A CHARACTERIZATION OF SUBSTRATES AND FACTORS INVOLVED IN YEAST NONSENSE-MEDIATED mRNA DECAY

A Dissertation Presented By

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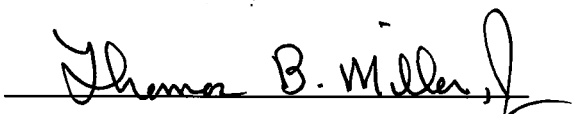

Dr. Thomas B. Miller, Jr., Dean of the
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Dept. of Molecular Genetics and Microbiology
December 8, 2002

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Abstract-

Many intricate and highly conserved mechanisms have evolved to safeguard organisms against errors in gene expression. The nonsense-mediated mRNA decay pathway (NMD) exemplifies one such mechanism, specifically by eliminating mRNAs containing premature translation termination codons within their protein coding regions, thereby limiting the synthesis of potentially deleterious truncated polypeptides. Studies in *Saccharomyces Cerevisiae* have found that the activity of at least three *trans*-acting factors, known as *UPF1*, *UPF2/NMD2*, and *UPF3* is necessary for the proper function of the NMD pathway. Further research conducted in yeast indicates that the degradation of substrates of the NMD pathway is dependent on their translation, and that the sub-cellular site of their degradation in the cytoplasm.

Although most evidence in yeast suggests that substrates of the NMD pathway are degraded in the cytoplasm while in association with the translation apparatus, some mammalian studies have found several mRNAs whose decay appears to occur within the nucleus or before their transport to the cytoplasm has been completed. In addition, study of the mammalian TPI mRNA found that this transcript was unavailable as a substrate for the NMD pathway once it had been successfully exported to the cytoplasm, further supporting the notion that the degradation of mammalian substrates of the NMD pathway occurs in association with the nucleus, or during export from the nucleus to the cytoplasm.

To determine if yeast cytoplasmic nonsense-containing mRNA can become immune to the NMD pathway we examined the decay kinetics of two NMDS substrate mRNAs in response to repressing or activating the NMD pathway. Both the *ade2-1* and *pgk1-UAG-2* nonsense-containing mRNAs were stabilized by repressing this pathway, while activation of NMD resulted in the rapid and immediate degradation of each transcripts. These findings demonstrate that nonsense-containing mRNAs residing in the nucleus are potentially susceptible to NMD at each round of translation.

The remainder of this thesis utilizes protein overexpression studies to gain understanding into the function of factors related to the processes of nonsense-mediated mRNA decay and translation in *Saccharomyces cerevisiae*. Overexpression of a C-terminal truncated form of Nmd3p was found to be dominant-negative for cell viability, translation and the normal course of rRNA biogenesis.

Overexpression studies conducted with mutant forms of the nonsense-mediated mRNA decay protein Upf1p, found that overexpression of mutants in the ATP binding and ATP hydrolysis region of Upf1p were dominant-negative for growth in an otherwise wild-type yeast strain. Furthermore, overexpression of the ATP hydrolysis mutant of Upf1p (DE572AA), resulted in the partial inhibition of NMD and a general perturbation of the translation apparatus. These results support previous studies suggesting a general role for Upf1p function in translation.

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CHAPTER 1

Introduction

The regulation of gene expression has been a fundamental area of research for many years. A great deal of effort has been directed toward understanding the mechansims by which an organsim's genome is expressed to generate functional proteins. The majority of research on gene expression has focused on the contribution of gene transcription to cellular protein levels. Until recent years, a somewhat overlooked area of gene expression has been the process of mRNA metabolism. The abundance of an mRNA, and its decay rates, play a significant role in establishing cellular protein levels, as well as the time required to reach new steady-state protein levels. In fact, several studies have suggested that organsims may modulate mRNA stability in response to enviormental stimuli (Ross, 1995; Gonzalez and Martin, 1996; Jarzembowski and Malter, 1997). The steady-state abundance of a given mRNA transcript is not solely dependent on gene transcription, but is determined by both the process of mRNA synthesis and mRNA degradation. Ultimately, it is the sum of these two processes that determines the abundance of a given mRNA (Hargrove and Schmidt, 1989). Therefore, the elucidation of the mechanism of mRNA turnover is critical to our understanding of gene expression.

Mechanisms of mRNA decay

To date, four pathways of mRNA decay have been identified in eukaryotes, with the characterization of the majority of these pathways occuring using the brewer's yeast, *Saccharomyces cerevisiae.*

Most yeast mRNAs, are degraded by pathways that require the prior deadenylation of a given transcript (Figure 1A). This observation is supported by transcriptional pulse-chase experiments studying the decay of both stable and unstable transcripts in yeast. These studies found that both classes of mRNAs were not degraded until the poly(A) tail was shortened (Decker and Parker, 1993). Additional research has found that this shortening event is mediated by the activity of the Ccr4 and Caf1 proteins (Tucker et al.,2001). This conclusion was supported by the observation that Ccr4 and Caf1 proteins localize to the cytoplasm, which is the expected subcellular location for proteins involved in mRNA turnover. More importantly, both *ccr4Δ* and *caf1Δ* strains exhibit defects in the extent and rate of deadenylation for many mRNAs, indicating that these proteins are major components of the cytoplasmic deadenylation pathway (Tucker et al., 2001).

The deadenylation event is followed by the removal of the 5' cap structure from the mRNA, through the enzymatic activity of Dcp1p. Dcp1p was determined to be the yeast decapping enyme due to the following observations: 1) Dcp1p is able to remove the 5' cap structure from synthetic RNAs (Stevens 1988; Beelman et al., 1996, LaGrandeur

and Parker, 1998), and 2) Deletion of the gene encoding Dcp1p, resulted in the accumulation of capped transcripts in yeast (Hatfield et al., 1996).

The deadenylated and decapped transcript is a substrate for degradation by Xrn1p, the major 5'→3' exoribonuclease in yeast (Decker and Parker, 1993; Muhlrad et al., 1994). The conclusion that degradation occurred primarily in a 5'→3' manner was derived from experiments using transcripts harboring poly(G) tracts. These poly(G) tracts create secondary structures that are resistant to the activity of exonucleases. Experiments using poly(G) containing transcripts found that the size of the decay intermediates detected was consistent with exonucleolytic degradation primarily initiating from the 5' end of the mRNA (Decker and Parker, 1993; Muhlrad et al., 1994). Characterization of a mutation in the *XRN1* gene found that strains harboring an *XRN1* mutation had more full length transcripts than those of wild-type cells, and that these transcripts lacked a detectable cap structure (Hsu and Stevens, 1993; Muhlrad et al., 1994). Additional studies using poly(G) containing *MFA2* mRNAs, showed that a *MFA2* transcript with a poly(G) tract in its 5' UTR was degraded up to the poly(G) insert, and then existed as a stable mRNA. These observations, in conjunction with experiments that showed *MFA2* mRNA existed as a complete and uncapped transcript in *xrn1Δ* cells, demonstrated that mRNA decapping is followed by exoribonucleoltyic digestion by Xrn1p (Muhlrad et al., 1994).

