH 7.5, 10 mM KCl, 5 mM MgCl₂, 2 mM DTT, 0.5 mM PMSF.
Post-mitochondrial extract (0.2 ml) in either 5 mM MgCl$_2$ or 10 mM EDTA-containing buffer was fractionated by sedimentation for 1 hr at 147,000 x g in a Beckman TL100 tabletop ultracentrifuge using a Beckman TLS55 rotor. Polysomes pellet to the bottom of the tube and mRNP complexes were collected at the interface between the 10 and 35% sucrose layers. The polysomes were dissolved in the buffer used for the sucrose gradients, and both the polysome and mRNP fractions were dialyzed against 10 mM HEPES-KOH, pH 7.5, 10 mM KCl, 2 mM MgCl$_2$, 1 mM DTT, 0.5 mM PMSF at 4 °C. Absorbance at 260 nm was measured using a Beckman DU 640 spectrophotometer to estimate the amount of recovered material. Aliquots were stored at -80 °C. Protein samples from each fraction were concentrated by trichloroacetic acid (TCA) precipitation and analyzed by Western blot. RNA was purified from fractions using TRIzol® reagent (Invitrogen), and analyzed by Northern blot, RT-PCR or ribonuclease protection assay.

2.10 Polysome profile analysis with linear sucrose density gradients

Linear gradients of 10%-40% (w/v) sucrose were prepared with 6.0 ml of 10% sucrose and 6.0 ml of 40% sucrose in 10 mM HEPES-KOH, pH 7.5, 10 mM KCl, 2 mM DTT, 0.5 mM PMSF containing either 5 mM MgCl$_2$ or 10 mM EDTA. Post-mitochondrial extracts were gently layered on top of the gradient, followed by centrifugation for 3.5 hrs at 228,000 x g in a Sorvall TH641 rotor. 0.5 ml fractions were collected from the bottom of the tube, and absorbance was
measured at 254 nm using a Beckman DU 640 spectrophotometer to obtain the polysome profile. Protein samples from each fraction were concentrated by TCA precipitation and analyzed by Western blot. RNAs were purified from fractions using TRIzol® reagent (Invitrogen), and analyzed by Northern blot, RT-PCR or ribonuclease protection assay.

2.11 Glycerol continuous gradient analysis of PMR-containing complexes

Cells were treated with 200 µg/ml puromycin (Sigma) for 30 min before harvest to disrupt ribosomes. A 12 ml of linear gradients containing 10%-40% (v/v) glycerol was prepared in buffer containing 10 mM HEPES-KOH, pH 7.5, 5 mM MgCl₂, 2 mM DTT, and 0.5 mM PMSF. Post-mitochondrial extracts were carefully layered on top of the gradient and separated by centrifugation for 20 hrs at 83,000 x g (4 °C) in a Sorvall TH641 rotor. Molecular size markers containing a mixture of thyroglobulin (MW = 669,000), ferritin (MW = 440,000), catalase (MW = 232,000), lactate dehydrogenase (MW = 140,000) and bovine serum albumin (MW = 67,000) were fractionated on a parallel gradient. 0.5 ml fractions were collected from the bottom of the tube. The protein concentration from 20 µl of each fraction from the gradient containing the molecular size marker was measured with Micro BCA™ Protein Assay Reagent Kit (Pierce). Data were plotted to identify those individual peaks corresponding to every protein standard. Protein samples from each fraction were concentrated by TCA precipitation and analyzed by Western blot. RNAs were purified from fractions using TRIzol®
reagent (Invitrogen), and analyzed by Northern blot, RT-PCR or ribonuclease protection assay. Fractions containing GFP and fusion proteins were transferred to a 96-well plate and measured with a Tecan fluorescence plate reader at excitation wavelength 470 nm.

2.12 IgG-sepharose selection of PMR1-TAP complexes

Cells were transiently transfected with 6 µg of vector (pcDNA3) alone, plasmid expressing myc-PMR60º or one of its various N- and C-terminal PMR1 deletions fused to TAP, plus 2 µg each of plasmids expressing full-length albumin (210) and luciferase mRNA. 250 µl of post-mitochondrial extract in lysis buffer containing 10 mM EDTA was mixed with 50 µl of drained IgG-sepharose™ 6 beads (Amersham Pharmacia Biotech) that were pre-blocked with lysate from non-transfected cells. This was incubated by end-over-end rotation for 1 hr at 4ºC. The beads were washed three times with 0.5 ml of washing buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% NP-40) and once with Tev buffer (10 mM Tris- HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.1%NP-40 and 1 mM DTT) at 4ºC. Bound complexes were eluted by cleavage with 50 U of Tev protease (Invitrogen) in 100 µl of Tev buffer at 4ºC for 2 hrs. Protein and RNA released from the resin were extracted using TRIzol® reagent, PMR1 was analyzed by Western blot using the monoclonal antibody against the myc epitope tag, and RNA samples were analyzed by semi-quantitative RT-PCR. For assay of endonuclease activity, protein recovered from IgG-sepharose by Tev cleavage was incubated with 250 fmol of a 5′-[32P]-labeled 160 nt transcript derived from
the portion of albumin mRNA bearing the mapped PMR1 cleavage sites. The reaction mixtures were separated on a denaturing 6% polyacrylamide-urea gel and analyzed by phosphorimager. Western blots were quantified by scanning densitometry of X-ray films using a Protein Databases 420oe scanning densitometer and ImageQuant™ software (Molecular Dynamics).

2.13 Immunoprecipitation of tyrosine phosphorylated PMR1

Twenty µl of drained SoftLink Avidin resin (Promega) was blocked with 200 µl Cos-1 cytoplasmic extract overnight at 4 °C and followed by 3 washes with washing buffer (10mM Tris-HCl, pH7.5, 150mM NaCl, 0.1% NP-40). Cells transiently transfected with 6 µg of vector (pcDNA3) or plasmid myc-PMR60°-TAP were harvested at 24 or 48 hrs after transfection. Twenty µg of cytoplasmic extracts were incubated with either 5 µl of RC-20:Biotin antibody (BD Transduction Laboratories) or 5 µl of pre-immune serum for 2 hrs at 4°C. The above mixtures were then incubated with blocked avidin resin for 2 hrs at 4°C. The resin was washed 5 times with washing buffer and boiled in SDS loading buffer. Samples were analyzed by Western blot using monoclonal antibody PY20 or monoclonal antibody against the myc epitope tag. Western blots were quantified by scanning densitometry of X-ray films using a Protein Databases 420oe scanning densitometer and ImageQuant™ software (Molecular Dynamics).
2.14 TCA protein precipitation

Prior to TCA precipitation, samples containing sucrose or glycerol were diluted to final concentration below 0.5 M. 100% TCA stock solution was added to each sample in a centrifuge tube to a final concentration of 10%. After incubation with TCA for 10 min on ice, samples were centrifuged at 14,000 x g in a microcentrifuge for 10 min. The pellet was washed once with acetone and 95% ethanol. After drying briefly in a speed vacuum for 5 min, each pellet was suspended in 1 X SDS sample buffer.

2.15 RNA recovery using TRIlzo® Reagent

TRIlzo® (Invitrogen) reagent is a phenol based solution used to extract nucleic acid from tissue or cultured cells in one step. The manufacturer’s protocol was modified for the purpose of producing clean total RNA from cultured Cos-1 cells. Two milliliters (ml) of TRIlzo® were added to a 60 mm dish and the cells were scraped off using a disposable cell lifter (Fisher Scientific). Cells were then split into two 1.5 ml microcentrifuge tubes and incubated at room temperature for 2 min. Tubes were centrifuged at 10,000 x g for 2 min to remove the cell debris, and incubated for another 5 min at room temperature to allow complete dissociation of nucleoprotein complexes. Then 200 µl of chloroform was added for each 1 ml of TRIlzo® used. The sample was mixed by vortexing for 15 sec, and incubated on ice for 5 min. The RNA was extracted into the aqueous phase that was completely separated by centrifugation at 14,000 x g for 5 min at 4°C. This RNA-containing aqueous layer was removed and transferred to a new
tube. The RNA was extracted with one volume of phenol:chloroform:isoamyl alcohol (25:24:1) and again with an equal volume of chloroform:isoamyl alcohol (24:1). Sodium acetate (pH 5.2) was added to a concentration of 0.3 M along with 2.5 volumes of ice cold 100% ethanol. The RNA was precipitated by incubating for 20 min at -80°C, followed by centrifugation at 14,000 x g for 15 min at 4°C. The RNA pellet was then washed once with 75% ethanol, air dried for 5 min and resuspended in DEPC treated H₂O. The recovered RNA was quantified with Beckman DU 640 spectrophotometer and stored at -80°C before further analysis.

2.16 RNA recovery using Absolutely RNA® RT-PCR miniprep kit

4.2 µl of 2-mercaptoethanol (β-ME) was added into 600 µl of lysis buffer from absolutely RNA® RT-PCR miniprep kit (Stratagene) prior to use. Cells collected with trypsin were washed once with PBS and then resuspended in lysis buffer. Cells were disrupted by vortexing or repeated pipetting. Up to 700 µl of homogenate was transferred to a prefilter spin cup that was seated in a 2 ml receptacle tube. The tube was centrifuged in a microcentrifuge at 14,000 x g for 5 min. The prefilter spin cup was removed from the receptacle tube and discarded. An equal volume of 70% ethanol was added to the filtrate and the tube was vortexed for 5 sec. Up to 700 µl of this mixture to transferred to a RNA binding spin cup that was seated in a fresh 2 ml receptacle tube. The mixture was centrifuged at 14,000 x g for 30 sec. The filtrate was discarded and the spin cup was washed with 600 µl of 1x low-salt wash buffer and incubated with DNase I
solution at 37°C for 15 min. A sequential wash with 600 µl of 1x high-salt wash buffer, 600 µl 1x low-salt wash buffer, and 300 µl of 1x low-salt wash buffer was performed before elution. Finally RNA was eluted with 50 µl of elution buffer.

2.17 Preparation of radiolabeled probe

2.17.1 Preparation of uniformly labeled DNA probe

Probes for the Northern blots were prepared using the Random Primer DNA labeling kit (Invitrogen). 25 ng of DNA dissolved in 20 µl of distilled water was denatured by heating for 5 min in a boiling water bath, and immediately cooled on ice. The DNA was kept on ice and mixed with 2 µl dATP solution (0.5 mM dATP in 3 mM Tris-HCl (pH 7.0), 0.2 mM Na₂EDTA), 2 µl dGTP solution (0.5 mM dGTP in 3 mM Tris-HCl (pH 7.0), 0.2 mM Na₂EDTA), 2 µl dTTP solution (0.5 mM dTTP in 3 mM Tris-HCl (pH 7.0), 0.2 mM Na₂EDTA), 15 µl Random Primers Buffer Mixture (0.67 M HEPES, 0.17 M Tris-HCl, 17 mM MgCl₂, 33 mM 2-mercaptopetethanol, 1.33 mg/ml BSA, 18 OD₂₆₀ units/ml oligodeoxyribonucleotide primers (hexamers), pH 6.8), 5 µl (approximately 50 µ Ci) [a⁻³²P]-dCTP, 3000 Ci/mmol, and distilled water to a final volume of 49 µl. 1 µl of Klenow Fragment (3 units) was added to the reaction and the sample was mixed gently but thoroughly and centrifuged briefly. The reaction mixture was incubated at 25 °C for 1 hr, and 5 µl of the stop buffer (0.2 M Na₂EDTA, pH 7.5) was added to stop the reaction.

The labeled probe was then purified using QIAquick nucleotide removal kit (Qiagen). 10 volumes of PN buffer was mixed with 1 volume of probe. The
QIAquick spin column was placed in a provided 2 ml collection tube. The sample mixture was applied to QIAquick column and centrifuged for 1 min at 6000 rpm to bind DNA. The radioactive flow-through was discarded and the QIAquick column was place onto a clean 2 ml collection tube. The QIAquick column was washed with 500 µl of Buffer PE and centrifuged for 1 min to discard the flow-through. The QIAquick column was placed back in the same tube, and centrifuged at =10,000 x g for an additional minute. The QIAquick column was placed in a clean 1.5 ml microcentrifuge tube, and the DNA probe was eluted in 100 µl of Buffer EB by centrifugation for 1 min at =10,000 x g. The amount of incorporated radioactivity was determined by spotting 1 µl of the flow through on a glass fiber filter, air dried and followed by washing with 50 ml of ice cold 10% (w/v) TCA containing 1% (w/v) of sodium pyrophosphate to remove unincorporated radioactive isotope. Then a scintillation counter was used to measure the radioactivity on the membrane to determine the incorporated radioactivity of the probe.

2.17.2 Preparation of 5' end-labeled RNA probe

Thirty pmol of DNA oligo was mixed with 1 x T4 polynucleotide kinase (PNK) Buffer, 10 U T4 PNK, 100 µCi γ-[32P]-ATP (3000 Ci/mmol), 10 U RNasin, 10 mM DTT and incubated at 37°C for 1 hr. The labelling mixture was then loaded on a 15% polyacrylamide/urea gel (acrylamide:bisacrylamide, 19:1, 1 x TBE, 7 M urea) to separate labeled RNA oligo from free nucleotide. The labeled RNA band was excised and soaked in a buffer containing 0.3 M sodium acetate,
0.1% SDS, 1 mM EDTA to elute the labeled RNA from the gel. The elution was carried out at 37°C for at least 1 hr. The eluted RNA was then extracted by phenol:chloroform:Isoamyl alcohol (25:24:1) (v/v) once and by chloroform:isoamyl alcohol (24:1) (v/v) once. Two volumes of ethanol and 1/10 volume of 3 M sodium acetate (pH 4.8) were added to the extracted RNA to precipitate the labeled RNA oligo, with 5-10 µg of glycogen added as the carrier.

2.17.3 Preparation of uniformly labeled RNA probe

MAXIscript™ kit (Ambion) was used for in vitro transcription. 1 µg of linearized DNA template was mixed with 1 × transcription buffer, 0.5 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 2.5 µM UTP, 30 µCi α-[³²P] UTP (3000 Ci/mmol), 5 mM DTT, 10 U RNasin and 1 µl of RNA polymerase solution (T7, T3 or SP6). The mixture was incubated at 37°C for 1 hr and the in vitro transcript was gel purified with a 6% polyacrylamide/urea gel. Probe elution and extraction were the same as described in 2.17.2.

2.18 Northern blot

Ten µg of total RNA was diluted to a final volume of 12 µl in sterile water. Three µl of deionized glyoxal and 2 µl of 0.1 M sodium phosphate (pH 7.0) buffer were added to the reaction. The mixture was heated at 50 °C for no more than one hr. The RNA samples were cooled to room temperature and 4 µl of 5X loading dye (50% glycerol, 0.01 M sodium phosphate, pH 7.0, 0.4% each bromophenol blue and xylene cyanole) was added. The gel boat and comb were
washed in 1 N NaOH for 20 min, and a 1% (w/v) agarose gel was prepared in 0.1 M sodium phosphate (pH 7.0) buffer. RNA samples were loaded onto the agarose gel equilibrated with 0.01 M sodium phosphate (pH 7.0) running buffer. Electrophoresis was performed at 100 V for approximately 3 hrs, until the bromophenol blue was about 1 cm from the bottom of the gel. Once both dyes had entered the gel, a stir bar was used to circulate buffer at low speed. The RNA samples were transferred from the gel onto a Nytran membrane using the Turbo Blotter (Scheicher and Schuell) as described by the manufacturer with 10X SSC. After blotting the blot was rinsed with 6x SSC and air dried for 30 min. The RNA was crosslinked to the Nytran membrane by exposing to a 254 nm UV-lamp (Stratalinker® 1800, at 120,000 µJ/cm²) for 45 sec.

The blot was prehybridized with 7 ml of Sigma Perfecthyb™plus solution containing 100 µg/ml sheared, heat denatured, salmon sperm DNA for at least 30 min at 68°C. The prehybridization buffer was then discarded and fresh Perfecthyb™plus solution with salmon sperm DNA was added. Ten million cpm of each riboprobe were then added directly to the Perfecthyb™plus solution and incubated at 68 ºC overnight. The radiolabeled DNA probe was previously heated at 100 ºC for 5 min and cooled on ice before use. The next morning the blot was washed twice with low stringency buffer (1% SDS, 2 x SSC) for 10 min at 25 ºC, twice with high stringency buffer (1% SDS, 0.5 x SSC) for 20 min at 68 ºC, and once with ultra high stringency buffer (1% SDS, 0.1 x SSC) for 2 hrs at 68°C. The blot was then dried briefly and visualized by phosphorimager analysis.
2.19 RT-PCR analysis of albumin and luciferase mRNA

Albumin and luciferase mRNA recovered from IgG-sepharose beads were analyzed by RT-PCR as described previously (159). Reverse transcription was performed with oligo (dT)$_{15}$ primer and Superscript II reverse transcriptase (Invitrogen). Five µg of RNA was mixed with 300 ng of oligo (dT)$_{15}$. The RNA-primer mixture was heated at 85°C for 5 min and quickly chilled on ice to anneal the oligo (dT) primer to the poly(A) tails of poly(A)$^+$ RNAs. Reverse transcription was performed with Superscript II reverse transcriptase (Invitrogen) in a reaction of 1 × first strand buffer, 10 mM DTT, 0.67 mM dNTPs, 5 U Rnasin and 400 U Superscript II reverse transcriptase at 42°C for 1.5 hrs. The reaction was then heated at 85°C for 10 min to inactivate Superscript II reverse transcriptase. The cDNA first strand was ready for PCR.

For the PCR reaction, albumin mRNA was amplified using 5'-CAAAGACCAGCCTTCAAACCTC-3' (sense) and 5'-CACAGTTGAATGCTCTAAGCA-3' (antisense). Luciferase mRNA was amplified using 5'-AGATCCACAACCTTCGCTTC-3' (sense) and 5'-CTGAGGAGCCTTCAGGATTAC-3' (antisense). In each set the sense primer was 5'-$[^{32}\text{P}]$-labeled by mixing the following: 1 µg primer, 1 × T4 PNK buffer, 30 µCi γ-$[^{32}\text{P}]$ ATP (3000 Ci/mmol) and 10 U T4 PNK (Roche), and incubating at 37°C for 1 hr. The 5’ end-labeled primers were heat-inactivated and diluted to 25 ng/µl before use.
Hot-start PCR was performed to the first strand cDNA as the following: 1/10 of cDNA from above reaction was mixed with 1 × PCR buffer, 6 mM MgCl$_2$, 0.2 mM dNTPs and 50 ng antisense primer in a HotStart 50° tube (Invitrogen). A small piece of wax in the tube was thawed by heating at 85°C for 30 sec and then cooled down at room temperature. Then 50 ng 5’-end labeled upstream primer and 1 U Taq DNA polymerase (Invitrogen) were added on the top of the wax. The thin layer of wax separates the cDNA from Taq DNA polymerase and the radiolabeled primer. PCR cycling was programmed as the following:

- 94°C × 2min — 1 cycle;
- 94°C × 45sec → 61°C × 45sec → 72°C × 1min — 18 cycles;
- 72°C × 2min — 1 cycle.

Serial dilutions of albumin and luciferase mRNA were analyzed in parallel to empirically determine that PCR conditions for the samples were within the linear range.

The radiolabeled PCR products were separated on a 6% polyacrylamide/urea gel. Electrophoresis was carried out at constant 60 watts for 1-1.5 hrs, followed by phosphorimager analysis.

2.20 Ribonuclease protection assay

2x10$^6$ cells were transfected as described in 2.3 with plasmids expressing albumin and luciferase mRNA, and co-transfected with plasmids expressing catalytically-active myc-PMR60 (161) or a form of the protein lacking the C-terminal 50 amino acids (?50C). Total RNA was isolated with TRIzol® reagent.
The antisense albumin riboprobe was synthesized as described in 2.17.3 by *in vitro* transcription (Ambion) using the T7 promoter from a pcRII-Topo plasmid containing exons 14 and 15 of albumin cDNA. The antisense firefly luciferase riboprobe was synthesized with the T3 promoter from a pBluescript (SK) plasmid containing first 153 nucleotides of firefly luciferase cDNA. Ribonuclease protection assay was carried out with 5 µg of total RNA hybridized to 40 pg of each riboprobe using the RPA III kit (Ambion) according to manufacturer’s instructions, and protected probe was separated on a denaturing 6% polyacrylamide/urea gel and quantified by phosphorimager analysis.

**2.21 Confocal microscopy**

Cos-1 cells were cultured on 18 mm diameter glass coverslips (Fisher Scientific) in 12-well plates. 4x10^4 cells were co-transfected with 0.2 µg of PMR600 and 0.2 µg of EGFP-Dbp5 or EGFP-hDcp1 plasmid DNA following the protocol described in 2.3. Forty hrs after transfection, cells were washed 3 times with PBS and fixed in freshly made fixation buffer (4% paraformaldehyde, 2 mM MgCl₂, 1.25 mM EGTA in PBS, pH 7.2) for 1 hr. The cells were washed with PBS for 4 times within 1 hr, and permeabilized in 0.2% Triton X100 in PBS for 15 min at 25°C. After rinsing 3 times with PBS and blocking for 1 hr in 5% normal rabbit serum in PBS, cells were incubated overnight at 4°C with the monoclonal antibody to the myc epitope, washed three times with PBS, and incubated with rabbit anti-mouse IgG antibody Alexa Fluor® 594 (Molecular Probes) for 1 hr at 25°C. Finally, cells were washed three times with PBS and incubated with 1
µg/ml of DAPI (4’, 6-Diamidine-2’-phenylindole dihydrochloride, Roche) in PBS for 15 min at 25°C. Cells were washed with PBS and mounted with ProLong (Molecular Probes) following the manufacturer’s protocol, and examined using a Zeiss 510 confocal microscope.
<table>
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<th>Plasmid</th>
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**Table 2.1 Primers used in cloning.**
3.1 Expression of PMR1 in mammalian cells

3.1.1 Expression of PMR60 and PMR80

The original cDNA clones of PMR1 were isolated by Elena Chernokalskaya, Ph.D, who screened a *Xenopus laevis* liver cDNA library using degenerate primers generated from PMR1 tryptic peptide sequences (212). The cDNA clone encodes an 80 kDa polypeptide (PMR80) that we believe is proteolytically processed to the mature 60 kDa form (PMR60). Molecular modeling of PMR60 to the crystal structure of human myeloperoxidase heavy chain identified His residues at positions 393 and 479 as likely to be involved in catalysis (unpublished data). Changing these residues to alanine generated a catalytically inactive form of PMR60 (termed PMR60°) that was used to examine polysome targeting without the confounding issue of mRNA degradation.

Myc epitope tagged recombinant mammalian expression plasmids were generated by cloning cDNA sequences corresponding to the *Xenopus* PMR80, PMR60 and PMR60° into the pcDNA3 vector (Invitrogen) and pTRE vector (BD
Biosciences). Each of the expression clones had an artificial Kozak sequence engineered into the DNA to facilitate translation of the PMR1 polypeptides.

To examine expression of PMR1 constructs in mammalian cells, each clone was transiently transfected into tet-off Cos-7 (BD Biosciences). Once the cells were transfected, recombinant protein was expressed and cell lysates were analyzed by Western blot with a monoclonal antibody against the myc epitope tag (Fig. 3.1A, B, and left panel of C), or a polyclonal antibody (Ab2) against *Xenopus* PMR1 (Fig 3.1C right panel) raised in our lab. These recombinant mammalian-expressed polypeptides approximately corresponded in size and sequence to the native PMR1. In Fig 3.1A, expression of PMR60, catalytically inactive PMR60º and PMR80 in the pcDNA3 vector are shown in lanes 1, 2 and 3 respectively. Fig 3.1B shows expression of PMR60, catalytically inactive PMR60º and PMR80 in the pTRE vector in lanes 1, 2 and 3 respectively. In both pcDNA3 and pTRE vector, PMR60 and PMR60º were expressed as a single band corresponding to a molecular size of 60 kDa approximately. Interestingly, PMR80 in both pcDNA3 and pTRE vectors was expressed as a doublet corresponding to molecular weight of 80 kDa. The reason for this is unknown.

To confirm the specific expression of PMR1 in transfected Cos-1 cells, the experiment shown in Fig. 3.1C examined the expression of a catalytically-inactive form of PMR1 (see below) bearing an N-terminal myc epitope tag (PMR60º) in transfected Cos-1 cells. Western blots with antibody to the myc tag identified only a single 60 kDa protein in cells transfected with a myc-PMR60º-expressing plasmid (left panel, lane 3). No band was observed in either untransfected (lane...
1) or pcDNA3 vector transfected (lane 2) Cos-1 cells. Western blots with polyclonal antibody to *Xenopus* PMR1 resolved the same major 60 kDa protein only in cells transfected with a PMR1-expressing plasmid (right panel, lane 5). While the polyclonal antibody to PMR1 weakly cross-reacted with a 48 kDa protein in untransfected (lane 4) or vector transfected cells (lane 5), the untransfected cells in lane 1 and vector transfected cells in lane 2 expressed no bands corresponding to PMR60 or 80. In addition, a 67 kDa protein cross reacting protein seen in MEL cells (161) was not observed even after prolonged exposure.

### 3.1.2 Expression of PMR1-TAP

The tandem affinity purification (TAP) approach has been successfully used for efficient recovery of large mRNP complexes in mammals. *Xenopus* PMR1 has previously been shown in a large complex (200). To help with future purification of the PMR1-containing complex, a TAP tag was added to the C-terminus of PMR1 constructs to generate PMR80-TAP, PMR60-TAP and PMR60º-TAP in both pcDNA3 and pTRE vector. The experiment in Fig. 3.2 examined expression of PMR60º bearing a C-terminal TAP affinity tag (PMR60º-TAP) in transfected Cos-1 cells. Similar to results observed in Fig. 3.1, Western blots with the myc epitope antibody (Fig. 3.2 A) identified only a single 85 kDa protein corresponding to PMR1 (60 kDa) plus TAP tag (~25 kDa) in cells transfected with PMR1-TAP expressing plasmids in the pcDNA3 vector (lane 3) and the pTRE vector (lane 4). No band was observed in either pcDNA3
transfected (lane 1) or pTRE vector transfected (lane 2) Cos-1 cells. Western blots with xPMR1 antibody (Fig. 3.2 B) showed the same specific protein only in cells transfected with a PMR1-TAP expressing plasmids (lane 3, 4) in addition to a cross-reactive 48 kDa protein in all lanes.

The TAP tag contains two affinity tags: protein A and calmodulin binding peptide. Both affinity tags are required for efficient affinity purification of the protein and associated protein complex. To examine the expression of protein A in TAP tag, I took advantage of the interaction between protein A and antibody. In Fig. 3.2 C, the membrane was directly blotted with a HRP-conjugated goat anti-rabbit secondary antibody without any primary antibody. Fig. 3.2 D examined the expression of Calmodulin binding peptide. The membrane was first blotted with biotinylated calmodulin and followed by streptavidin conjugated alkaline phosphatase. Although three additional cross-reactive proteins were observed in all lanes, specific protein corresponding to 85 kDa was only seen in cells transfected with PMR1-TAP expression plasmids in pcDNA3 (lane 3) or pTRE vector (lane 4).

The experiments in Fig. 3.1 and 3.2 confirmed the specific expression of PMR1 and PMR1-TAP in Cos-7 cells. These cells expressed no PMR1-like protein or activity, making them good system for further study.

3.1.3 In vitro ribonuclease activity of PMR1

Having successfully expressed PMR1 in mammalian cells, I wished to examine whether these recombinant proteins have ribonuclease activity as
Xenopus PMR1 does. An *in vitro* ribonuclease activity assay originally developed for xPMR1 was adapted to test mammalian expressed recombinant PMR1s. Lysates from cells transfected with plasmids pcDNA3-TAP, pTRE-TAP and plasmids expressing PMR60-TAP were tested for the presence of ribonuclease activity *in vitro* using a radiolabeled *in vitro* albumin transcript probe. To avoid the endogenous ribonucleases in Cos-1 cells, cell lysates were partial affinity purified with IgG-sepharose beads. Figure 3.3 A demonstrates that PMR60-TAP proteins from both vectors, generated from affinity purified transfected cell lysate, had significant ribonuclease activity compared to the purified lysates from cells transfected with plasmids expressing only TAP (compare lanes 4, 5 to 2 and 3, respectively). While the PMR60 polypeptide had significant nuclease activity, the characteristic doublet cleavage fragments of xPMR1 (double arrow in Fig. 3.2A) were not observed in the mammalian expressed PMR1 polypeptide. The doublet generated by the purified xPMR1 (lane 6) represents specific cleavage fragments of xPMR1 at nucleotides 120 and 124 of the Xa160 transcript. This experiment confirmed the previous result from baculovirus expression of PMR1 in which ribonuclease activity was observed without the production of the doublet product. In addition, a plasmid expressing the catalytically-active 60 kDa form of PMR1 (PMR60) selectively destabilized human β-globin mRNA when transfected into MEL cells (161).
3.1.4 *In vivo* ribonuclease activity of PMR1

In order to examine the ribonuclease activity of PMR1 in a system similar to the physiological condition, I established a stable cell line to express PMR1 in a regulated manner to avoid possible over-expression in transient transfection. Previously our lab has made a tet-off LM(TK⁻) cell line harboring a tTA plasmid. A PMR1-expression plasmid driven by tetracycline regulated promoter in the pTracer vector was stably expressed in tet-off LM(TK⁻) cells. The plasmids were maintained in the presence of doxycycline to repress expression of PMR1 and the potential toxic effects. Western blot with myc epitope antibody (Fig. 3.3B) showed that the expression of PMR1 in the cell line was regulated by the concentration of doxycycline in the medium. While the maximum expression of PMR1 was achieved in the medium containing no doxycycline, protein expression was shut off at the presence of 1000 ng/ml of doxycycline. Plasmids expressing the reporters albumin and ferritin mRNA were transiently transfected into cells grown in medium with or without doxycycline. Albumin is the native substrate of PMR1. Ferritin was chosen as transfection control because its sequence contains no PMR1 cleavage sites. Total RNA was isolated from cells at different time points after transfection. Fig. 3.3C showed a Northern blot carried out with probes against albumin, ferritin and endogenous GAPDH mRNAs. The data were quantified and plotted in Fig. 3.3D after normalizing albumin to ferritin. The steady state level of albumin mRNA was significantly lower 20 hours post transfection, when PMR1 was expressed in these cells ([dox]-) compared to when PMR1 was not expressed ([dox]+).
3.2 Polysome association of recombinant PMR1 in transfected cells

A major finding in the previous study was that PMR1 is present in a relatively latent form in a >670 kDa polysome-bound mRNP complex. The initial goal of this project was to identify domains of PMR1 involved in targeting this mRNA endonuclease to polysomes. Because Xenopus hepatocytes are difficult to culture and transfect, I examined mammalian cell lines for their ability to recapitulate the process of PMR1 targeting to polysomes. The selected cell line had to meet two criteria. First, to minimize interference with polysome targeting by exogenous PMR1 or its mutant forms, and to restrict changes in albumin mRNA to the activity of the expressed endonuclease, these cells should not express the 67 kDa PMR1-like endonuclease activity that was identified previously in murine erythroleukemia (MEL) cells (161). Second, transfected cells should display similar distribution of PMR1 and albumin mRNA on sucrose density gradients as Xenopus hepatocytes (200). The results in Fig. 3.1 and 3.2 showed that no 67 kDa PMR1-like protein was identified in Cos cells. And there was no evidence for PMR1-like endonuclease activity in Cos-1 cells (data not shown). Therefore Cos-1 cells fit the first criterion.