A less frequently utilized pathway for degradation of deadenylated transcripts, involves the degradation of the mRNAs in a 3'→5 fashion. In this pathway the deadenylated transcript is subject to degradation by the nucleolytic activity of a complex

comprised of Ski2p, Ski3p, Ski6p/Rrp41p, Ski8p and Rrp4p proteins that are components or regulators of the exosome (Anderson and Parker 1998). This was demonstrated by experiments showing that an mRNA fragment previously known to degraded in a 3'→5' manner, accumulated in strains harboring mutations in exosome components (Anderson and Parker, 1998). This observation was supported by an experiment that found that the 3'→5' decay of some transcripts, which occurred in the decapping mutant *dcp1-2*, was no longer observed when this mutant was combined with an exosome mutant (Anderson and Parker, 1998)

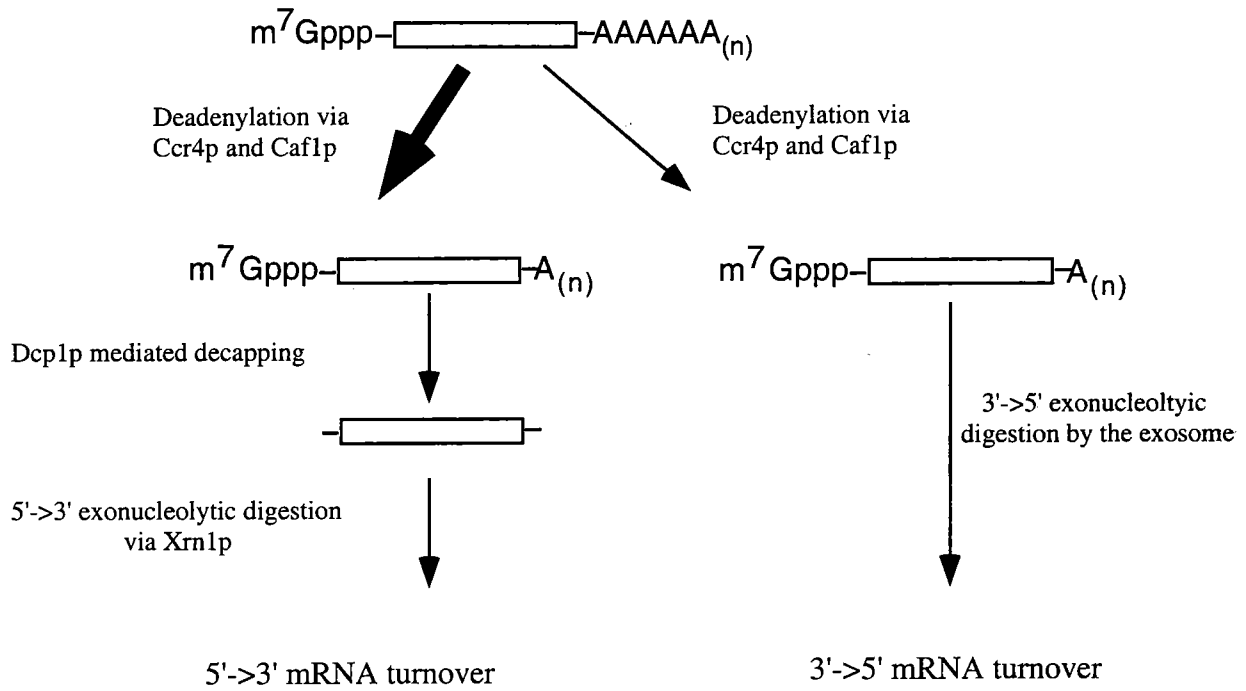
The remaining well-characterized mechanisms of mRNA degradation do not require prior deadenylation of their substrate transcripts. In the process of nonsense-mediated mRNA degradation (NMD), an mRNA that is determined to be aberrant due to the presense of a premature termination codon, is rapidly decapped and degraded without prior poly(A) shortening (Mulhrad and Parker 1994;Figure 1B). Another example of deadenylation-independent decay, found in higher eukaryotes, is intitiated by site-specific endonucleoltyic cleavage of a substrate mRNA, followed by its exoribonucleolytic degradation (Bernstein et al., 1992; Brown et al., 1993; Binder et al., 1994;Figure 1B).

Although the detailed characterization of most of these degradation mechanisms has occurred primarily in yeast, these pathways appear to be evolutionarily conserved. Studies in mammalian cells have found that initial step in the degradation of some mRNAs is deadenylation (Shyu et al., 1991). Mammalian NMD experiments examining the metabolic fate of substrates of the this pathway, have detected the accumulation of 5' shortened, polyadenylated decay intermediates, supporting the yeast observation that

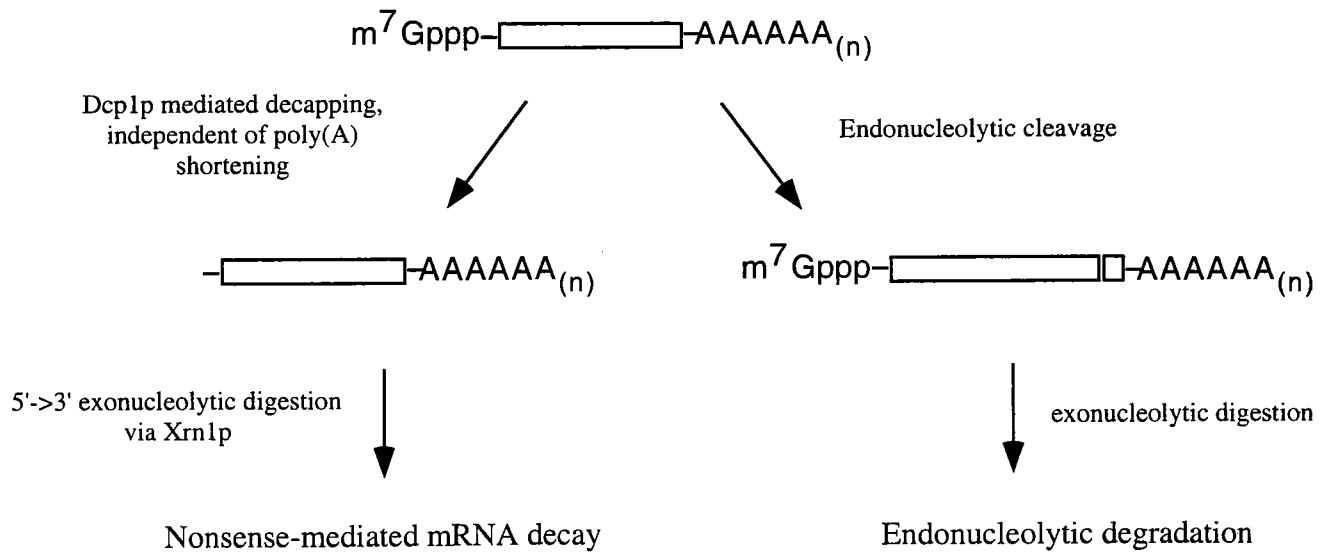
decay initiates at the 5' end of the mRNA without significant poly(A) shortening (Belgrader et al., 1993).

Figure 1. Pathways of mRNA degradation in eukaryotes. (A) Deadenylation dependent mRNA degradation mechanism. Deadenylation occurs via the activity of the Ccr4p and Caf1p, once the poly(A) tail has been shortened to a critical length, degradation proceeds primarily via the 5'->3' mRNA turnover pathway, or alternatively by the 3'->5' decay pathway. **(B)** Two deadenylation independent mechanisms that have been identified in eukaryotes. In the NMD pathway (left), recognition of a premature termination codon stimulates decapping independent of poly(A) shortening via the Dcp1p, in yeast. The decapped message is then degraded in a 5'->3' fashion by Xrn1p, in yeast. In the exonucleolytic degradation (right), such as occurs in TRS1 mRNA, a cleavage site in the 3'UTR of the message, triggers endonucleolytic clevage. The resulting products are then degraded by the activity of exonucleases.