3.2.1 Polysome association of PMR1 in linear gradient

To examine whether Cos-1 Cells also qualify for the second criterion, i.e. whether PMR1 in transfected cells displays similar distribution of PMR1 and albumin mRNA on sucrose density gradients as Xenopus, cells were transfected
with PMR60° plus a plasmid that expresses full length *Xenopus* albumin mRNA (210) (Fig. 3.4). Polysome profile analysis was performed using linear sucrose gradients in buffer containing MgCl₂ to maintain ribosome integrity (Fig. 3.4A), or EDTA to dissociate ribosomes (Fig. 3.4B). The 254 nm absorbance patterns for each gradient are shown at the top of each dataset. In gradients containing MgCl₂ PMR60° had a distribution similar to that in *Xenopus* hepatocytes (200), with a significant portion sedimenting with polysomes. The polysome fractions were identified by distribution of ribosomal protein S6 and absorbance at 254 nm. Albumin mRNA co-sedimented with PMR60° on polysomes on these gradients. The sedimentation of albumin mRNA on these gradients was also similar to that seen in *Xenopus* hepatocytes. As with *Xenopus* liver (200), ribosome dissociation with EDTA released polysome-bound PMR1 as a complex that sedimented with the mRNP fraction (Fig. 3.4B). Final confirmation that PMR60° bound to polysomes was achieved by treating cells with puromycin for 30 min prior to harvest (Fig. 3.4C). Like EDTA, this treatment resulted in PMR60° sedimenting with mRNP fractions. These results indicate that polysome targeting of PMR1 is accurately reproduced by expression of a catalytically-inactive form of the enzyme in transfected Cos-1 cells, thus confirming that this model system satisfies the second criterion.

The polysome association of PMR1 was similar to other polysomal associated proteins such as PABP and eIF4E shown in Fig. 3.5. PABP was associated with polysome as well as other light mRNP complexes in the top of gradient containing MgCl₂ (Fig. 3.5A). Interestingly, only a small portion of eIF4E
was associated with polysomes (Fig. 3.5A). EDTA treatment was able to
dissociate polysomes and releases both polysome-bound PABP and eIF4E to
light mRNP complexes (Fig. 3.5B).

3.2.2 Polysome association of PMR1 in step gradient

To facilitate the identification of polysome targeting domains, I employed
discontinuous sucrose density gradients, a technique our lab and others have
used successfully to study the distribution of proteins and mRNAs between
polysome and mRNP-bound complexes (201,213). In the experiment shown in
Fig. 3.6, cells were transfected with plasmids expressing PMR60\(^\circ\) and albumin
mRNA. Cytoplasmic extracts were applied to gradients consisting of equal
volumes of 10 and 35% sucrose. Following centrifugation, polysomes pellet to
the bottom of the tube and mRNP complexes accumulate at the interface
between the different sucrose concentrations, as shown in Fig. 3.6A. Material
recovered at the top, interface (mRNP) and bottom (polys) was analyzed by
Western blot using antibody to the myc epitope tag. The ability of these
gradients to separate polysomes from smaller complexes was confirmed by
analyzing the distributions of eIF4E and ribosomal protein S6 in each portion of
the tube (bottom of Fig. 3.6B). PMR60\(^\circ\) was present in the polysome pellet as
well as lighter fractions in gradients containing MgCl\(_2\). Invariably, PMR60\(^\circ\)
disappeared from the polysome fraction following EDTA treatment. Similar to
what was seen in the linear gradient, albumin mRNA co-sediments with PMR60\(^\circ\).
In the Northern experiment shown in Fig. 3.6C, 63.4% of albumin mRNA was
found in polysomes and 34.5% was in mRNP before EDTA treatment, and only 7.6% of albumin was found in polysomes and 71.9% was in mRNPs after EDTA treatment. This was confirmed in a separate experiment using RT-PCR (Fig. 3.6D).

3.3 Identification of polysome targeting domains of PMR1

3.3.1 Expression of deletion PMR1 in mammalian cells

To identify regions of PMR1 involved in targeting to polysomes, I took a “loss of function” approach. Deleting a domain important in targeting PMR1 to polysome-bound mRNP complexes should be apparent by a change in the gradient distribution of the protein. Since an active RNase is capable of degrading polysome-bound mRNAs to artificially release PMR1 from the mRNP complex, I chose a catalytically inactive form of PMR1 (PMR60⁰) as the study subject. I made a series of PMR60⁰ deletion expression plasmids covering all coding sequences of PMR1. 50, 100 or 150 amino acids were removed from the N or C termini of PMR60⁰ (Fig. 3.7A) or PMR60⁰-TAP (Fig. 3.8A). A plasmid containing a deletion of the middle part of PMR1 and expresses only the N-terminal and the C-terminal 150 amino acids was also made (data not shown). All constructs have a myc-epitope tag at the N-terminus to facilitate detection of PMR1, and were driven by the CMV promoter in the pcDNA3 expression vector. It is possible that some recombinant proteins may not fold properly and degrade rapidly after translation. Therefore, I first examined the expression levels of each deletion construct. A Western blot of the PMR1 deletions expressed in Cos-1
cells is shown in Fig. 3.7B. Expression of PMR1-TAP deletions was shown in Fig. 3.8B. All deletion proteins were stably expressed at similar level in Cos-1 cells.

### 3.3.2 Analysis of PMR1 targeting domain deletions using discontinuous sucrose density gradient

In experiments shown in Fig. 3.9, cells were transfected with equal amounts of plasmid expressing full-length PMR60º or one of the deletion mutants. I was looking for changes in the gradient distribution and disappearance of the deleted protein in polysome fraction. Meanwhile the full-length PMR60º should not be affected by deleted protein, and its presence in polysome fraction was used as a control for the step gradient.

Cytoplasmic extracts were applied to gradients consisting of equal volumes of 10 and 35% sucrose. Following centrifugation, polysomes pellet to the bottom of the tube and mRNP complexes accumulate at the interface between the different sucrose concentrations. In each experiment extracts were separated on MgCl₂ or EDTA-containing gradients, and material recovered at the top, interface (mRNP) and bottom (polys) was analyzed by Western blot using antibody to the myc epitope tag.

The ability of these gradients to separate polysomes from smaller complexes was confirmed by analyzing the distribution of ribosomal protein S6 in each portion of the tube (bottom of Fig. 3.9C). Invariably, full-length PMR60º (identified with arrow on each autoradiogram) was present in the polysome pellet.
in every MgCl$_2$-containing gradient, and disappeared from the polysome fraction following EDTA treatment.

Deleting 50 or 100 amino acids from the N-terminus (?50N, ?100N) had no effect on the binding of PMR60° to polysomes. However, polysome association was lost when 150 amino acids were removed from the N-terminus (?150N). A more immediate effect was seen with C-terminal deletions. In this case, polysome binding was lost after deleting as few as 50 amino acids (?50C).

3.3.3 Sedimentation on linear sucrose density gradient confirms the function of the C-terminal targeting domain

The impact of the ?50C deletion on the sedimentation of PMR1 was examined further using linear sucrose density gradients (Fig. 3.10). In Figs. 3.10A and B cells were treated without and with puromycin prior to harvest and separated on MgCl$_2$-containing gradients. The polysome fractions were identified by antibody against ribosomal protein S6 (bottom panels in Fig. 3.10A). As expected, puromycin treatment blocked translation and dissociated polysomes (bottom panels in Fig. 3.10B). However in all cases ?50C PMR1 sedimanted with RNP complexes at the top of the gradient. For comparison, extracts were fractionated on a gradient containing EDTA (Fig. 3.10C). Again, ?50C PMR1 sedimanted at the top of the gradient. These data confirm that the C-terminal 50 amino acids are necessary for polysome targeting by PMR1.
3.4 Identification of sequences required for targeting PMR1 to polysomes

3.4.1 Polysome association of GFP fusions of PMR1

The “loss of function” approach successfully identified two possible targeting domains in N- and C-termini of PMR1. However, it remained possible that N- or C-terminal deletions could indirectly alter mRNP binding due to improper protein folding. This was addressed by a “gain of function” approach using portions of PMR1 added to a protein that does not target to polysomes. Polysome binding by the fusion protein will indicate that I have correctly identified the mRNP-targeting domain. Green fluorescent protein (GFP) does not form a stable complex with polysomes (see below). This made GFP a good candidate to test the ability of the domains of PMR1 identified above to target a heterologous protein to polysomes. The individual portions of PMR60° deleted in Fig. 3.7A were fused to the N- or C-termini of GFP as indicated in Fig. 3.11A. These were co-transfected with a plasmid expressing native GFP, and the distribution of both proteins on discontinuous gradients was monitored as in Fig. 3.9 by Western blot using a polyclonal antibody to GFP. As noted above, native GFP was never found in the polysome pellet (Fig. 3.11B, filled circles). The N-terminal 100 amino acids of PMR60° were not sufficient to bring GFP to polysomes; however, polysome binding was observed for a fusion bearing the N-terminal 150 amino acids (Fig. 3.11B, 100N). These data confirm the presence of a polysome targeting domain between positions 100 and 150 of PMR1. In contrast to the results in Fig. 3.9, the C-terminal 50 amino acids of PMR60° were not sufficient to target a GFP fusion protein to polysomes (Fig. 3.11B, 50C).
However, efficient polysome targeting was achieved with the C-terminal 100 or 150 amino acids, indicating either that the C-terminal targeting domain spans the region at position –50, or that it is located nearby and the ?50C deletion disrupted the folding of this portion of the protein.

3.4.2 Further characterization of C-terminal targeting domain required for polysome association of PMR1

The behavior of the C-terminal deletions (Figs. 3.9 and 3.10) and GFP fusions (Fig. 3.11) suggested either that the C-terminal targeting domain was disrupted by removal of the last 50 amino acids, or that amino acid residues involved in targeting lie just upstream of this deletion, and loss of the C-terminal 50 amino acids interfered with proper folding of the protein. To further characterize this targeting domain, I constructed two C-terminal deletions where first 30 or 40 amino acids from the C-terminus were removed from the full length PMR1. Cytoplasmic extracts from cells transfected with plasmids expressing ?30C or ?40C proteins were used to study the sedimentation of the deletion mutants on linear sucrose density gradients. The experiment in Fig. 3.12 compared the impact of the ?30C, ?40C and ?50C deletion on the sedimentation of PMR1. The polysome identities were determined by antibody against ribosomal protein S6 (bottom panels of Fig. 3.12A, B and C) and by disappearance after puromycin treatment that blocked translation and dissociated polysomes to mRNP complexes (right panels of Fig. 3.11A and B). Fig. 3.12C shows data that confirm the result shown in Fig. 3.10 that all of ?50C
PMR1 sedimented with mRNP complexes at the top of the gradient and none appeared in the polysome fraction. In experiments shown in Figs. 3.12A and B, cells were treated without (left panel), and with puromycin (right panel) respectively prior to harvest and separated on MgCl₂-containing sucrose gradients. Most of ?40C sedimented with mRNP complexes, but a small fraction appeared in the polysome fraction. A profound effect was observed in ?30C PMR1 gradient where large fraction of ?30C PMR1 sedimented with polysomes, similar to behavior observed for full length PMR1 (Fig. 3.4A). Comparing polysome profiles of ?30C, ?40C and ?50C deletions showed a trend toward greater binding to polysomes with increasing amounts of the C-terminal portion of PMR1.

**3.4.3 Association of PMR1 with the 680 kDa complex**

The above results raised the question whether the same domains that target PMR1 to polysomes are also responsible for its presence in a ~680 kDa polysome-associated complex (200). In the experiment in Fig. 3.13, cytoplasmic extracts from cells transfected with PMR60⁰ or ?50C were treated for 30 min with EDTA to dissociate ribosome-bound complexes prior to gradients, then separated on glycerol gradients and analyzed by Western blot for PMR60⁰ (Fig. 3.13B) or ?50C PMR1 (Fig. 3.13C). A mixture of protein markers was also separated on a glycerol gradient under the same conditions, and analyzed for protein concentration with the BCA method (Fig. 3.13A). The sizes of protein complexes were identified by peaks of absorbance at 595 nm. As observed for
Xenopus PMR1, PMR60° sedimented in a ~680 kDa complex. A portion also sedimented between the 67 and 232 kDa size standards. Complexes formed by ?50C PMR1 predominantly sedimented between the 67 and 232 kDa size standards, and none was seen as 680 kDa complex.

Final confirmation that PMR60° association to 680 kDa complex was achieved by transfecting cells with PMR60°, ?50C PMR1, GFP, GFP-50C and GFP-100C and treating cells with puromycin to dissociate ribosome-bound complexes prior to harvest. Cytoplasmic extract was then separated on glycerol gradients and analyzed by Western blot for PMR60° or ?50C PMR1 (Fig. 3.14A), and by fluorescence for GFP (Fig. 3.14B). A ~680 kDa complex was observed with PMR60° but not with ?50C PMR1. Both GFP-50C and ?50C PMR1 sedimented in a 67-140 kDa complex. This contrasted with the sedimentation of GFP-100C, which was similar to that of PMR60°, with about half of the protein in a ~680 kDa complex and the remainder in a complex that sedimented at ~140 kDa. These data indicate that the C-terminal portion of PMR1 responsible for targeting the enzyme to polysomes is also responsible for its integration into the ~680 kDa complex.