A.


m7Gppp–AAAAAA(n)
Deadenylation via Ccr4p and Caf1p
Deadenylation via Ccr4p and Caf1p
m7Gppp–A(n)
m7Gppp–A(n)
Dcp1p mediated decapping
3'->5' exonucleoltyic digestion by the exosome
5'->3' exonucleolytic digestion via Xrn1p
5'->3' mRNA turnover
3'->5' mRNA turnover

B.


m7Gppp–AAAAAA(n)
Dcp1p mediated decapping, independent of poly(A) shortening
Endonucleolytic cleavage
AAAAAA(n)
m7Gppp–AAAAAA(n)
5'->3' exonucleolytic digestion via Xrn1p
exonucleolytic digestion
Nonsense-mediated mRNA decay
Endonucleolytic degradation

Additional the evidence supporting the conservation of these decay mechanisms, is provided by the identification of mammalian homologues to yeast proteins that are essential for function of mRNA metabolism in *Saccharomyces cerevisiae*, such as Xrn1p, Dcp1p, the exosome, as well as Upf1p,Upf2p/Nmd2p, and Upf3p (Bashkirov et al., 1997; Dunckley and Parker 1999; Anderson and Parker 1998; Allmang et al., 1999; Perlick et al., 1996; Applequist et al., 1997; Serin et al., 2001)

Nonsense-mediated mRNA decay pathway

Many cellular proofreading mechanisms exist to protect organsims from the accumulation of aberrant transcripts and proteins (Yarus 1992; Friest et al., 1996; Gottesman et al., 1997). In some cases these pathways exist not only to safeguard cells from the production of deleterious products, but also to augment the regulation of cellular processes (Welch and Jacobson, 1999; Lew et al., 1998; Morrison et al., 1997;Ross 1995). Nonsense-mediated mRNA decay, or NMD, is an example of such a mechanism. The process of nonsense-mediated mRNA decay has evolved to aid in the removal of mRNAs which lack complete open reading frames, typically transcripts whose defects result from the presence of a premature termination codon within the open reading frame of the transcript (Leeds et al., 1991, 1992; Peltz et al., 1993a,b).

A. NMD in yeast

Although the process of nonsense-mediated mRNA decay has been shown to function in a wide variety of eukaroytes, the mechanism of NMD has been characterized in detail in the yeast *Saccharomyces cerevisiae* (Losson and Lacroute, 1979; Leeds et al., 1991, 1992; Peltz et al., 1994; Caponigro and Parker, 1996; for reviews see Hilleren and Parker 1999; Jacobson and Peltz, 2000)

Substrates of the NMD pathway

A primary function of the NMD pathway is the rapid removal of mRNAs containing premature termination codons. However, subsequent research has revealed that NMD also assumes a physiological role in the cell, specifically, by modulating cellular levels of other classes of transcripts. One class of substrates in yeast found to be regulated by NMD are intron-containing pre-mRNAs that are transported to the cytoplasm prior to their splicing. These unspliced mRNAs are targets of NMD due to in-frame premature termination codons resulting from intron retention (He et al., 1993). Some mRNAs with upstream open reading frames are also a class of transcripts subject to NMD degradation (Cui et al., 1995). This class of mRNAs is atypical, in that they harbor no premature translation termination codon, but rather a single or multiple short uORFs before the true mRNA coding region. This class of mRNAs was identified in a screen for mutations that would suppress a mutation in the *CYC1* mRNA, which resulted in the generation of an out of frame initiator codon upstream of the true initator (Cui et al., 1995). This upstream initation codon effectively reduced the levels of translation

initiation at the true inititation codon. Several mutations known as *sua* (suppressor of upstream aug) mutants, were found to suppress this upstream initatior (Cui et al., 1995). Two of these *sua* mutants, known as *sua1* and *sua6*, were later found to be *UPF2/NMD2* and *UPF3* respectively, which are factors known to be essential for NMD (Cui et al., 1995). A third class of mRNAs, whose stability is regulated by NMD, includes some transcripts that undergo leaky scanning (Welch and Jacobson, 1999). In these transcripts, initiation occurs at an out of frame intiator codon (AUG) that is downstream of the true translation start site. Initation at this out of frame AUG, results in rapid termination of translation, mimicking the effect of a premature termination codon within the true reading frame. This mechanism has been shown to have consequences for the expression of both the leaky scanned transcipt, as well as genes regulated by its protein product (Welch and Jacobson, 1999). An additional class of abberant mRNAs subject to degradation via the NMD pathway, is comprised of transcripts with extended 3'UTRs (Pulak and Anderson 1993; Muhlrad and Parker 1999; Das et al., 2000). In contrast with the majority of the aformentioned NMD substrates, degradation of this mRNA substrate is not dependent upon the presence of a premature termination codon. These transcripts were identified as substrates for NMD since they were stabilized when NMD was inactivated (Pulak and Anderson, 1993; Muhlrad and Parker, 1999; Das et al., 2000). The mechanism by which these transcripts are detected as aberrant has not been determined, but possible modes of their detection will be discussed later.

mRNA degradation via the yeast NMD pathway is independent of prior poly (A) shortening, but requires translation and the activity of several trans-acting factors

As mentioned earlier, a distinguishing feature of NMD is that degradation of nonsense-containing mRNAs does not begin with shortening of the 3'-poly(A) tail. Nonsense-containing transcripts are recognized as aberrant by the translation apparatus, decapped by Dcp1p, independent of their poly (A) status, and subsequently degraded by the 5'→3 exoribonuclease activity of Xrn1p (Figure 1B).

The fact that decapping occurs without prior poly(A) shortening is surprising, but is supported by the following facts: 1) both wild-type and nonsense-containing transcripts are stabilized in strains harboring deletions of Xrn1p, the major cytoplasmic exoribonuclease (Mulhrad et al., 1994); 2) nonsense and wild-type transcripts stabilized in *xrn1Δ* strains lack a 5'-cap structure, indicative of processing by Dcp1p, the yeast decapping enzyme (Hatfield et al., 1996). These findings indicate that NMD is independent of deadenylation, but utilizes downstream degradation mechanisms identical to that of deadenylation depedent decay, once a mRNA has been targeted for removal.

Although the mechanisms by which a nonsense-containing mRNA is degraded are well characterized, the events that target nonsense transcripts for degradation are not completely understood. For example, a premature termination codon is essential for NMD regulated degradation of most substrates characterized to date. However, the mere presence of this premature termination codon is not sufficient to promote degradation.

An obvious, and convincing piece of evidence, that NMD activity requires more

than the presence of a premature termination codon, is the observation that expression of a suppressor tRNA, which enhances the read through of a premature termination codon, is able to stabilize nonsense substrates, indicating that recognition of a premature stop codon is dependent on translation of the mRNA (Losson and Lacroute, 1979; Gozalbo and Hohmann, 1990). A similar experiment confirming this observation involved the use of the translation elongation inhibiting drug, cycloheximide. Addition of cycloheximide to levels that inhibit translation results in the stabilization of nonsense-containing mRNAs (Herrick et al., 1990; Peltz et al., 1992). Further evidence that nonsense transcript recognition is dependent on translation was shown in experiments involving translation initiation. Mutation of the initiation codon of a nonsense-containing transcript results in stabilization of the mRNA (Peltz and Jacobson, 1996). Similar results were obtaining studying the translation initiation mutant, *prt1-1*. The *PRT1* gene, encodes the p90 subunit of translation initiation factor eIF3. A conditional allele of this gene, known as *prt1-1*, has been shown to strongly inhibit translation initiation (Welch and Jacobson, 1999; Naranda et al., 1994). Experiments conducted with this allele at non-permissive temperatures, reveal that nonsense-containing transcripts are stabilized to levels equivalent to those observed in well-characterized mutants of the NMD pathway (Welch and Jacobson, 1999). Another significant contribution to the body of evidence demonstrating that the processes of NMD and translation are linked, is the observation that factors involved in mRNA decay are cytoplasmic, and that these factors preferentially associate with the translation apparatus, as evidenced by their co-localization with polyribosomes and 80S ribosomes on sucrose density gradients (Peltz et

al., 1993b; Atkin et al., 1995,1997; Mangus and Jacobson 1999). This observation is further supported by experiments that have found nonsense-containing transcripts also co-sediment with polyribosomes on sucrose density gradients. The sedimentation of these nonsense-substrates correlates with a polyribosome whose size is consistent with the position of the premature stop codon within the given ORF (He et al., 1991; Zhang at al., 1997). Complementary experiments have shown that the cycloheximide-mediated stabilization of nonsense-containing transcripts, is rapidly reversed upon the removal of the drug (Zhang at al., 1997). While the body of evidence detailed above clearly illustrates the intimate link between mRNA translation and decay, additional support for this argument can be found in studies involving the characterization of the trans-acting factors that regulate NMD. Identification and subsequent characterization of factors involved in the NMD pathway have shown that deletion of any of the genes critical for the function of this pathway, results in increased levels of nonsense codon readthrough (Leeds et al., 1992; Cui et al., 1995; Weng et al., 1996a,b; Maderazo et al., 2000). This result is strengthened by biochemical data that indicates critical components of the NMD pathway (i.e., *UPF1*, *UPF2/NMD2*, and *UPF3*) are able to interact with either one or both of the polypeptide release factors eRF1 and eRF3 (Czaplinski et al., 1998; Wang et al., 2001).

The results presented above show a clear association between the processes of mRNA translation and degradation. This is not a trivial observation, as this evidence suggests that NMD in yeast is indeed a cytoplasmic event. The fact that NMD in yeast appears to be a cytoplasmic event is significant because is contradicts some experiments

in mammalian cells which suggest a nuclear localization of NMD. This discrepancy will be examined in greater detail within the body of this thesis.

Characterization of transacting factors required for nonsense-mediated mRNA decay

Several factors have been identified that mediate the function of the NMD pathway, including several factors intially identified and best characterized in *Saccharomyces cerevisiae*. Factors which mediate NMD in yeast were discovered by genetic studies focusing on allosuppression, omnipotenet suppressors, regulation of frameshifting and translation, suppressors of uORFs as well analysis of two-hybrid interactors with factors discovered by these traditional genetic screens (Culbertson et al., 1980; Hamsey et al., 1991; Dinman and Wickner 1994; Cui et al., 1995; He and Jacobson 1995; Lee and Culbertson, 1995; He et al., 1997;Welch and Jacobson 1999). Subsequent analysis of these genes verified their function in the NMD pathway, as mutations in *UPF1*, *UPF2/NMD2/SUA1*, *UPF3/SUA6*, *PRT1*, *HRP1*, *MOF2*, *MOF5*, *MOF8* and *DBP2* preferentially stabilize NMD substrates without significantly altering the abundance or stablity of most wild-type transcripts (Leeds et al., 1991, 1992; He and Jacobson 1995; Lee and Culbertson 1995; Cui et al., 1995, 1999a,b; He et al., 1997; Gonzalez et al., 2000; Bond et al., 2001).

The main thrust of research has focused on the characterization of the protein products of *UPF1*, *UPF2/NMD2*, and *UPF3*. This is due in large part to the fact that

although these genes are non-essential, they have been shown to be highly evolutionarily conserved proteins that are critical components of the NMD pathway in yeast, as disruption of any of these three genes leads to a dramatic stabilization of nonsense-containing transcripts (Leeds et al., 1991,1992; He and Jacobson 1995; Cui et al., 1995; Lee and Culbertson, 1995; He et al., 1997).

The first of these factors to be identified, *UPF1* (up-frameshift suppressor 1), was originally identified by Culbertson and colleagues as a suppressor of a specific frameshift mutation at the *his4* locus in *S. cerevisiae* (Culberston et al., 1980), a result which ultimately suggested a role for Upf1p in translational fidelity in yeast. Subsequent studies of the *UPF1* gene showed that frameshift, or nonsense mutations, in the *HIS4* or *LEU2* mRNAs, which typically render these transcripts unstable, are stabilized in *upf1Δ* strains (Leeds et al., 1992). Sequence analysis found that the *UPF1* gene encodes a 109 kD protein with cysteine and histidine rich domains near its N-terminus, suggestive of two putative zinc fingers, as well as nucleotide binding sites and seven motifs which are characteristic of members of RNA/DNA helicase superfamily I proteins (Altamura et al., 1992; Koonin, 1992; Leeds et al., 1992). Biochemical analysis using wild-type *UPF1*, and alleles harboring mutations which correspond with highly conserved residues within helicase superfamily I, have confirmed the nucleic acid binding, ATPase and helicase activities suggested by Upf1p's high degree of homology to members of this helicase family (Weng et al., 1996a,b).

NMD2 was originally identified in a two-hybrid screen using *UPF1*, as well as in a separate genetic screen (He and Jacobson, 1995; Cui et al., 1995). Disruption of *NMD2*

resulted in a decay defect similar to that of a *upf1Δ* strain. Sequence analysis found that Nmd2p is an acidic protein with a predicted molecular weight of 127 kD. Nmd2p is not significantly homologous to any known proteins, but does appear to have a putative nuclear localization sequence, three NIC motifs found in translation initiation factors (Aravind et al., 2000), as well as a putative transmembrane region. Deletion of either of these two regions resulted in a defect identical to an *nmd2Δ,* indicating that these regions are critical for the structure or function of wild-type Nmd2p (Maderazo 2000). Further studies have shown that targeted overexpression of a carboxy-terminal fragment of Nmd2p to the cytoplasm, but not the nucleus, results in dominant negative inhibition of NMD (He and Jacoboson, 1995).

Genetic and molecular biological techniques identified a third protein responsible for the regulation of NMD in yeast. *UPF3* is a 45 kD protein that contains three putative nuclear localization signals, as well as two sequence elements which resemble nuclear export signal domains (Lee and Culbertson, 1995; He et al., 1997; Shirley et al., 1998). Work by Shirley and colleagues found that Upf3p is primarily a cytoplasmic protein,that shuttles between the nucleus and cytoplasm (Shirley et al., 1998). Mutations within the putative nuclear localization and nuclear export sequence elements, disrupt Upf3p localization and aborgate NMD function, suggesting that shuttling of this protein is critical for the proper activity of the NMD pathway. However, it is important to note that chimeric experiments, which replaced the Upf3p NES with that from HIV-1 Rev were able to restore Upf3p localization, but not wild-type NMD function (Shirley et al., 1998).

This suggested that the previously mutated residues may function as targeting sequences, or may simply serve a critical role in the proper function of this protein.

The UPF/NMD genes provide the link between translation and NMD

The observation that single or multiple deletions of the *UPF/NMD* genes has similar mRNA decay and suppression phenotypes suggests that these proteins function in a common pathway (He et al., 1997; Maderazo et al., 2000; Wang et al., 2001). This hypothesis is supported by directed two-hybrid analysis, showing that Upf1p interacts with Nmd2p, and Upf2p/Nmd2p is able to interact with Upf3p, indicating that these proteins may function as a complex to regulate NMD (He et al., 1997).

Despite these observations, current genetic and biochemical studies suggest that it is unlikely that the *UPF/NMD* factors function as a complex. The cellular concentrations of the *UPF/NMD* factors were determined by comparing the levels of these factors in cell extracts, to known levels of the purified protiens. These experiments found that Upf1p is ten-fold more abundant than Upf2p/Nmd2p, which is in turn two-fold more abundant than Upf3p (Maderazo et al., 2000). These stoichometric data strongly argue against the NMD factors functioning as a complex to mediate mRNA decay.