3.5 PMR1 forms a specific complex with its substrate mRNA

3.5.1 PMR1 can be efficiently recovered by TAP

The tandem affinity purification (TAP) approach, originally developed by Seraphin and co-workers at EMBL (214), has been successfully used to recover complexes from mammalian cells (58). The TAP tag consists of a C-terminal
fusion of protein A with the calmodulin-binding protein, separated by a Tev protease cleavage site (Fig. 3.15A). Lysate from cells expressing a TAP fusion protein is passed through an IgG-sepharose column, which can be washed under different stringencies depending on the strength of the interactions under study. Bound complexes are released by cleavage with Tev protease, and the recovered proteins can then be processed through a second affinity purification step on calmodulin agarose. Protein is recovered from calmodulin agarose by elution with EGTA for yeast and EDTA for mammalian cells. For the purpose of my study, only first step of affinity purification was used. The experiment in Fig. 3.15B examined the recovery efficiency of this approach. About 44% of input PMR1 can be successfully recovered by TEV cleavage (compare lane 2 to lane 1 in Fig. 3.15B). A separate experiment confirmed this efficiency when TAP was used to recover GFP bearing C-terminal TAP tag (data not shown).

3.5.2 Albumin mRNA can be recovered by PMR1-TAP

The results presented in 3.4 raised the possibility that PMR1 participates in a polysome-bound complex containing its substrate mRNA. If so, I reasoned that substrate mRNA should be selectively recoverable only by those forms of PMR1 that showed targeting to polysomes on sucrose density gradients. To test this, I transfected plasmids expressing PMR60º-TAP protein or empty vector with a plasmid expressing albumin mRNA into Cos-1 cells. Postmitochondrial extracts were recovered by TAP affinity using IgG-sepharose beads. RNA was then extracted from the material bound to the beads using TRIzol reagent and
analyzed by RT-PCR. The experiment shown in Fig. 3.15C showed that PMR1 specifically recovered 4 times more albumin mRNA than vector (compare lane 4 to 3 in Fig. 3.15C). Albumin mRNA was present equally in both input samples (input in lane 1 and 2). The bands shown in autography are not from cDNA contamination, as no band was seen in lane 5 and 6 where no RT was performed.

3.5.3 Identification of a region of PMR1 required for formation of a complex with its substrate mRNA

To further confirm the above result and identify the region of PMR1 that is required for formation of a complex with its substrate albumin mRNA, I added a C-terminal tandem affinity (TAP) tag (214) to each of the PMR60° deletion constructs in Fig. 3.8A, co-transfected these with plasmids expressing *Xenopus* albumin and luciferase mRNAs, and examined the recovery of albumin and luciferase mRNAs on IgG-sepharose. The overall expression of each PMR1-TAP fusion in post-mitochondrial extracts of transfected cells was first determined by Western blot (Fig. 3.16A, top panel). Proteins lacking 100 or 150 amino acids from the N-terminus of PMR60° (?100N, ?150N) reproducibly expressed 30% less protein than the other constructs. In contrast, quantitative RT-PCR showed that there was no difference in the overall expression of albumin or luciferase mRNAs in any of the transfected cells (Fig. 3.16A, bottom panels). For unknown reasons, in this particular experiment, albumin mRNA yielded a doublet RT-PCR product.
The postmitochondrial extracts were treated with EDTA to release PMR1-containing complexes from polysomes, following which IgG-sepharose was added to recover complexes containing the different TAP-tagged forms of PMR1. After extensive washing, the bound complexes were released by cleavage within the TAP tag by Tev protease. Since Tev protease cleavage generates calmodulin-binding protein (CBP) from the TAP tag, the released products are denoted in Fig. 3.16B as PMR1-CBP. Differences in the amount of each of the PMR1-CBP deletion constructs recovered on IgG-sepharose matched the differences observed for input protein in Fig. 3.14A, indicating that the individual PMR1 deletions had no effect on the efficiency of binding of these complexes to IgG-sepharose.

However, the recovery of albumin mRNA differed significantly depending on the presence of polysome targeting domains present in the TAP fusion proteins. Albumin mRNA was recovered only by PMR1-TAP fusion proteins that retained both the N- and C-terminal targeting domains identified in Fig. 3.9. In other words, only full-length PMR60, and the ?50N and ?100N forms of the protein were present in the albumin mRNA-containing mRNP complexes. The amount of albumin mRNA recovered with the other proteins matched that which was recovered nonspecifically in cells transfected with empty vector (pcDNA3).

Background recovery of luciferase mRNA was observed for each of the PMR1-TAP fusion proteins, proving that PMR1 selectively targeted the albumin mRNA-containing mRNP. Results with the N-terminal deletions also indicated that features of PMR1 modulate the efficiency of this process. The same amount of
albumin mRNA was recovered by full-length PMR60° and the N-terminal 100 amino acid deletion (?100N), even though there was significantly less of the latter. The increase in the ratio of albumin mRNA to PMR1 -CBP bound to IgG-sepharose (shown beneath the figure) indicates that the N-terminal portion of PMR1 may determine the efficiency of targeting of PMR1 to the substrate mRNA-containing complex. Thus, the presence of PMR1 on polysomes results from the selective formation of an mRNP complex containing both PMR1 and its substrate mRNA.

3.6 Endonuclease-mediated mRNA decay involves the selective targeting of PMR1 to the polysome-bound substrate mRNP complex

Having shown that polysome targeting involves the formation of a specific complex between PMR1 and its substrate mRNA, I sought to determine if such targeting was required for endonuclease-mediated mRNA decay. For these experiments PMR60° was replaced with the catalytically-active form of the protein. Cells were transfected with PMR60 or the ?50C form of the protein together with vectors expressing albumin and luciferase mRNA, and the impact of these proteins on steady state levels of target (albumin) and non-target (luciferase) mRNA was determined by RNase protection assay. The Western blot in Fig. 3.17A (upper panel) shows that PMR60 and ?50C PMR1 were expressed equally in transfected cells. Luciferase, as a control for transfection efficiency, was expressed equally in transfected cells in Fig. 3.17A (middle panel). PMR60 selectively targeted and degraded albumin mRNA, such that its steady-
state level dropped 11-fold in cells expressing catalytically-active enzyme compared to cells transfected with vector alone (Fig. 3.17A, lower panel). Luciferase mRNA was unaffected by the presence of PMR60 in the cell, thus confirming that selectivity in mRNA decay involves the selective targeting PMR1 to substrate mRNP. Removing the C-terminal targeting domain reduced the ability of PMR1 to degrade albumin mRNA by half.

To insure that the ?50C deletion did not alter the catalytic activity of PMR1 I examined the ability of PMR60 and ?50C PMR1 recovered from transfected Cos-1 cells to degrade albumin mRNA in vitro. In the experiment in Fig. 3.17B cells were transfected with TAP fusions of PMR60, ?50C, or GFP and harvested 30 min after puromycin was added to the cells to dissociate polysome-bound complexes. Protein recovery following Tev protease cleavage of complexes bound to IgG-sepharose was evaluated by Western blot (Fig. 3.17B, upper panel), and catalytic activity was determined using a 5'-[32P] labeled albumin mRNA substrate transcript (Fig. 3.17B, lower panel). PMR60 and ?50C were similarly active in degrading albumin mRNA, indicating that the stabilization observed with ?50C PMR1 in Fig. 3.17A was in fact due to loss of polysome targeting and not to disruption of the active site of the enzyme. These data confirm that endonuclease-mediated mRNA decay requires the targeting of both PMR1 and its substrate mRNA to polysomes.
3.7 A 5' stem-loop blocks polysome binding of albumin mRNA

To confirm that the degradation of albumin mRNA required its association with polysome-bound PMR1, a 24 nt stem-loop (Fig. 3.18A) was inserted 34 bp upstream of AUG (SL-albumin). This stable stem-loop, with a calculated \(?G = -42.9\) kcal/mol, was shown previously to inhibit translation initiation when it was present <60 nt upstream from AUG (211). The experiment in Fig. 3.18B examined the distribution of SL-albumin in each fraction after step gradient. Shown are the samples from the top, middle (mRNP) and bottom (polysome) fractions. Ninety-five percent of wild-type albumin mRNA was recovered in the polysome pellet, and the same percent of SL-albumin was recovered in the mRNP fraction. These data indicate that the inserted stem-loop effectively prevented ribosome loading onto albumin mRNA.

Then I examined the degradation of this SL-albumin in Fig. 3.18C. Cos-1 cells were transiently transfected with either an albumin minigene plasmid that expresses full-length Xenopus albumin mRNA (210), or the plasmid that expresses SL-albumin. A plasmid expressing luciferase mRNA was also transfected as control. Cytoplasmic extracts were prepared 24 hrs after transfection and applied to discontinuous gradients. Polysomes were collected from the bottom of the tube and mRNP complexes were collected at the interface between the 10 and 35% sucrose layers. RNA was extracted from these fractions using TRIzol reagent (Invitrogen), and analyzed by ribonuclease protection assay using an antisense probe to exons 14 and 15 of albumin mRNA. The experiment in Fig. 3.18C repeated the result in Fig. 3.17A in that PMR60
selectively degraded albumin mRNA such that its steady-state level dropped 7-fold in cells expressing catalytically-active enzyme compared to cells transfected with vector alone (compare lanes 1 and 2 in Fig. 3.18C), while luciferase mRNA was unaffected by the presence of PMR60 in the cell. In agreement with the requirement for targeting PMR1 to polysomes, the steady-state level of SL-albumin in the presence of PMR60 remained the same as in the absence of PMR60 (compare lane 3 to 4). In summary, this element prevented the loading of ribosomes onto albumin mRNA, and blocked its degradation by PMR1.

3.8 PMR1 is tyrosine phosphorylated

Previous studies in our lab have suggested that the endogenous PMR1 in *Xenopus* liver is tyrosine phosphorylated (unpublished data). To test whether PMR1 expressed in Cos-1 cells is tyrosine phosphorylated, Cos-1 cells were transfected with TAP fusions of the full length protein (PMR60°). Transfected cells were incubated for 6 hrs in the absence or presence of genistein, a tyrosine kinase inhibitor, prior to harvest. Polysome-bound complexes were dissociated by treating cells with puromycin for 30 min prior to harvest. Cytoplasmic extracts from transfected cells in the absence of genistein were incubated for 1 hr in the absence or presence of calf intestinal alkaline phosphatase (CIAP), and the PMR1-TAP fusion proteins from untreated, CIAP or genistein treated extracts were recovered on IgG-sepharose. Input, unbound, and bound fractions eluted with SDS sample buffer were separated by SDS-PAGE and analyzed by Western blot with the phosphotyrosine specific antibody PY20, or antibody to the myc tag.
PMR1 from untreated extract strongly reacted to the PY20 antibody (Fig. 3.19A) as well as to antibody to the myc tag (Fig. 3.19B), whereas PMR1 from CIAP and genistein treated extracts reacted only to antibody to the myc tag, indicating that PMR1 is tyrosine phosphorylated in Cos-1 cells. In addition, the bound fraction treated with CIAP showed an extra band which migrated faster than the original band, suggesting that this might be a dephosphorylated form of PMR1. The overall expression and recovery of PMR1 in genistein treated extract was less than untreated due to the cell toxicity of genistein (Fig. 3.19B, input and bound). Comparing the relative signal density of bound samples, the differences between untreated sample and CIAP or genistein treated samples in PY20 blot (Fig. 3.19A) were much larger in the myc epitope tag blot (Fig. 3.19B). Since signals in the myc epitope tag blot reflected the amount of total PMR1 and signals in the PY-20 blot only showed the amount of phosphorylated PMR1, these results indicated that CIAP or genistein treatment reduced the phosphorylation state of PMR1. Therefore, PMR1 is phosphorylated in transfected Cos-1 cells.

Signature motifs within the sequence of PMR1 are shown schematically in Fig. 3.20A. PMR1 is translated as an 80 kDa precursor that is processed to the catalytically-active ~60 kDa form (212). The large open box shows the region of similarity to the peroxidases, with the polysome targeting domains bracketed beneath this. Notable in this analysis were three proline-rich domains identified as potential SH3 ligands, and a single predicted site for tyrosine phosphorylation (black rectangle). The C-terminal targeting domain lies within the last 100 amino acids of PMR1, and previous results indicated this domain was interrupted by a
deletion that removed the terminal 50 amino acids (?50C, Fig. 3.10A). The location of the putative tyrosine phosphorylation site at position -69 was therefore consistent with a possible role in the C-terminal targeting domain of PMR1.

The experiment shown in Fig. 3.20B studied PMR1 phosphorylation in transfected Cos-1 cells. Previous work in 3.3 and 3.4 mapped the general locations of domains involved in endonuclease targeting to polysomes, and showed that one is located in the C-terminal 100 amino acids. The use of proteins bearing a C-terminal tandem affinity (TAP) tag facilitated the recovery of PMR1-containing complexes on IgG-sepharose in 3.5. To test whether PMR1 expressed in Cos-1 cells is tyrosine phosphorylated within the C-terminal 100 amino acids, Cos-1 cells were transfected with TAP fusions of the full length protein (PMR60⁰) or deletions that removed 50 (?50C) or 100 (?100C) amino acids from the C-terminus. Polysome-bound complexes were dissociated by treating cells with puromycin for 30 min prior to harvest, and the PMR1-TAP fusion proteins were recovered on IgG-sepharose. Western blot with antibody to the myc tag showed that all 3 forms of PMR1 were recovered with equal efficiency (Fig. 3.20B left panel). In contrast, only full-length PMR60⁰ and the ?50C deletion showed appreciable tyrosine phosphorylation (Fig. 3.20B, right panel). Therefore, PMR1 is tyrosine phosphorylated in transfected Cos-1 cells as in *Xenopus* liver (unpublished data), and as predicted, the tyrosine phosphorylation site lies within the last 100 amino acids.

The sequence **RDGD RFYY** located between amino acids 643 and 650 matches a consensus tyrosine phosphorylation sequence R/KXXD/GXXXY. The
presence of adjacent tyrosines within this site raised the possibility that PMR1 might be phosphorylated on either or both of these residues. To pinpoint the residue that is phosphorylated each of these tyrosines was changed to phenylalanine (Y649F, Y650F) or both were changed together (Y649, 650F). TAP fusions of these proteins were transfected together with GFP-TAP into Cos-1 cells, and protein recovered on IgG-sepharose was analyzed as in Fig. 3.17B. All 4 forms of PMR60° were expressed and recovered with equal efficiency (Fig. 3.20C, left panel), but only those forms that retained a tyrosine at position 650 (Y650) were phosphorylated (Fig. 3.20C, right panel). Interestingly, GFP was also tyrosine phosphorylated, and the similar recovery of this in each sample served as an internal control for protein binding to IgG-sepharose. These data map the phosphorylation site to the tyrosine at position 650 within the C-terminal targeting domain.

3.9 Most of the PMR1 expressed in Cos-1 cells is tyrosine phosphorylated

To estimate the extent of PMR1 tyrosine phosphorylation I compared the efficiency of recovery of total PMR1 versus tyrosine phosphorylated PMR1 by immunoprecipitation with the phosphotyrosine-specific antibody RC20:biotin. In the experiment in Fig. 3.21, Cos-1 cells were transfected with PMR60°-TAP. Forty-eight hrs later puromycin was added dissociate polysome-bound complexes, and cytoplasmic extracts were incubated with RC20:biotin or nonimmune IgG prior to binding onto immobilized avidin. Bound proteins were eluted with SDS sample buffer, and input, unbound and bound fractions were
separated by SDS-PAGE. The blot in the left panel was probed with PY20 to determine the efficiency of recovering tyrosine phosphorylated PMR60\(^\circ\)-TAP with RC20:biotin. Tyrosine phosphorylated proteins were only recovered from extract that received RC20:biotin, and PMR60\(^\circ\)-TAP was the only \(\sim\)75 kDa tyrosine phosphorylated protein expressed in these cells (indicated with an arrow). Scanning densitometry of the PY20 blot showed that 67\% of input tyrosine phosphorylated PMR60\(^\circ\)-TAP was recovered with RC20:biotin on the avidin resin. A blot of the same fractions probed with antibody to the myc tag showed that 40\% of the input PMR60\(^\circ\)-TAP was recovered in the bound fraction. Normalizing this percent recovery to the 67\% binding efficiency one obtains a value of 60\% for tyrosine phosphorylated PMR60\(^\circ\)-TAP. This probably represents a minimum value because some degree of dephosphorylation likely occurred in the course of this experiment.