Further evidence that the *UPF/NMD* factors do not typically exist as a heterologous complex comes from studies of the role of these factors in nonsense suppression. Recently, a quantitative assay for translation suppression was devloped, using the naturally occurring nonsense substrate *can1-100* (Maderazo et al., 2000).

Readthrough of this nonsense allele of the *CAN1* gene results in the production of a functional arginine permease. The level of nonsense suppression, or readthrough, was experimentally determined by monitoring the sensitivity of yeast strains to the toxic arginine analog, canavanine. The sensitivity of yeast strains to the drug canavanine correlates to the level of arginine permease production and therefore, the relative level of nonsense suppression (Maderazo et al., 2000). This assay allowed Maderazo and colleagues to study the role of *UPF/NMD* mutants in translational fidelity. Studies with these mutant strains revealed that deletion of *UPF1* resulted in higher levels of suppression (canavanine sensitivity) than deletion of *UPF2/NMD2* or *UPF3*, implying that Upf1p is the most critical of the decay factors in regulating translational fidelity, with Upf2p/Nmd2p and Upf3p acting as factors which modulate the activity of Upf1p (Maderazo et al., 2000).

This model is strongly supported by immunoprecipitation studies conducted with the *UPF/NMD* factors. Work by Czaplinski and colleagues, using immunoprecipiation has shown that Upf1p interacts with the release factors eRF1 and eRF3, suggesting that Upf1p exerts its effects on translational fidelity through these termination factors (Czaplinski et al., 1998,1999). Additional immunoprecipitation studies involving Upf2p/Nmd2p and Upf3p have found that eRF3 co-immunoprecipitates with Upf2pp/Nmd2p and Upf3p in a specific manner. Interestingly, Upf2p/Nmd2p and Upf3p were unable to successfully co-immunoprecipitate eRF1p (Wang et al., 2001). GST-pull down experiments were performed to confirm co-immunoprecipation of eRF3 by Upf2/Nmd2p and Upf3p and showed that the observed results were due to a specific

interaction between eRF3 and these proteins. These studies found that Upf2p/Nmd2p interacted with eRF3 at levels comparable to those of Upf1p, but that Upf2/Nmd2p and Upf3p were unable to interact with eRF1p in conditions that detected an interaction between Upf1p and eRF1. Wang et al. hypothesized that Upf1p forms a complex with both eRF1 and eRF3 to mediate peptide release. The authors suggest that Upf2p/Nmd2p or Upf3p may bind to a Upf1p-eRF3 complex, once eRF1 has dissociated from the Upf1-eRF1-eRF3 complex, and that this binding may induce the dissociation of eRF3 from Upf1p. This dissociation event would allow the formation of an Upf1p-Upf2p/Nmd2p-Upf3p complex that would then mediate NMD through the ATPase/helicase activity of Upf1p.

Collectively, the present data in the field suggest the following: 1) Upf1p, Upf2p/Nmd2p, Upf3p play a critical role in mediating NMD and nonsense suppression in yeast, 2) Upf1p is the primary effector in these pathways and its function is mediated by the activity of the Upf2/Nmd2 and Upf3 proteins, and 3) The *UPF/NMD* factors function in nonsense suppression by modulating the recognition of the premature termination codon.

NMD is modulated by the presence of cis-acting sequences

Downstream elements

As mentioned earlier, the mere presence of a premature termination codon is insufficient to trigger the NMD pathway. Since recognition of the premature termination

codon is dependent on translation of the NMD substrate, the question arises, how does the ribosome distinguish between a premature and normal termination codon? An obvious difference between the two termination codons is their position within the reading frame of an mRNA. A number of studies have been conducted to address this particular question. In these studies premature nonsense codons were introduced at regular intervals within the ORF of several genes. These studies found that a nonsense codon inserted within the first three-quarters of the ORF were able to activate NMD, while mutations introduced beyond this region appeared to be recognized as normal termination codons, since they had no significant effect on mRNA stability (Losson and Lacroute, 1979; Peltz et al., 1993; Hagan et al., 1995; Yun and Sherman, 1995; Zhang et al., 1995; Hennigan and Jacobson, 1996). These experiments suggest that the spatial positioning of a termination codon is a key discriminating factor in determining its recognition as either a normal or premature termination codon.

Additional studies characterizing *PGK1* nonsense alleles, have found that NMD requires a downstream sequence in addition to the presence of a premature termination codon. Characterization of various *PGK1* nonsense alleles found that deletion of a large portion of the region 3' of the early nonsense codon resulted in the inactivation of NMD (Peltz et al., 1993a,b). Portions of this deleted sequence were then reinserted downstream of the termination codon to determine if there was a specific element which aided in mediating NMD. These experiments identified a 106 segment of *PGK1* (referred to as a DSE or downstream sequence element), which was able to activate NMD when positioned within roughly 150 nucleotides downstream of a premature termination codon

(Peltz et al., 1993a,b; Ruiz-Echevarria et al., 1996,1998). It is hypothesized that these elements are masked in wild-type mRNAs, presumably because the DSE is inactivated by the traversal of translating ribosomes through its coding region. Similar experiments have been conducted to locate DSE-like elements in other transcripts. These studies were able to identify similar *cis*-elements in other mRNAs. A very loosely conserved DSE consensus sequence was found (5'-YGCUGAUGYYYYY-3') by comparing these new *cis*-elements to the *PGK1* DSE (Peltz et al., 1993a,b; Hagan et al., 1995; Zhang et al., 1997). Database analysis using this consensus sequence found the presence of DSE-like elements in the coding regions of upwards of 75% of yeast mRNAs. This raises the possiblity that these elements are incorporated into the mRNA as a way to aid in determining the quality of the transcript.

Studies using the *GCN4* mRNA have demonstrated that the DSE can also function in the regulation of another class of NMD substrates, specifically mRNAs containing short uORFS. The 5' UTR of the *GCN4* mRNA contains four uORFs, which mediate its expression (Hinnebusch, 1997,1994). Although this transcript contains several uORFs it is normally stable, and not a substrate of the NMD pathway. However, if the *PGK1* DSE is inserted 3' of the initiation codon of the *GCN4* uORF1, the mRNA is destabilized, indicating this sequence is able to target a message for degradation (Ruiz-Eschevarria and Peltz 1996).

Stabilizer elements

Research on *cis*-acting elements which mediate NMD have also identified regions within mRNAs which can protect a given transcript from degradation when positioned downstream of a termination codon. As mentioned earlier, only premature termination codons residing with in the 5' proximal two-thirds to three-quarters of an mRNA are able to trigger NMD, while mutations beyond this spatial barrier have little effect on mRNA stability (Losson and Lacroute, 1979; Peltz et al.,1993a,b; Hagan et al., 1995). The hypothesis that stablization beyond the first three-quarters of the mRNA was due to the lack of a functional DSE was investigated by Peltz and colleagues using the well-characterized *PGK1* mRNA. To test this hypothesis the *PGK1* DSE was inserted 3' proximal to late nonsense mutations in *PGK1* mRNAs, which had previously been shown to be stable transcripts. Insertion of the DSE downstream of "late" nonsense codons in stable *PGK1* transcripts did not significantly alter the stability of these mRNAs (Peltz et al., 1993a). This suggested that lack of a functional DSE was not the root cause of the inherent stability of these *PGK1* "late" nonsense-containing transcripts (Peltz et al., 1993a,b). Peltz and co-workers suggested that sequences, termed stabilizer elements, might exist within given mRNAs and that the transition of a translating ribosome though these sequences inactiviated its ability to respond to DSEs. Although these stability regions appear in many mRNAs examined, the detailed examination of several mRNAs suggested to harbor such stabilizing sequences has been unsuccessful in detecting any significant sequence or structural homology between these regions.

Evidence for stabilizing elements has also been found studying the translational regulation of the *GCN4* transcript. The 5' UTR of the *GCN4* mRNA appears to harbor a sequence element that is capable of inactiving NMD. A 68 nucledotide region 3' of uORF4 called a STE (Stabilizer element) appears to confer immunity to NMD to the *GCN4* transcript, since deletion of this region from the mRNA triggers its degradation by the NMD pathway (Ruiz-Eschevarria et al., 1998). The STE appears to be a *bona-fide* stabilizing element, since it can function to stabilize other known nonsense-containing mRNAs, provided it is inserted downstream of the premature termination codon, but upstream of a functional DSE (Ruiz-Echevarria et al., 1998). Further study of stabilizer elements has detected a similar element within the uORF of the *YAP1* mRNA, which is also capable of rendering a mRNA immune to NMD (Ruiz-Echevarria and Peltz, 2000). These elements appear to promote the binding of Pub1p to the uORFs of their given transcripts. Pub1p binding to the stabilizer region appears to confer immunity to NMD, since these STEs fail to inhbit NMD in *pub1Δ* strains (Ruiz-Echevarria and Peltz, 2000). These results were further confirmed by stability experiments conducted with the *CPA1* transcript. Experiments assaying this transcript found the abundance of *CPA1* mRNA was unaltered in both wild-type and *pub1Δ* strains (Ruiz-Echevarria and Peltz, 2000). Since this mRNA also contains a uORF, but does not appear to habor a STE, one possible interpretation of these results is that Pub1p is only capable of mediating mRNA stability in conjunction with a cis-acting STE.

B. Mammalian NMD

Substrates of NMD

As previously mentioned, NMD is a highly conserved mechanism that allows cells the ability to target and remove potentially deleterious transcripts derived from errors in gene expression. A number of NMD substrates have been clearly detailed in the preceding text dealing with NMD in *Saccharomyces cerevisiae*. At this point I would like to catalog the major substrates of NMD that have been identified in mammalian systems.

Like yeast, a fair number of nonsense substrates arise from mutation events, such as insertions, deletions, and translocations within the genome. A second class of NMD substrates in mammals, result from inaccurate or incomplete pre-mRNA splicing. Improper splicing can result in the production of transcripts that retain introns. Typically, the retention of such an intron results in the introduction of a premature termination codon within the ORF of a given transcript, thus making it a substrate of the NMD pathway (Maquat 1995,1996; Lozano et al., 1994). Additional cellular processes, specific to higher eukaryotes, which generate substrates for the NMD pathway, are somatic rearrangements and hypermutations that result from cellular events necessary for the production of fuctional immunoglobins and T-cell receptors (Li and Wilkinson 1998). These rearrangements are essential to generate the diversity needed to recognize a large range of protein targets. Unfortunately the majority of these rearrangements result in the production of non-functional transcripts, primarily through the generation of frameshift

and premature nonsense containing transcripts. Another interesting class of mammalian transcripts that are regulated by the NMD pathway are selenoprotein mRNAs. In these substrates, a premature UGA codon may be recognized as an NMD substrate, or alternatively as a signal to incorporate a rare selenocysteine amino acid at this codon position. The presence of a premature termination codon results in these selenocysteine mRNAs being recognized by the NMD pathway at an appreciable rate, suggesting their expression may be regulated by NMD (Moriarty et al., 1998; Sun et al., 2000).

NMD requires translation and the activity of *trans*-acting factors

Despite some controversy as to the cellular compartmentalization of NMD in mammals (see below), a fair amount of evidence suggests that translation is necessary for the recognition of many mammalian NMD substrates. In fact, mammalian NMD appears indistinguishable from yeast in its sensitivity to perturbance of normal translation. Structures or mutations that interfere with translation intiation appear to affect nonsense recognition, suggesting that translation is a critical element of substrate recognition in mammalian systems (Belgrader et al., 1993; Thermann et al., 1998). Furthermore, treatment of mammalian cells lines with translation elongation or fidelity altering drugs such as anisomycin, cycloheximide, and puromycin appear to effectively inhibit NMD function (Carter et al., 1995).

Research in yeast has determined that NMD requires the activity of at least three *trans*-acting factors known as *UPF1*, *UPF2/NMD2*, and *UPF3*. Since mammalian NMD appeared to harbor many similarities to the yeast process, experiments were conducted to

determine if *UPF/NMD* factor homologues existed, and if these factors played a pivotal role in NMD in mammals. Human homologues of these *UPF/NMD* factors, termed hUpf1p, hUpf2p, hUpf3p, and hUpf3-X were cloned based on their sequence similarity to their *S. cerevisae* and *C. elegans* homologues (Perlick et al., 1996; Applequist et al., 1997; Lykke-Andersen et al., 2000; Serin et al., 2001).

Further genetic and biochemical characterization of hUpf1p has demonstrated that it is a functional homologue of the yeast protein. Biochemical studies have shown that like *S. cerevisiae* Upf1p, human Upf1p demonstrates RNA-dependent ATPase and 5' → 3' helicase activities, as well as an RNA-binding activity that is modulated by ATP (Bhattacharya et al., 2000). Separate studies have found that expression of a chimeric allele of hUpf1p, which contained the central portion of hUpf1p, flanked by the amino and carboxy terminal regions of *S. cerevisiae* Upf1p, was able to complement a yeast *upf1Δ* (Perlick et al., 1997). Additional studies by Sun and collegues have shown that a mutant form of hUpf1p, harboring an arginine-cysteine mutation corresponding to a well characterized yeast mutation known to be dominant negative for NMD was able to inhibit NMD in COS cells in a dominant-negative manner (Sun et al., 1998). These genetic and biochemical studies, in conjunction with the observation that Upf1p is known to interact with *in vitro* synthesized forms of release factors RF1 and RF3, suggests that hUpf1p functions in NMD in a similar manner to its yeast counterpart (Czaplinki et al., 1998).

Human Upf2p, Upf3p, Upf3p-X were more recently identified, and therefore are less completely characterized. However, several interesting results have come out of their limited characterization of these proteins. Indirect immunofluorescence has found

that hUpf1p, hUpf2, hUpf3p are localized primarily to the cytoplasm, while hUpf3p-X appears to shuttle to the cytoplasm, but is primarily a nuclear localized protein (Serin et al., 2001). Subsequent co-immunoprecipitation studies with the human UPF factors, confirmed previous two-hybid interactions detected with their yeast counterparts, i.e., hUpf1p interacted with hUpf2p as well as hUpf3p, and that hUpf2p is able to interact with hUpf1p and hUpf3p (Serin et al., 2001). Furthermore, amino acid residues in hUpf2p found to be necessary for interaction with hUpf1p, were similar to those that were predicted by yeast two-hybrid mapping with their *S. cerevisiae* counterparts, further reinforcing the highly conserved nature of this cellular pathway (Serin et al., 2001).