**3.10 Tyrosine phosphorylated PMR1 sediments with polysomes and mRNP complexes on sucrose gradients**

When cytoplasmic extracts are fractionated on sucrose density gradients PMR1 is localized to two complexes (Fig. 3.4). One is a polysome-bound complex that contains PMR1 together with its translating substrate mRNA. The second, more slowly sedimenting complex, is similar in size to mRNPs. To examine the phosphorylation state of PMR1 in each of these complexes Cos-1 cells were transfected with PMR60\(^\circ\)-TAP, and cytoplasmic extracts were separated on a 10-40\% sucrose gradient. Since Western blot with PY20 would
identify PMR1 and all other tyrosine-phosphorylated proteins I instead used binding to IgG-sepharose to selectively recover PMR60°-TAP in each fraction. Bound protein was eluted with SDS sample buffer and analyzed by Western blot using antibody to the myc tag and PY20 (Fig. 3.22). Both antibodies revealed the same distribution of protein across the gradient, with one population bound to polysomes and another sedimenting with mRNPs. This distribution matched that of PMR60°, and support results in Fig. 3.21 showing that the majority of PMR1 expressed in Cos-1 cells is tyrosine phosphorylated.

3.11 Phosphorylation at tyrosine 650 is required for targeting PMR1 to polysomes

The results in 3.10 suggest either that tyrosine phosphorylation is not required for targeting PMR1 to polysomes, or that the smaller PMR1-containing complex is a precursor to the polysome-bound complex. If the first option is true, then inactivating the tyrosine phosphorylation site with the Y650F mutation should have no effect on the gradient distribution of PMR1. Alternatively, if tyrosine phosphorylation is required for targeting PMR1 to polysomes, protein with the Y650F mutation should sediment only in the smaller complex. To examine whether Y650 is required for targeting PMR1 to polysomes, I performed a step gradient with cell extracts from cells transfected with plasmid bearing the Y650F mutation. Protein with the mutation did not sediment with polysomes (lane 11, Fig. 3.23) while wild type PMR1 did (lane 4, Fig. 3.23). The presence of
polysomes in the pellet fraction was confirmed by Western blot for ribosomal protein S6 in Mg^{++} and EDTA-containing gradient.

A linear gradient was also performed to accurately analyze the polysome profile of PMR1 bearing the Y650F mutation. The gradient profile in Fig. 3.24A showed that PMR60° with the Y650F mutation is restricted to the slowly-sedimenting complex. Adding EDTA to dissociate the polysomes had no impact on the sedimentation of the Y650F (Fig. 3.24B), and the sedimentation profiles in Figs. 3.24A and B are identical to that of PMR1 released by EDTA dissociation of polysomes.

Since most of the PMR1 in the cell is tyrosine phosphorylated it was formally possible that the Y650F mutation disrupted polysome targeting by replacing a negatively-charged residue with a hydrophobic residue. This was examined by substituting aspartic acid for tyrosine at position 650 (Y650D, Fig. 3.24C). As with the Y650F mutation, PMR60° with the Y650D mutation did not target to polysomes and was instead restricted to the mRNP complex like PMR60° with the Y650F mutation. Therefore, tyrosine phosphorylation, and not a negative charge at this position, is required for targeting PMR1 to polysomes.

3.12 The Y650F mutation blocks formation of the complex of PMR1 with substrate mRNA

A central observation in 3.5 and 3.6 was that PMR1 forms a selective complex with its translating, polysome-bound mRNA, and it is in this context that endonuclease cleavage initiates mRNA decay. A key piece of data supporting
this is shown in Fig. 3.16 where albumin mRNA could only be recovered on IgG-
sepharose by TAP-tagged forms of the protein that retained the ability to
associate with polysomes on sucrose density gradients. The similarity between
the results in Fig. 3.24 and those obtained with N- or C-terminal targeting domain
deletions raised the possibility that tyrosine phosphorylation is required to form
the complex of PMR1 with polysome-bound substrate mRNA. The first test of this
hypothesis compared the recovery of albumin mRNA on IgG-sepharose by
PMR60°-TAP with PMR60°-TAP bearing the Y650F mutation. In the experiment
shown in Fig. 3.25, Cos-1 cells were transfected with vectors expressing albumin
mRNA, luciferase mRNA, and PMR60°-TAP, the Y650F mutant form of the
protein, PMR1 with the C-terminal targeting domain deleted (?150C -TAP). The
?150C deletion was shown previously to inactivate PMR1 targeting to polysomes
and block the recovery of albumin mRNA by PMR1. Polysomes were
dissociated by treating cells with puromycin for 30 min prior to harvest, and
PMR1-containing complexes recovered on IgG-sepharose were analyzed by
Western blot for recovered protein, and by RT-PCR for recovered mRNA.
Somewhat more of the Y650F mutation and the ?150C deletion forms of PMR60°
than the full-length protein were recovered from IgG-sepharose (left panel)
Equal amounts of albumin and luciferase mRNA were expressed in each of the
transfectants; however, albumin mRNA was only recovered with full length
PMR60°-TAP (right panel). The selective nature of this process was confirmed
by the absence of luciferase mRNA recovered with any form of the protein.
Together with the results in Fig. 3.24 these data indicate that the mutation that
blocks tyrosine phosphorylation of PMR1 prevents incorporation of this protein into a complex with its substrate mRNA.

3.13 Tyrosine phosphorylation is required for PMR1 to join the functional ~680 kDa polysome complex with its substrate mRNA

The functional unit of endonuclease-mediated mRNA decay is a ~680 kDa mRNP complex containing PMR1 and its polysome-bound substrate mRNA, and deletions that inactivate PMR1 targeting to polysomes also prevent its binding to this complex, block the recovery of albumin mRNA with PMR1, and stabilize albumin mRNA to degradation by PMR1 in vivo. Therefore, if tyrosine phosphorylation is required for PMR1-mediated mRNA decay, the Y650F mutation should block formation of the ~680 kDa complex. In the experiment shown in Figs. 3.26A and B, PMR60º-TAP or PMR60º-TAP with the Y650F mutation were co-transfected into cells along with plasmids expressing albumin and luciferase mRNA, and cytoplasmic extracts prepared from puromycin-treated cells were separated on glycerol gradients. Western blots using antibody to the myc tag showed that PMR60º-TAP with the Y650F mutation was restricted to the complex that sediments at =140 kDa, whereas wild-type PMR60º-TAP was present both in this and the ~680 kDa complex (Fig. 3.26A). For the rest of this dissertation I will refer to the ~680 kDa complex as complex I and the =140 kDa as complex II.

Gradient fractions corresponding to complex I and complex II were pooled as indicated in Fig. 3.23A and divided into two portions. RNA was extracted from
one portion to determine the distribution of albumin mRNA and luciferase mRNA in the two complexes (Fig. 3.26B, input). The other portion was used to determine which of these complexes contained both PMR1 and albumin mRNA. This was accomplished by binding the pooled fractions to IgG-sepharose and extracting RNA from material recovered by cleavage with Tev protease (Fig. 3.26B, bound). Albumin mRNA and luciferase mRNA in the input and bound fractions were then assayed by semi-quantitative PCR. The input samples showed that albumin mRNA and luciferase mRNA were both present in complex I. For unknown reasons albumin mRNA was also in the smaller complex II in cells expressing PMR60º-TAP with the Y650F mutation, but not in cells expressing wild type PMR60º-TAP. A different picture emerged for RNA recovered on IgG-sepharose. Here albumin mRNA present in complex I was only recovered with wild type PMR60º-TAP, and no mRNA was recovered with Y650F mutant. These data indicate that the inability of PMR60º-TAP with the Y650F mutation to target to polysomes (Fig. 3.24) or recover albumin mRNA (Fig. 3.25) resulted from a block in the formation of complex I containing PMR1 and its substrate mRNA.

The results described above point to tyrosine phosphorylation at position 650 as a critical modification required for targeting PMR1 to its polysome-bound substrate mRNA. However, PMR1 has two independent polysome targeting domains, and the above experiments were all performed with the full-length protein. Previous work showed that the C-terminal 100 amino acids of PMR1 are sufficient to target a GFP fusion protein both to polysomes and to the ~680 kDa
complex I. The role of a phosphotyrosine at position 650 in this process was examined on glycerol gradients, which compared the sedimentation of GFP with that of a fusion protein with the wild-type C-terminal 100 amino acids (GFP-100C-WT) or a fusion protein with the Y650F mutation (GFP-100C-Y650F). The results in Fig. 3.27 show that the Y650F mutation inactivated the targeting of the GFP fusion protein to complex I. These data point to phosphorylation of Y650 as a critical determinant for the function of the C-terminal targeting domain in binding PMR1 to complex I.

3.14 The Y650F mutation stabilizes albumin mRNA to PMR1-mediated degradation

A deletion that disrupted the C-terminal polysome targeting domain of PMR1 stabilized albumin mRNA to degradation by PMR1. Since tyrosine phosphorylation is required for targeting PMR1 to polysome-bound substrate mRNA, the Y650F mutation should have the same stabilizing effect as that deletion. To test this Cos-1 cells were co-transfected with empty vector (pcDNA3), or catalytically-active forms of PMR60 or PMR60 with the Y650F mutation, and plasmids expressing albumin and luciferase mRNA. The Western blot in Fig. 3.28A (left panel), shows that PMR60 and the Y650F mutant were equally expressed. Compared to vector control, PMR60 catalyzed a 12-fold decrease in the steady-state level of albumin mRNA without affecting the level of luciferase mRNA (Fig. 3.28A, right panel). The Y650F mutation stabilized albumin mRNA, such that its level returned to ~50% of that seen with the vector
control. This degree of stabilization is the same as that seen in Fig. 3.16 with the ?50C mutation.

To insure that the Y650F mutation did not alter the catalytic activity of PMR1, I examined the ability of wild type and mutant protein to degrade albumin mRNA in vitro. In the experiment shown in Fig. 3.28B, cells were transfected with TAP fusions of PMR60, PMR60 with the Y650F mutation or GFP. Cytoplasmic extracts from puromycin-treated cells were applied to IgG-sepharose, and bound proteins were recovered by Tev protease cleavage. The Western blot in the upper panel of Fig. 3.28B shows that both forms of PMR60 were recovered with equal efficiency. A 5'-[\(^{32}\)P]-labeled albumin mRNA substrate transcript was degraded equally well by both proteins, whereas no ribonuclease activity was observed with GFP (Fig. 3.28B, lower panel). Thus, the Y650F mutation stabilized albumin mRNA by preventing tyrosine phosphorylation of PMR60 and consequent targeting of this endonuclease to its polysome-bound substrate mRNA.

### 3.15 Subcellular localization of PMR1 in Cos-1 cells

#### 3.15.1 PMR1 is distributed throughout the cytoplasm

A number of proteins involved in 5’-3’ mRNA decay (Dcp1, Dcp2, Xrn1, Lsm1-7), are concentrated in cytoplasmic foci (42,103,107). It has been suggested that those cytoplasmic foci are the places where 5’-3’ mRNA decay happens. To determine if PMR1 co-localizes with these proteins, Cos-1 cells were transfected with plasmids expressing PMR60\(^{\circ}\) and EGFP-Dbp5, which is
distributed throughout the cytoplasm (215) (Fig. 3.29A), or EGFP-Dcp1 (Fig. 3.29B). Fixed cells were stained with DAPI and a monoclonal antibody to the myc epitope and Alexa Fluor® 594 conjugated goat anti-mouse secondary antibody (Molecular Probes), and examined by confocal microscopy for direct fluorescence of DAPI, EGFP, and immunofluorescence of PMR60°. The distribution of PMR60° was indistinguishable from that of Dbp5 (Fig. 3.29A), and contrasted with the focal staining of Dcp1 (Fig. 3.29B). A similar cytoplasmic staining pattern was also seen for MEL cells that stably express a low level of PMR60 (161).

3.15.2 The Y650F mutation does not change the subcellular distribution of PMR1

Phosphorylation of Y650 of PMR1 was shown to be required for its polysome association. However, little is known about the mechanism and the biological functions of the phosphorylated Y650. An interesting question was whether the Y650F mutation would change the subcellular distribution of PMR1. To address this question, Cos-1 cells were transfected with plasmids expressing PMR60° with the Y650F mutation and EGFP-Dbp5, or EGFP-Dcp1 using the same conditions as in the experiment shown in Fig. 3.29. Y650F was distributed evenly in cytoplasm (Fig. 3.30), and was indistinguishable from the distribution of PMR60° (compared with Fig. 3.29). This data indicated that Y650F mutation did not alter the subcellular distribution of PMR1.
Fig. 3.1. Expression of PMR1 in Cos-7 cells. A. Cos-7(Tet-off) cells were transfected with plasmids expressing the catalytically active form of PMR1 (PMR60), the catalytically inactive form of PMR1 (PMR60⁰), and the PMR1 precursor (PMR80) with a myc epitope tag in the pcDNA3 vector. Cytoplasmic extracts were analyzed by Western blot with a monoclonal antibody to the myc epitope tag. B. Cos-7(Tet-off) cells were transfected with plasmids expressing the catalytically active form of PMR1 (PMR60), the catalytically inactive form of PMR1 (PMR60⁰), and the PMR1 precursor (PMR80) with a myc epitope tag in the pTRE vector. Cytoplasmic extracts were analyzed by Western blot with a monoclonal antibody to the myc epitope tag. C. Cos-1 cells were transfected with vector (pcDNA3) or plasmid expressing a catalytically inactive form of PMR1 (PMR60⁰) with a myc epitope tag. Cytoplasmic extracts were analyzed by Western blot with a monoclonal antibody to the myc epitope tag (left panel) or a polyclonal antibody to *Xenopus* PMR1 (right panel).
Fig. 3.2. Expression of PMR1-TAP in Cos-7 cells. Cos-7(Tet-off) cells were transfected with pcDNA3 vector, pTRE vector, and plasmids expressing the catalytically active form of PMR1 with an N-terminal myc epitope tag and a C-terminal TAP tag in pcDNA3 (pcDNA3-PMR60-TAP), and in pTRE (pTRE-PMR60-TAP). Cytoplasmic extracts were analyzed by Western blot with a monoclonal antibody to the myc epitope tag (A), a polyclonal antibody to *Xenopus* PMR1 (B), a HRP-conjugated goat anti-rabbit secondary antibody without any primary antibody (C), or biotinylated calmodulin and followed by streptavidin conjugated alkaline phosphatase (D).
Fig. 3.3. Mammalian recombinant PMR1 activity assay. A. To test whether recombinant PMR1 retains endonuclease activity, Cos-7(Tet-off) cells were transfected with pcDNA3-myc-TAP or pTRE-myc-TAP vectors, or plasmids expressing C-terminal TAP fusions of catalytically active PMR1 (pcDNA3-myc-PMR60-TAP and pTRE-myc-PMR60-TAP). TAP-tagged protein was recovered from EDTA treated postmitochondrial extract by binding to IgG-sepharose. Catalytic activity of the proteins recovered by Tev cleavage was assayed by incubation with 5′-[32P]-labeled albumin mRNA substrate transcript for 15 min, followed by electrophoresis on a 6% polyacrylamide/urea gel. B. LM(TK')(tet-off) cells harboring stably transfected tet-regulated PMR1-TAP and constitutive GFP were cultured in the medium containing decreasing concentrations of doxycycline. Cytoplasmic extracts were analyzed by Western blot with monoclonal antibodies to the myc epitope tag (upper panel) or GFP (lower panel). C. The above cell line was transfected with plasmids expressing albumin and ferritin mRNA. Cytoplasmic RNAs were analyzed by Northern blot with probes to albumin mRNA (upper panel) or ferritin mRNA (lower panel). D. Data in C were quantified with ImageQuant™, and the plotted data show the amount of albumin mRNA normalized to ferritin mRNA.
Fig. 3.4. Polysome binding of PMR1 and albumin mRNA. A. Cos-1 cells were transfected with plasmidss expressing PMR60° and albumin mRNA. Postmitochondrial extracts from transfected cells were separated on 10-40% sucrose density gradient containing 5 mM MgCl₂ and fractions were analyzed by absorbance at 260 nm, for distribution of PMR60° by Western blot with antibody to the myc tag, and by Northern blot for albumin mRNA. The direction of sedimentation is indicated with an arrow. The identity of polysome-containing fractions was confirmed by Western blot with a polyclonal antibody to ribosomal protein S6. B. Postmitochondrial extract from transfected cells was treated with 10 mM EDTA, and separated on a 10-40% sucrose density gradient containing 10 mM EDTA, and individual fractions were analyzed for the distribution of RNA, PMR60°, and ribosomal protein S6 as in A. C. Transfected cells were treated with puromycin for 30 min prior to harvest and sedimentation on a gradient containing 5 mM MgCl₂.
Fig. 3.5. Polysome binding of PABP and eIF4E. A. Postmitochondrial extract from Cos-1 cells was separated on a 10-40% sucrose density gradient containing 5 mM MgCl₂ and fractions were analyzed by Western blot with antibody to PABP (upper panel), or eIF4E (lower panel). The direction of sedimentation is indicated with an arrow. B. Postmitochondrial extract from Cos-1 cells was treated with 10 mM EDTA, and separated on a 10-40% sucrose density gradient containing 10 mM EDTA, and individual fractions were analyzed by Western blot with antibody to the PABP (upper panel), or eIF4E (lower panel).
**Fig. 3.6. Co-sedimentation of PMR1 and albumin mRNA in discontinuous sucrose gradients.**

A. Scheme for rapid separation of mRNP complexes and polysomes. Cell lysate is fractionated by sedimentation on a discontinuous 10% and 35% sucrose gradient. After centrifugation at 145,000 g for 1 hr, mRNP complexes accumulate at the interface between 10% and 35% sucrose, while polysomes pellet to the bottom.

B. Cos-1 cells were transfected with plasmid expressing PMR60° and albumin mRNA. Postmitochondrial extract from transfected cells was separated on the discontinuous sucrose density gradient containing 5 mM MgCl₂ or 10 mM EDTA, and proteins recovered from the pellet (Polysomes), interface (mRNPs) and top of the gradient were analyzed by Western blot with antibody to myc tag (upper panel) or ribosomal protein S6 (lower panel). Cytoplasmic RNA was extracted from each fraction and analyzed by Northern blot with a cDNA probe to albumin mRNA (C), RT-PCR with primers specific to albumin mRNA (D). Data in C and D were quantified with ImageQuant™.
Fig. 3.7. Expression of N- and C-terminal deletions of PMR1.  

A. Sequential 50 amino acid deletions were prepared from the N- and C-termini of PMR60° in a manner that retained the myc epitope tag on the N-terminus.  

B. Expression of each of the individual PMR60° deletions in transfected Cos-1 cells is shown by Western blot using the monoclonal antibody to the myc epitope tag.
Fig. 3.8. Expression of C-terminal TAP fusions of N- and C-terminal deletions of PMR1.  

A. Sequential 50 amino acid deletions were prepared from the N- and C-termini of PMR60° in a manner that retained the myc epitope tag on the N-terminus and TAP tag on the C-terminus.  

B. Expression of each of the individual PMR60° deletions in transfected Cos-1 cells is shown by Western blot using the monoclonal antibody to the myc epitope tag.
Fig. 3.9. N- and C-terminal domains of PMR1 required for targeting to polysomes. Each of the N- and C-terminal deletions was cotransfected with full-length PMR60° into Cos-1 cells, and cytoplasmic extracts from transfected cells were separated on discontinuous 10 and 35% sucrose density gradients containing either 5 mM MgCl₂ or 10 mM EDTA. The top, middle (mRNP) and bottom (polys) fractions were collected and analyzed by Western blot using the monoclonal antibody to the myc epitope tag. Full-length PMR60° is indicated on each blot with an arrow. The sedimentation of polysomes was confirmed by Western blot with antibody to ribosomal protein S6.
Fig. 3.10. Impact of a targeting domain deletion on PMR1 binding to polysomes. A. Postmitochondrial extract of Cos-1 cells transfected with a plasmid expressing the ?50C deletion of PMR60° was separated on linear 10-40% sucrose gradients containing 5 mM MgCl$_2$ (upper panels). B. Cells transfected as in A were treated with puromycin for 30 min prior to harvest. C. Extract from transfected cells was separated on a gradient containing 10 mM EDTA. Fractions were analyzed by Western blot for PMR60° using antibody to the myc epitope tag, or for ribosomal protein S6. The arrow indicates the direction of sedimentation.
Figure A shows the effect of MgCl₂ on protein distribution measured by Western blot. The band at 50 kDa, labeled as Δ50C, is visible in the presence of MgCl₂, while S6 is present at lower molecular weights.

Figure B displays the effect of puromycin and MgCl₂ on protein distribution. The Δ50C band is prominently visible at 75 kDa, and S6 is also present at lower molecular weights.

Figure C illustrates the effect of EDTA on protein distribution. The Δ50C band is again visible at 75 kDa, and S6 is present at lower molecular weights.
Fig. 3.11. Domains of PMR1 that can target GFP to polysomes. A. A schematic is shown of constructs where the indicated amino acid residues from the N- or C-terminal portions of PMR60° were fused to the N- or C-termini of GFP, respectively. B. Cos-1 cells were transfected with equal amounts of each of the GFP fusion proteins indicated in A and GFP. Postmitochondrial extracts were separated on discontinuous sucrose density gradients and fractions collected from the top, interface (mRNP) and bottom (polys) were analyzed by Western blot using a polyclonal antibody to GFP. GFP is indicated with a filled circle.
Fig. 3.12. Fine mapping of polysome targeting domain of PMR1. Cos-1 cells were transfected with plasmids expressing ?30C PMR60° (A), or ?40C PMR60° (B), or ?50C PMR60° (C). Postmitochondrial extract from cells with (left panel) or without (right panel) puromycin treatment prior to harvest was separated on a 10-40% sucrose density gradient containing 5 mM MgCl₂ and fractions were analyzed for distribution of PMR60° by Western blot with antibody to the myc epitope tag, and by Northern blot for albumin mRNA. The direction of sedimentation is indicated with an arrow. The identity of polysome-containing fractions was confirmed by Western blot with a polyclonal antibody to ribosomal protein S6.
**Fig. 3.13. PMR1 is associated with ~680 kDa large complex.** Cos-1 cells were transfected with PMR60°. Post-mitochondrial extracts with (B) without (C) EDTA treatment were separated on 10-40% glycerol gradients, and individual fractions were analyzed for PMR60° distribution by Western blot. Molecular size markers consisting of a mixture of bovine serum albumin (M_r = 66,000), lactate dehydrogenase (M_r = 140,000), catalase (M_r = 232,000), ferritin (M_r = 440,000) and thyroglobulin (M_r = 669,000) were fractionated on a parallel gradient. Protein concentration in each fraction was assayed by BCA and plotted in A.
Fig. 3.14. Domains of PMR1 required for joining the ~680 kDa complex. A. Cos-1 cells transfected with PMR60° or ?50C were treated with puromycin for 30 min prior to harvest. Post-mitochondrial extracts were separated on 10-40% glycerol gradients, and individual fractions were analyzed by Western blot for PMR60° and ?50C. B. Cos-1 cells transfected with GFP-50C, GFP-100C or GFP alone were treated with puromycin for 30 min prior to harvest. Post-mitochondrial extracts were separated on 10-40% glycerol gradients, and individual fractions were analyzed for GFP fluorescence.
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B

Fluorescence (units)

Fraction number

- GFP
- GFP50C
- GFP100C
Fig. 3.15. Application of TAP purification in PMR1 transfected Cos-1.  

A. Schematic view of TAP purification. B. Cos-1 cells were transiently transfected with plasmid expressing PMR60°-TAP and albumin mRNA. Post-mitochondrial extract was treated with EDTA to dissociate polysomes and then loaded onto an IgG-sepharose column. The beads were washed extensively, and bound material was eluted with TEV protease. Protein and RNA were extracted separately from the recovered fraction. Equal amount of samples collected from input, flow through, beads bound before and after Tev cleavage, and Tev elute fractions were analyzed by Western blot with antibody to myc epitope tag. Lane 1: Total input; Lane 2: TEV elute; Lane 3: Beads before TEV; Lane 4: Beads after TEV; Lane 5: Unbound. C. The recovered RNAs with PMR1-TAP or pcDNA3 vector by IgG-sepharose column were analyzed for albumin mRNA by RT-PCR with albumin specific primers.
Fig. 3.16. PMR1 forms a specific complex with its substrate mRNA.  **A.** Full-length PMR60° and each of the deletion constructs with the C-terminus fused with a tandem affinity (TAP) tag, or vector (pcDNA3) were transfected into Cos-1 cells together with plasmids expressing albumin mRNA and luciferase. The relative expression level of each of the fusion proteins in total cytoplasmic extract (input) was determined by Western blot using antibody to the myc epitope tag. These are denoted as PMR1-TAP. Beneath this is a Western blot for luciferase (luc) expression. The relative amount of albumin and luciferase mRNA in each sample was determined by quantitative RT-PCR and the products were separated on a denaturing 6% polyacrylamide/urea gel and quantified by phosphorimager analysis. The results were compared to a standard curve of serial dilutions of *in vitro* transcripts for each mRNA (indicated with a triangle). In this particular experiment albumin mRNA yielded a doublet product. **B.** Each of the cytoplasmic extracts in **A** was bound to IgG-sepharose and eluted by Tev protease cleavage (bound). Because this step generates products with a C-terminal calmodulin binding protein tag these are denoted PMR1-CBP. Recovered protein was analyzed by Western blot using the monoclonal antibody to the myc epitope tag, and albumin and luciferase mRNA recovered in each sample were analyzed by quantitative RT-PCR, and phosphorimager analysis. The relative amount of PMR1-containing construct in each of the input and bound fractions was determined by scanning densitometry, and these data were used to determine the relative recovery of albumin mRNA by each of these columns.
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- **Luc**
- **Albumin mRNA**
- **Luc mRNA**

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- **PMR-CBP**
- **Albumin mRNA**
- **Luc mRNA**

| .04 | 1 | .93 | 1.5 | .11 | .06 | .11 | .06 |

| Albumin mRNA |
| PMR-CBP |
Fig. 3.17. Endonuclease-mediated decay requires polysome binding of PMR1.  

A. Cos-1 cells were transfected with catalytically-active PMR60 or ?50C PMR1, together with plasmids expressing luciferase and albumin mRNA. The relative expression of each protein in cytoplasmic extracts was determined by Western blot in the upper panels, and albumin and luciferase mRNA were quantified by RNase-protection assay (lower panel).  

B. To test whether ?50C PMR1 retained the activity of the parent protein Cos-1 cells were transfected with C-terminal TAP fusions of both proteins or with GFP-TAP. Cells were treated with puromycin for 30 min prior to harvest and TAP tagged protein was recovered from post-mitochondrial extract (input) by binding to IgG-sepharose and elution with Tev protease (bound). Western blots of input and bound protein are in the upper panel. Catalytic activity of the recovered proteins was assayed by incubation with 5'-[\textsuperscript{32}P]labeled albumin mRNA substrate transcript for 0, 5, 10 and 30 min, followed by electrophoresis on a 6% polyacrylamide/urea gel. The lane 1 is input RNA only, and lane 2 is RNA recovered following degradation by PMR1 present in Xenopus liver polysome extract.
Fig. 3.18. Endonuclease-mediated decay requires polysome binding of substrate mRNA. A. The 24 bp stem-loop shown here was cloned into the 5’ UTR of albumin mRNA (SL-albumin) to block translation. B. Cytoplasmic extract from cells transfected with plasmid expressing wild type albumin or SL-albumin was separated on a discontinuous sucrose gradient. RNA was extract from top, mRNP and polysome fractions and analyzed for albumin mRNA distribution by RT-PCR. C. Cos-1 cells were transfected with catalytically-active PMR60 or pcDNA3 (vector) together with plasmids expressing luciferase and wild type albumin or SL-albumin. Albumin and luciferase mRNA were quantified by ribonuclease-protection assay.
G = -42.9 kcal/mol
**Fig. 3.19. PMR1 is tyrosine phosphorylated in Cos-1 cells.** Cos-1 cells were transfected with PMR60°-TAP, and incubated with presence or absence of genistein for 6 hrs prior to harvest. PMR60°-TAP was recovered by IgG-sepharose beads. Cytoplasmic extracts from cells that were not given genistein were incubated for 1 hr in the presence or absence of CIAP prior to binding to IgG-sepharose beads. Input, unbound, and bound fractions eluted with SDS sample buffer were separated by SDS-PAGE and analyzed by Western blot with PY20 (A) or antibody to the myc epitope tag (B).
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C-Myc
Fig. 3.20. Mapping the tyrosine phosphorylation site on PMR1.  A. A schematic of the 80 kDa unprocessed PMR1 is shown with the mature 60 kDa form (PMR60) indicated, sequences with potential for binding to SH3 domains shown in grey boxes, a leucine-rich variant repeat in the box with horizontal lines, a potential tyrosine kinase phosphorylation site in the black box, and the locations of the two polysome targeting domains in brackets.  B. To map the general location of the tyrosine phosphorylation site, Cos-1 cells were transfected with plasmids expressing TAP-tagged forms of the full-length protein (PMR60º) or deletions lacking 50 (?50C) or 100 (?100C) amino acids from the C-terminus. Protein recovered on IgG-sepharose was extracted with SDS sample buffer and analyzed by Western blot with antibody to the N-terminal myc epitope tag on PMR60º or PY20.  C. The tyrosine residues at positions 649 (Y649F), 650 (Y650F) or both sites (Y649,650F) in PMR60º-TAP were mutated to phenylalanine, and the phosphorylation state of each protein expressed in transfected cells was determined as in B.
Fig. 3.21. Estimating the population of PMR1 that is tyrosine phosphorylated. Cytoplasmic extracts from cells transfected with PMR60°-TAP were incubated with RC20:biotin or nonimmune IgG prior to binding to immobilized avidin. Input, unbound, wash and bound fractions eluted with SDS sample buffer were separated by SDS-PAGE and analyzed by Western blot with PY20 or antibody to the myc epitope tag on PMR60°-TAP. The blot in the left panel probed with PY20 (lanes 1-8) determined the specificity and efficiency for recovering tyrosine phosphorylated protein, and the blot in the right panel probed with myc epitope antibody (lanes 9-12) determined the efficiency of recovering PMR60°-TAP. The data was quantified by scanning densitometry as shown beneath the figure. The recovery of myc epitope staining protein on avidin resin was normalized to the efficiency of recovery for tyrosine phosphorylated PMR60°-TAP determined with PY20.
percent recovery of PMR60⁰-TAP