Additional evidence suggesting that the hUPF factors play a critical role in regulating mammalian NMD, comes from a series of complementary experiments from several research groups. Work by Lykke-Andersen and colleagues has found that tethering of any of the hUPFs to the 3' UTR of beta-globin mRNA elicits NMD and that multiple isoforms of hUpf3p, appear to selectively associate with spliced beta-globin mRNA *in vivo*, suggesting a link between the nucleus and NMD (Lykke-Andersen et al., 2000). Additional experiments suggesting a role for nuclear splicing in NMD come from a series of immunoprecipitation and tethering experiments. Collectively, recent research has shown that multiple forms of hUpf3 and possibly hUpf2, interact with a series of proteins (SRm160, DEK, RNPS1, Y14, and REF), all of which appear to be deposited at exon-exon junctions after completion of splicing (Lykke-Andersen et al., 2001; Kim et al., 2001; Le Hir et al., 2001). Suprisingly, the tethering of RNPS1 to the 3'UTR of an mRNA appears to trigger its degradation via the NMD pathway, suggesting that RNPS1

provides a portion of the framework to which factors such as hUpf3p and hUpf2p may bind to mediate NMD. However, an alternative interpretation of these results, is that tethering of the protein to the 3' UTR, creates an artificial substrate for the NMD pathway, and is thus not reflective of the true process of NMD.

cis-acting sequences and mammalian NMD

As is the case in yeast, an initial question of interest in the study of NMD in higher eukaryotes was determining what factor or factors allowed the cell to discriminate an aberrant translation termination codon from a normal termination codon. Nonsense-mediated mRNA decay in mammals also appears to be dependent upon *cis*-acting sequences that mediate NMD in a spatially dependent manner. Unlike yeast, there does not appear to be a conserved DSE sequence which is unmasked by a premature termination codon, aiding the ribosome in recognizing this aberrant stop codon. Rather, nonsense recognition appears to involve events surrounding the proper processing of introns from pre-mRNA transcripts. This was a somewhat surprising observation, as yeast and mammalian NMD seem to be regulated by identical factors and to target similar cellular substrates. Upon deeper reflection, the relationship between the two systems is still very strong. One major difference between yeast and mammalian cells is the prevalence of introns, as these intervening sequences are quite commonplace in mammals and comprise a component of most mammalian pre-mRNAs. In contrast, there are a very limited number of yeast mRNAs that harbor even a single intron. One of the first observations suggesting that NMD in mammals might be regulated by intron processing

was that a nonsense containing transcript derived from an intronless mRNA was stable (Cheng et al., 1994). Recent studies have found that the naturally occurring intronless mammalian transcripts of the heat shock p70 and histone H4 genes are also immune to NMD (Maquat and Li 2001). Further research has found that mammalian NMD requires the presence of a premature termination codon as well as the presence of at least one intron downstream of the aberrant stop to initiate NMD. A series of detailed mapping experiments have found that the premature termination codon typically must be positioned greater than 50-55 nucleotides upstream of the 3' most exon-exon junction (Cheng et al., 1994; Nagy and Maquat, 1998; Thermann et al., 1998; Zhang et al., 1998a,b; Sun et al., 2000a,b). The spatial rule defined by these experiments is strongly supported by analysis of a pool of wildtype genes that have one or two 3' untranslated exons downstream of their normal termination codon. This study found that the terminator in 98% of these genes, was less than 50-55 nucleotides upstream of the 3' most exon junction. Since the mRNAs analyzed were wild-type transcripts which do not respond to NMD, these results strongly support the observation that the distance between a premature termination codon and the exon-exon junction is critical for activation of NMD (Nagy and Maquat., 1998).

Controversy over the cellular site of NMD in mammalian systems

As previously mentioned, a large body of evidence in yeast suggests that NMD occurs in association with the cytoplasm and that this pathway is dependent on mRNA

translation for the identification of NMD substrates. Complementary work in mammalian systems has found a similar dependence on the proper function of translation for nonsense substrate recognition. For example, the addition of drugs that alter translational elongation such as anisomycin, cycloheximide, emetine, puromycin and pactamycin, have been shown to inhibit NMD (Qian et al., 1993; Menon and Neufeld 1994; Carter et al., 1995). The introduction of structures that block translation initiation also have been demonstrated to interfere with NMD function (Belgrader et al., 1993; Thermann et al., 1998). In addition, introduction of a suppressor tRNA to a mammalian cell line has been shown to inactivate the NMD of specific nonsense-containing substrates (Belgrader et al., 1993; Li et al., 1997).

While these observations strongly suggest that translation is necessary for NMD, consequently implying that NMD occurs in the cytoplasm, several observations suggest that NMD may take place in the nucleus, or before mRNA transport from the nucleus to the cytoplasm has been completed. Analysis of mRNA fractions from the nucleus and cytoplasm obtained by subcellular fractionation has found that the degradation of some mRNAs appears to occur in both the nuclear and cytoplasmic fractions (Cheng and Maquat, 1993; Lozano et al., 1994; Kessler and Chasin, 1996). Furthermore, additional studies have found mRNAs that appear to degrade specifically in association with the nucleus, as the cytoplasmic abundance and stability of these transcripts appears unaltered by NMD activity (Cheng and Maquat, 1993; Lozano et al., 1994; Carter et al., 1996). The best-characterized example of these transcripts is the mammalian TPI mRNA. Several studies have shown that premature termination codons within the coding region

of the TPI mRNA are capable of reducing the abundance and half-life of this mRNA in several cell lines. Further research has shown that TPI mRNA is only found at a reduced abundance and half-life in subcellular fractions that correspond to the nucleus, but not in fractions that represent the cytoplasm, suggesting that decay of this transcript occurs in association with the nucleus (Cheng and Maquat 1993; Belgrader et al., 1994a,b). One explanation of the immunity of the TPI mRNA to degradation in the cytoplasm is that the mRNA fraction observed in the cytoplasm may be unable to be translated. To test this hypothesis, wild-type and nonsense alleles of the TPI mRNA were transfected into mammalian cell lines and tested for the ability to associate with polyribosomes. These experiments found that TPI mRNA was indeed able to associate with polyribosomes at a size that is consistent with that of its open reading frame (Stephenson and Maquat 1996). These experiments demonstrated that TPI mRNA is able to associate with the translation apparatus, and that its failure to undergo NMD is not a consequence of an inability to initiate translation. However, these experiments come with the following *caveat*. By necessity the constructs used in this study were selected post-transfection with the antibiotic hygromycin B. This aminoglycocide has been shown to interfere with translation elongation and fidelity (Velazquez book). Presumably, the activity of this potent translational inhibitor has been deactivated by the activity of the resistance gene carried by the plasmids used in this study. However, one cannot rule out the possibility that the resistance gene allows translation to occur at acceptable levels for cell growth, but may not inactivate the drug to a level sufficient to allow for normal NMD function. It is conceivable that most alterations in codon recognition could dramatically alter the

ability of the translation apparatus to recognize a premature termination codon, and subsequently to trigger NMD.

Figure 2. Models of NMD function. **(A)**The surveillance complex model for NMD function in yeast. **(B)** The faux UTR model for NMD function in yeast.

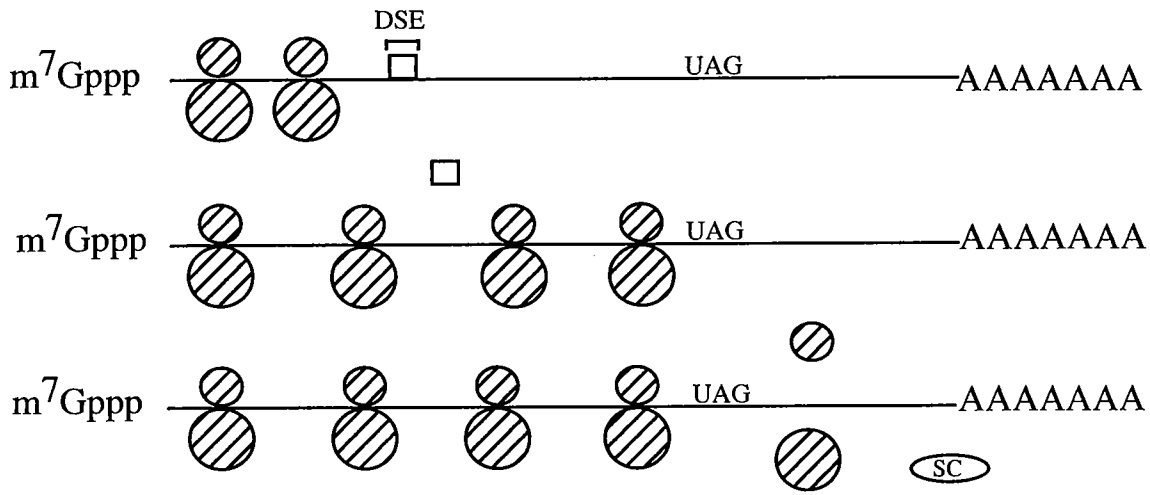
A.