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Fig. 3.22. Tyrosine-phosphorylated PMR1 sediments with polysomes and mRNP complexes on sucrose gradient. Cos-1 cells were transfected with PMR60º-TAP and cytoplasmic extracts were separated on a 10-40% sucrose gradient that contained MgCl₂ to maintain ribosome integrity. Even-numbered fractions were treated with EDTA to dissociate polysome-bound complexes, applied to IgG-sepharose and eluted with SDS sample buffer. The recovered proteins were analyzed by Western blot with antibody to the myc epitope tag (upper panel) or with PY20 (middle panel). The sedimentation of polysomes was determined by Western blot of unselected gradient fractions with a monoclonal antibody to ribosomal protein S6 (lower panel).
Fig. 3.23. **Y650F does not sediment with polysome on a discontinuous sucrose gradient.** Cos-1 cells were transfected with plasmid expressing PMR60° or Y650F. Post-mitochondrial extract from transfected cells was separated on discontinuous sucrose density gradients containing 5 mM MgCl₂ or 10 mM EDTA, and proteins recovered from the pellet (polysomes), interface (mRNPs) and top of the gradient were analyzed by Western blot with antibody to myc epitope tag (upper panel) or ribosomal protein S6 (lower panel).
Fig. 3.24. Mutating Y650 blocks the targeting PMR1 to polysomes.  A. Cos-1 cells were transfected with plasmid expressing PMR60° with the Y650F mutation, and cytoplasmic extracts were separated on a 10-40% sucrose gradient. Even-numbered fractions were analyzed by Western blot with antibody to the myc epitope tag or with antibody to S6 to map the sedimentation of polysomes on the gradient.  B. The sample analyzed in A was sedimented on a gradient containing 10 mM EDTA, and even-numbered fractions were analyzed as in B with antibodies to the myc epitope tag or S6.  C. Cells were transfected with PMR60° in which Y650 was changed to aspartic acid (Y650D), and the sedimentation of PMR1 was analyzed on a gradient containing MgCl₂ as in A.
Fig. 3.25. The Y650F mutation prevents recovery of substrate mRNA by PMR1. Full-length PMR60⁰-TAP, PMR60⁰-TAP with the Y650F mutation (Y650F), or PMR60⁰-TAP deleted of the C-terminal 150 amino acids (?150C) were transfected into Cos-1 cells together with plasmids expressing albumin and luciferase mRNAs. Extracts from puromycin-treated cells were bound to IgG-sepharose and eluted by cleavage with Tev protease. Protein recovered in the bound complexes was analyzed by Western blot with antibody to the myc epitope tag (A) and RNA extracted from the bound complexes was analyzed by quantitative RT-PCR and phosphorimager analysis (B).
Fig. 3.26. The Y650F mutation blocks targeting of PMR1 to the functional ~680 kDa complex. A. Cos-1 cells transfected with PMR60° or PMR60° with the Y650F mutation plus plasmids expressing albumin and luciferase mRNAs were treated with puromycin prior to harvest, and cytoplasmic extracts were separated on 10-40% glycerol gradients. Even-numbered fractions were analyzed by Western blot with antibody to the myc epitope tag. B. The fractions identified with brackets in A were pooled and identified as complex I and complex II. Half of each pooled complex was bound to IgG-sepharose and eluted by cleavage with Tev protease, and RNA extracted from the bound and unbound complexes was analyzed by quantitative RT-PCR.
Fig. 3.27. The Y650F mutation fails to target GFP fusion to ~680 kDa complex. The C-terminal 100 amino acids of PMR1 bearing the native sequence or the Y650F mutation were fused to the C-terminus of GFP. Cytoplasmic extracts from Cos-1 cells transfected with these or GFP alone were fractionated on a 10-40% glycerol gradient and individual fractions were assayed for fluorescence.
Fig. 3.28. The Y650F mutation stabilizes albumin mRNA to degradation by PMR1. A. Cos-1 cells were transfected with pcDNA3 vector, pcDNA3 expressing catalytically-active PMR60, or pcDNA3 expressing PMR60 with the Y650F mutation, together with plasmids expressing albumin and luciferase mRNAs. The relative expression each protein was determined by Western blot (left panel) and their impact on steady-state levels of albumin and luciferase mRNA were determined by RNase protection (right panel). The relative amount of albumin mRNA normalized to luciferase mRNA determined by phosphorimager analysis is shown beneath the autoradiogram. B. The catalytic activity of PMR60 and PMR60 with the Y650F mutation was determined by assaying the degradation of 5’-[^32P]labeled albumin mRNA by PMR60-TAP, PMR60-TAP with the Y650F mutation, or GFP-TAP that were expressed in transfected cells and recovered on IgG-sepharose. The upper panel is a Western blot of input protein and protein recovered from IgG-sepharose Tev protease cleavage, and the lower panel is a time course of 0, 5, 10, 20 and 30 min of incubation with substrate RNA.
Fig. 3.29. **Cytoplasmic distribution of PMR1.**  

**A.** Cos-1 cells were transfected with a plasmid expressing myc-PMR60° and a plasmid expressing a Dbp5-EGFP fusion protein. Fixed cells were stained with monoclonal antibody to the myc epitope and DAPI, and examined by confocal microscopy for Dbp5-EGFP (panel a), PMR60° (panel b) and DAPI (panel c). The merged images are presented in panel d.  

**B.** Cells were transfected as above with plasmids expressing myc-PMR60° or Dcp1-EGFP.
Fig. 3.30. Cytoplasmic distribution of PMR1 with Y650F mutation. A. Cos-1 cells were transfected with a plasmid expressing myc-PMR60º-Y650F and a plasmid expressing a Dbp5-EGFP fusion protein. Fixed cells were stained with monoclonal antibody to the myc epitope and DAPI, and examined by confocal microscopy for Dbp5-EGFP (panel a), PMR60º (panel b) and DAPI (panel c). The merged images are presented in panel d. B. Cells were transfected as above with plasmids expressing myc-PMR60º-Y650F or Dcp1-EGFP.
4.1 Development of PMR1 cell culture model system

Our understanding of PMR1 function has been hampered not only by a lack of a suitable *Xenopus* hepatocyte cell line but also by lack of an expression cell culture system. While primary cell culture has been proven useful to answer basic questions regarding the need for translation during mRNA decay and the role of the estrogen receptor in regulated mRNA decay, it has not been a suitable model system to investigate the molecular interactions of PMR1, regulation of its activity or its subcellular localization. The main focus of this study was to identify domains in PMR1 that are responsible for targeting it into polysome associated complexes. Much of the work in this study used recombinant PMR1 as a tool to identify interacting proteins and to recover complexes bearing these proteins. However, *Xenopus* hepatocytes are difficult to culture and have low transfection efficiency, limiting their use for this study. Therefore, I sought to develop a cell culture system for PMR1. I found that Cos-1 cells do not express the mammalian ortholog of PMR1, minimizing potential competition with an exogenous protein. More importantly, PMR1 sedimented on a sucrose density gradient in the same
manner in these cells as the endogenous protein in frog liver. The absence of a
cross-reacting protein and appropriate sucrose gradient sedimentation made
Cos-1 cells a useful model system for future studies. The Cos-1 cell culture
model developed in this study made it possible to address many issues related to
*Xenopus* hepatocyte cells. For example, by using a myc tagged expression clone
I tracked PMR1 and studied its localization *in vivo*; by using a TAP tagged form I
could efficiently isolate PMR1 containing complexes and identify domains
involved in targeting PMR1 to its substrate mRNA. Furthermore, the development
of a stable cell line expressing PMR1 will allow us to apply the TAP recovery
technique together with Matrix-assisted Laser Desorption/Ionization (MALDI) and
LC-MS/MS analysis, leading to characterization of PMR1 containing complexes
and identification of interacting proteins in these complexes. By combining
microarray analysis with this technique, it will be possible to identify all potential
mRNA cellular targets of PMR1. These results will help to define the molecular
basis for targeting and substrate selectivity in mRNA decay.

Recombinant PMR1 expressed in Cos-1 cells has significant ribonuclease
activity and could generate many of the non-consensus albumin degradation
fragments generated by *Xenopus* PMR1. However, it failed to generate the
consensus PMR1 cleavage products at motif AYUGA. These results mirror
observation made by a previous member of our laboratory using a baculovirus
expression system. One possible explanation is that the cleavage products fail to
accumulate and get rapidly degraded by exoribonucleases in these cells. A
similar observation was made when purified native PMR1 was assayed following
renaturation, with loss of AYUGA sequence cleavage but not loss of ribonuclease activity (data not shown). All of these observations suggest that correct folding of the mature 60 kDa protein may be important for sequence selective cleavage and that this folding does not occur in mammalian cells. Alternatively, the folding may be correct but the cells may not correctly modify the protein to generate this particular cleavage property. This suggested that co- or post-translational modification may be important for proper enzyme function. Further characterization of the Cos-1 cell expressed PMR60 protein will be necessary to understand the post-translational modifications PMR1 undergoes for its function.

4.2 Polysome association of PMR1

PMR1 is found on polysome as well as in low molecular weight complexes that are not associated with ribosomes (Fig. 3.4). PMR1, although bound to mRNP particles, does not appear to degrade the mRNA until it is associated with polysomes, suggesting that polysome association of PMR1 is the key step for mRNA degradation. My data show that the N- and C-terminal domains of PMR1 each play a role in targeting the protein to polysomes. Both domains are required for polysome binding by the full length protein (Fig. 3.9), but each can also act independently to target a GFP fusion to polysomes (Fig. 3.11). The N-terminal targeting domain lies between the first 100 and 150 amino acids, although the exact mechanism of how this domain works is currently unknown. An interesting observation (Fig. 3.16B) showed that 50% more albumin mRNA was recovered by PMR1 lacking the N-terminal 100 amino acids than either the
full-length protein or a deletion lacking 50 amino acids. In regarding this, albumin mRNA is present in vast excess over PMR1 in liver, and it has previously been shown that the estrogen activation of mRNA decay may involve changes in both unit enzyme activity (200) of substrate mRNA targeting. These data raise the possibility that the N-terminus of PMR1 contains a region that modulates the efficiency of targeting of the endonuclease to substrate mRNP.

The C-terminal targeting domain is complex. The behavior of the C-terminal deletions (Figs. 3.9 and 3.10) seems to conflict with the observation made in GFP fusions (Fig. 3.11). The deletion of the C-terminal 50 amino acids disrupts the targeting to polysome, whereas this fragment cannot target GFP to polysomes and only the C-terminal 100 amino acids can when fused to GFP. In other words, the C-terminal 50 amino acids are necessary but not sufficient for polysome targeting, and the C-terminal 100 amino acids are efficient for targeting PMR1 to polysomes. This observation suggests that the C-terminal targeting domain lays just upstream of ?50C and was disrupted by removal of the last 50 amino acids. An alternative explanation is that loss of the C-terminal 50 amino acids interfered with proper folding of the protein. The following-up study (Fig. 3.20) showed the phosphotyrosine at position 650 located 14 amino acids upstream of ?50C is involved in targeting to polysomes. This result supports the former hypothesis, as the C-terminal 50 amino acids do not include the tyrosine phosphorylation site and therefore are not sufficient for polysome targeting. It is clear, however, that the last 50 amino acids contain additional sequences required for polysome targeting, as ?50C disrupted the polysome association.
By adding sequences back to ?50C (Fig. 3.12), I have shown that polysome targeting of PMR1 was partially recovered with ?40C and almost completely restored with ?30C. This result indicates that the additional 20 amino acids located downstream of ?50C from the last 30 to 50 amino acids are required for efficient targeting to polysomes. Fusing this fragment to GFP successfully targeted the fusion protein to polysomes (R. Singh, unpublished). Further experiments with alanine scanning mutagenesis will identify these amino acids that are required for polysome targeting.

4.3 PMR1 forms a specific complex with its substrate mRNA

One puzzle in PMR1 mediated mRNA decay is how PMR1 finds its substrates. Unlike the G3BP endonuclease, which has a single RRM motif in the carboxy-terminus (177), PMR1 is a paralog of the peroxidase gene family (212), and lacks recognizable RNA binding domains. The presence of multiple polysome targeting domains provided a useful tool to test whether the association of PMR1 to polysomes involved the formation of a specific complex between the endonuclease and its substrate mRNA. To accomplish this I added a TAP tag to PMR1 and deletions that either retained or lost the ability to target to polysomes, and examined the recovery on IgG-sepharose of substrate (albumin) and control (luciferase) mRNA following EDTA dissociation of polysomes. The results in Fig. 3.16B show that albumin mRNA was recovered only by forms of PMR1 that retained the ability to target to polysomes, thus proving that PMR1 is part of a complex with its polysome-bound substrate mRNA.
The selective nature of this process was underscored by the observation that only background levels of luciferase mRNA were recovered with all of the PMR1-TAP constructs.

Although the data presented in here suggest that PMR1 and its substrate mRNA are in the same complex, direct binding of PMR1 to substrate mRNA has not been observed (unpublished). This suggests that PMR1 associates with albumin mRNA through mRNPs. Glycerol gradient analysis played a key role in identifying these mRNPs associated with PMR1-mediated mRNA decay. The glycerol gradient data in Fig. 3.13 and Fig. 3.14 indicate that the C-terminal sequences involved in targeting PMR1 to polysomes are necessary and sufficient for incorporating PMR1 or a GFP fusion protein into a ~680 kDa polysome-associated complex that was previously identified in *Xenopus* liver (200). The results in Fig. 3.17 indicated that this ~680 kDa polysome-associated complex is likely the functional unit of PMR1 *in vivo*, or at least the initial site for endonuclease-mediated mRNA degradation; because a) albumin mRNA was degraded in cells expressing catalytically-active PMR1 but luciferase mRNA was not, and the steady-state level of albumin mRNA was reduced 11-fold compared to cells transfected with vector alone (Fig. 3.17), and b) loss of a targeting domain by deleting the C-terminal 50 amino acids had no effect on catalytic activity, yet reduced the efficiency of this process by 50% (Fig. 3.17), and c) albumin mRNA was stabilized to PMR1 degradation by a 5’-UTR stem-loop that blocked its incorporation into polysomes (Fig. 3.18).
There is precedent to suggest that this association of PMR1 with mRNPs is not unique. It has been shown that β-globin mRNA in mouse sarcoma (S-180) ascite cells associates with an endonuclease activity that cleaves the mRNA at AU and AG sites within the 3’UTR (216). Interestingly, like PMR1, the majority of ribonuclease activity was found to be associated with polysomes, although low levels of endonuclease activity were detectable on mRNPs. It is not known if the endonuclease binds directly to mRNA, however a tight association is suggested by the fact that dilution of mRNPs does not alter the rate that the mRNA is degraded (216). In human proerythroleukemic K562 cells and rat liver, an endonuclease activity was found to associate with cytoplasmic and nuclear mRNPs that could degrade mRNA in vitro (217). Thus, the association of PMR1 with mRNP particles may represent a common means for endonucleases to target their substrates as well as associate with factors that may regulate nuclease activity.

4.4 Tyrosine phosphorylation of PMR1

It has been reported that phosphorylation participates in the regulation of nuclease activity. Serine hyperphosphorylation of the 52 kDa G3BP endonuclease in quiescent but not serum-stimulated hamster fibroblasts was responsible for activation of the endonuclease activity (177). In serum-stimulated cells, G3BP is associated with the membrane-bound GTPase-activating protein, RasGAP, and does not appear to be involved in endonucleolytic decay. The mechanism for this interaction has yet to be defined. It was suggested that once
the cells become quiescent, G3BP becomes hyperphosphorylated, dissociates from RasGAP and targets proto-oncogene mRNAs, such as myc, to regulate cell proliferation (177). In this way, the ras signaling pathway regulates cell proliferation not only through activation of signal transduction cascades, but also through the modulation of mRNA stability.

The Scansite analysis identified a potential tyrosine phosphorylation site within the C-terminal polysome targeting domain upstream of the ?50C deletion. I confirmed that PMR1 is tyrosine phosphorylated in our model system and mapped the phosphorylation to position 650 (Fig. 3.20C). Although much of the PMR1 expressed is tyrosine phosphorylated (Fig. 3.21), the state of tyrosine phosphorylation does not seem to change in response to estrogen (data not shown). Thus, tyrosine phosphorylation per se is not the primary means of activating PMR1 unit activity following estrogen treatment. However, additional phosphorylation sites may exist on PMR1 and a role for phosphorylation of PMR1 on other residues in responding to estrogen and activating PMR1 cannot be excluded. There are examples of phosphorylation playing an important role in regulating protein-protein or protein-RNA interactions, and changes in the phosphorylation of RNA-binding proteins modulating the turnover of unstable mRNAs (133,203,218).

To study the role of tyrosine phosphorylation, I performed a series of experiments looking at PMR1-containing complexes and PMR1-mediated mRNA decay. The functional consequences of tyrosine phosphorylation became immediately apparent on sucrose density gradients. The phosphorylation of Y650
is required for targeting PMR1 to polysomes, and targeting was lost when the phosphorylated tyrosine was mutated to phenylalanine (Figs. 3.23 and 3.24A). Several additional lines of evidence support the conclusion that phosphorylation of Y650 in the C-terminal targeting domain is required for endonuclease mediated mRNA decay. First, aspartic acid at position 650 (Y650D) did not substitute for tyrosine, so it is unlikely that a negative charge at this location was the primary determinant for polysome binding (Fig. 3.24B). Next, the targeting of PMR1 to ~680 kDa complex was inactivated by mutations that changed tyrosine at position 650 to phenylalanine (Y650F) (3-26A). Changing Y650 to phenylalanine inactivated the recovery of albumin mRNA by the TAP-labeled protein as effectively as deletion of the C-terminal targeting domain (Fig. 3.25).

The formation of a complex containing PMR1 and substrate mRNA lies at the core of endonuclease-mediated mRNA decay, and the inability of PMR60° with the Y650F mutation to recover albumin mRNA indicates that this single amino acid alteration prevented the formation of this complex. In the experiment shown in Fig. 3.26, albumin mRNA was present in complex I, but it was not recovered on IgG-sepharose because the Y650F mutation restricted PMR1 to the lighter complex II (Fig. 3.26B). These data provide the biochemical explanation for data in Fig. 3.25. The behavior of the parent protein extended to a GFP fusion bearing the C-terminal 100 amino acids of PMR1 (Fig. 3.27). Whereas the wild-type sequence targeted GFP to complex I, protein bearing the Y650F mutation was restricted to the slowly-sedimenting complexes (complex II). Based on the result that PMR1 is found in both large complex (~680 kDa
complex I) and smaller complex (~140 kDa complex II), we hypothesized that the targeting of PMR1 to polysomes involves a stepwise process beginning with the formation of complex II (which sediments with the mRNP fraction on sucrose gradients), and then tyrosine phosphorylation leads to the formation of polysome-bound complex I with its substrate mRNA. The results obtained with the Y650F mutation on sucrose and glycerol gradients support that PMR1 joins complex I either at the time of tyrosine phosphorylation or thereafter. The complex II appears to serve as the precursor for forming the ~680 kDa polysome-bound complex I.

The above data confirm that tyrosine phosphorylation activates the C-terminal polysome targeting domain, thus licensing the endonuclease to join the polysome associated complex containing its translating substrate mRNA. As shown in Figs. 3.17 and 3.18, PMR1-mediated mRNA decay pathway is blocked and mRNA is stabilized by insertion of a secondary structure in the 5′-UTR that blocks polysome entry of substrate mRNA or by deletions that inactivate targeting of PMR1 to polysome. These evidences together indicate that PMR1-mediated mRNA decay process likely occurs in the context of polysome association and tyrosine phosphorylation appears to be the key step in the process of endoribonuclease-mediated mRNA decay.

Since the targeting of PMR1 to polysomes is required for endonuclease-mediated mRNA decay, the Y650F mutation should stabilize albumin mRNA as effectively as the ?50C deletion. The results in Fig. 3.28 show this to be the case. PMR60 catalyzed a 12-fold decrease in albumin mRNA without affecting
the steady-state level of luciferase mRNA (Fig. 3.28A). The Y650F mutation had no impact on the catalytic activity of PMR1 (Fig. 3.28B), but nonetheless stabilized albumin mRNA to endonuclease-mediated degradation (Fig. 3.28A). In agreement with all of the preceding data, the degree of stabilization observed in Fig. 3.28 was similar to that of the ?50C mutation (Fig. 3.17). The tyrosine at position 650 is both present and phosphorylated in the ?50C deletion of PMR1 (Fig. 3.19B), but the ?50C deletion removed additional downstream sequence that is needed for targeting to polysomes. This is supported by the polysome profiles of ?40C and ?30C where ?30C is fully capable to be targeted to polysome and ?40C is only partially associated with polysomes (Fig. 3.12). The sequences required in addition to Y650 phosphorylation for targeting to polysome lie between 30 and 40 amino acids from C-terminus of PMR1.

Previous work in our lab showed that bacterial- and baculovirus-expressed PMR1 had less enzymatic activity than expected to efficiently cleave albumin mRNA. Also, a yeast 2-hybrid screen failed to identify any PMR1 interacting proteins. One possible explanation for both observations is maybe the absence of proper post-translational modifications in these recombinant expression systems. Data presented in this study indicate that tyrosine phosphorylation is a required post-translational modification for endonuclease-mediated decay. Tyrosine phosphorylation and protein tyrosine kinases (PTKs) are key effectors of signal transduction in cells. Phosphorylation of Y650 of PMR1 suggests that PTKs may directly function in mRNA decay. Data presented in Fig. 3.25 showing that changing tyrosine at position 650 to
phenylalanine stabilized albumin mRNA provide direct evidence linking the activity of one or more tyrosine kinases to endonuclease-mediated mRNA decay. Nevertheless, because tyrosine phosphorylation at Y650 targets PMR1 to the polysome associated complex, the identification of a tyrosine kinase seems to be the key step to dissect the signal transduction pathway that leads to activation of PMR1 and to understand endonuclease-mediated mRNA decay. Furthermore, it is estimated that ~ 67% of PMR1 is tyrosine phosphorylated, and the rest is in dephosphorylated form. There are many examples that the cycle of phosphorylation and dephosphorylation play a critical role in regulating biological function. We hypothesize that a tyrosine phosphatase may be involved in dephosphorylating Y650 of PMR1, which releases PMR1 from polysomes and recycles PMR1 to form new complexes that may re-associate with a new transcript and initiate another round of mRNA decay.

PMR1 is found to form a complex with its translating substrate mRNA in a ~680 kDa mRNP (complex I). However, PMR1 has no known RNA binding motif or RNA binding activity. Thus, proteins present in the ~680 kDa complex must selectively bind both substrate mRNA and PMR1. The latter interaction is through a phosphotyrosine-binding protein. Scansite analysis of PMR1 revealed the sequences flanking the tyrosine phosphorylation site are a potential ligand for group I SH2 domains characteristic of Lck, Abl and Nck (219). This sequence is conserved in the mouse, rat and human orthologs. The work in our lab has previously confirmed that the mouse ortholog is tyrosine phosphorylated (unpublished). Therefore, an SH2-containing protein is potential target for
directing PMR1 to complex I and determining PMR1 substrate specificity. Mena and VASP have been recently identified as proteins that interact with PMR1 (Y. Peng, unpublished). Mena and VASP act primarily to modulate dynamic changes in the actin cytoskeleton. However, there are several steps where Mena may play a role in PMR1 mediated mRNA decay. The proline-rich domain of Mena serves as a hub for different signal transduction pathways and as a binding partner for several PTKs. Therefore, it may function in bringing together PMR1 and a PTK, a result that is consistent with the presence of Mena in complex II (Y. Peng, unpublished). Of course, the real biological function of Mena and VASP in mRNA decay still awaits experimental results to confirm. Nevertheless, this study shows that phosphorylation of Y650 is one of the key post-translational modifications that are required for enzyme function. To the best of our knowledge this is the first demonstration of tyrosine phosphorylation regulating the function of an effector nuclease involved in the catalysis of mRNA decay. This study also suggests that tyrosine kinases have a previously unappreciated impact on the turnover of mRNAs that are targeted by endonuclease-mediated mRNA decay and might become the potential therapeutic targets for cancer and other diseases.

4.5 Endonuclease-mediated decay is distinct from exonuclease decay pathway

Results presented in this study underscore the fundamental differences between endonuclease-mediated mRNA decay and the more generalized exonucleolytic pathway(s) of mRNA decay. While recent characterization of the
exonuclease-mediated pathways has indicated that 5’-3’ decay acts on non-translating, deadenylated mRNA in discrete subcellular foci (P-bodies), PMR1 selectively targets and degrades its translating substrate mRNA on polysomes. Therefore endonuclease-mediated mRNA decay is distinctly different from the exonuclease-mediated pathways.

In addition to differences in the enzymatic mechanisms of mRNA degradation, the decay factors themselves show differences in subcellular distribution. Cellular PMR1 is found on polysomes and smaller complexes (Fig. 3.4) and confocal microscopy shows diffuse PMR1 staining throughout the cytoplasm (Fig. 3.29). The same diffuse staining is seen for Dbp5; however, there is no evidence that these proteins are in the same complex. The generalized distribution of PMR1 is distinctly different from the focal concentration of Dcp1 (Fig. 3.29B). In yeast these focal complexes are sites for decapping and Xrn1-mediated 5’-3’ degradation of the mRNA body (65). This contrasts with endonuclease-mediated mRNA decay, where PMR1 and its substrate mRNA come together in a complex on the translating mRNP (200). In this context endonucleolytic cleavage within the body of the mRNA has the same functional effect as deadenylation by effectively separating the cap from the poly(A) tail. This may explain earlier observations that deadenylation is not required for endonuclease-mediated mRNA decay (163). Furthermore, this mechanism has the potential to promote more rapid inactivation of a target mRNA than a deadenylation-dependent process, since it requires only a single cleavage event.
Although the full scope of endonuclease-mediated mRNA decay has yet to be determined, it is thought to be restricted to specific groups of mRNAs (63,199). Examples include avian apo-VLDL II (165), all of the *Xenopus* serum protein mRNAs (195), and transferrin receptor mRNA (163). While the degradation of these mRNAs is activated by some external stimulus (e.g. estrogen, iron status), it has been recently shown that nonsense-containing β-globin mRNA undergoes endonuclease-mediated mRNA decay in erythroid cells (160). Notably, the endonuclease responsible for this is strikingly similar to PMR1, even to the point of cleaving at precisely the same sites *in vivo* (161). Given the central role of ribosome scanning in NMD, the presence of an mRNA endonuclease on the translating mRNP provides a direct mechanism for the rapid inactivation and degradation of mRNA upon detection of a premature termination codon.

### 4.6 Concluding remarks

In conclusion, I would like to propose a model of endonuclease-mediated mRNA decay (Fig. 4.1), which incorporates much of the data and hypotheses presented above. In this model the binding of one or more specific proteins to a substrate (e.g. albumin) mRNA generates a platform for recruiting PMR1 to a ~680 kDa complex that comprises the translating mRNP. PMR1 is originally associated with its interacting proteins in a ~140 kDa light complex (complex II). Upon phosphorylation at tyrosine 650 by a specific tyrosine kinase, it is moved to the ~680 kDa large complex (complex I) containing substrate mRNA, and becomes associated with membrane-bound polysomes as part of the large...
complex. Under conditions when estrogen is not present, PMR1 will have a basal level of nuclease activity that modulates the half-life of transcripts such as albumin. PMR1 is subsequently activated by estrogen through a signal transduction pathway to generate decay intermediates with free 3’ ends (199). Such products would be good substrates for subsequent degradation by the exosome. Once target mRNA is degraded, PMR1 is dephosphorylated by a tyrosine phosphatase and released from polysome-bound complex I as free protein or as complex II, and recycled to form new complexes that may re-associcate with a new transcript. The phosphorylation and dephosphorylation cycle regulates association and dissociation of PMR1 with polysomes and is necessary for initiation of another round of mRNA decay. In contrast, mRNAs lacking this constellation of binding proteins (eg. luciferase) are not targeted by PMR1 and instead undergo deadenylation-dependent decay. This can take the form of 3’-5’ degradation by the exosome, or decapping followed by 5’-3’ degradation of the mRNA body. This form of endonuclease targeting provides considerable flexibility for rapidly changing the production of a protein product in response to various stimuli.
Fig. 4.1. Model for endonuclease-mediated mRNA decay. I propose that mRNAs are specifically targeted for degradation by exonuclease or endonuclease-mediated mRNA decay by their complement of specific mRNA binding proteins. mRNAs targeted for exonuclease-mediated mRNA decay first undergo deadenylation, which triggers the loss of ribosome binding and degradation either by the cytoplasmic exosome or by decapping and 5’-3’ decay in P-bodies. In contrast, mRNAs that are targeted for endonuclease-mediated mRNA decay are bound by one or more proteins (shown in purple) that recruit PMR1 to the ~680 kDa polysome-bound substrate mRNP complex. Activation of the endonuclease in this context (lightning bolt) results in cleavage within the mRNA body without the need for prior deadenylation. The resulting decay intermediates are then most likely degraded by the exosome, with the scavenger decapping enzyme DcpS acting to remove the cap from the 5’ limit digest.
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