Normal Termination

mRNA enters cytoplasm with Hrp1p bound to DSE

Hrp1p is displaced by translating ribosome and shuttles back to the nucleus

Efficient termination; surveillance complex does not detect DSE/Hrp1p complex

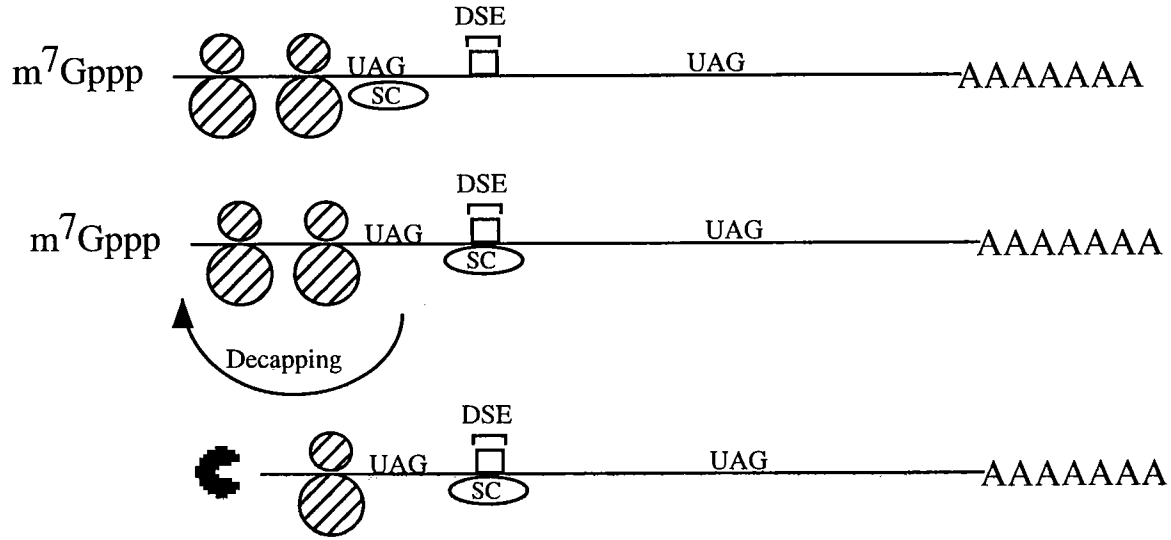


Premature Termination

Surveillance complex recognizes premature termination initiates 3' scanning

Surveillance complex interacts with DSE/Hrp1p complex and stimulates decapping via Dcp1p

Transcript is degraded 5'->3' via Xrn1p

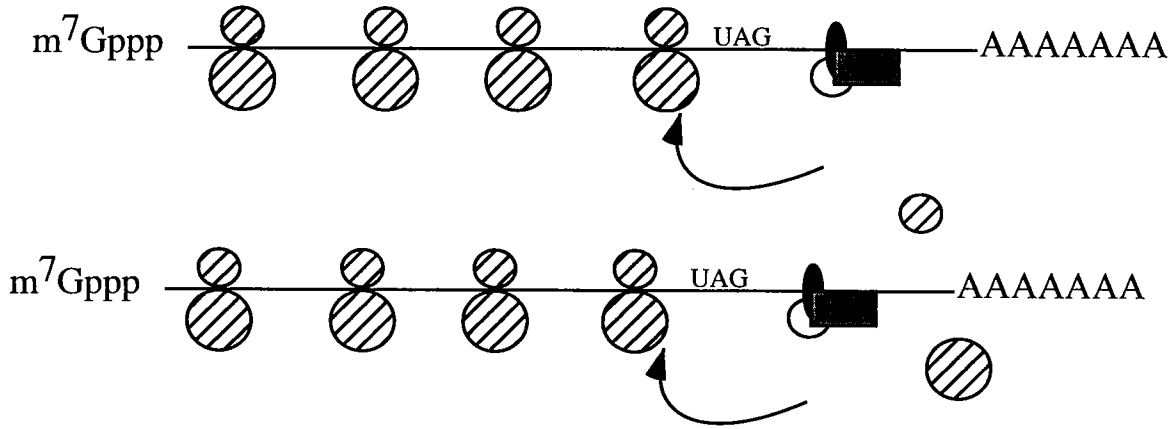


B.

Normal Termination

Full complement of complement of regulatory factors assemble at the 3' UTR

Termination is efficient; decapping is not triggered by Upf1p

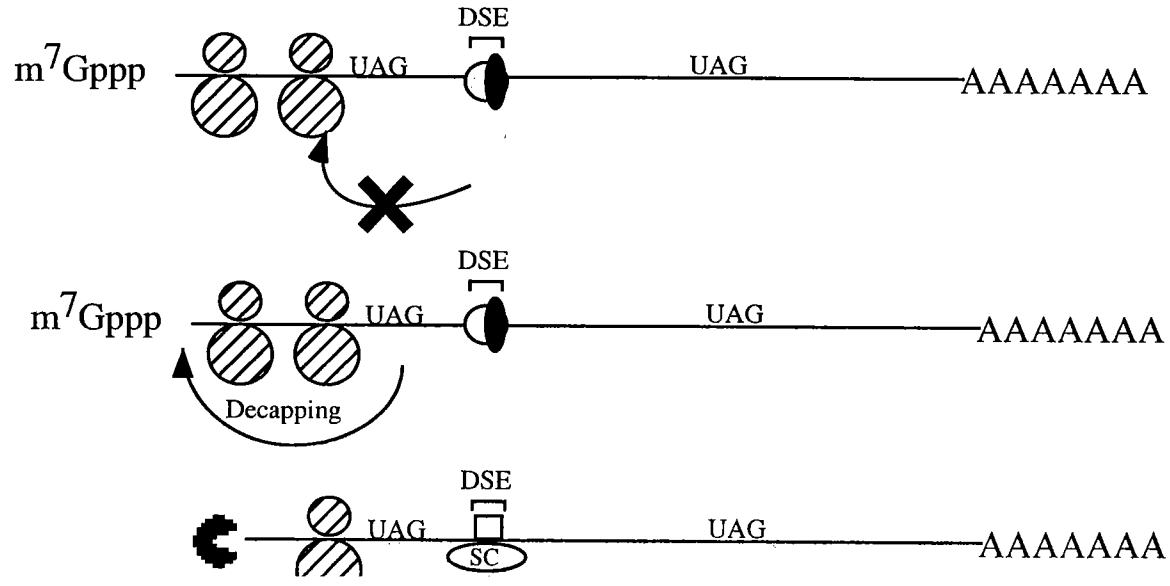


Premature Termination

DSE mimics 3'UTR but fails to recruit all factors necessary for proper termination

Termination is inefficient; Upf1p stimulates decapping via Dcp1p

Transcript is degraded 5'->3' via Xrn1p



Models of NMD function

Two popular models of NMD function have evolved from the extensive studies of NMD in yeast (Figure 2). In one model NMD occurs as an active response to recognition of a *cis*-acting sequence and it complementary factors that reside downstream of a premature termination codon (Czaplinski et al., 1998,1999; Jacobson and Peltz 2000; Gonzalez et al., 2001). The second model suggests that NMD is the result of not terminating in close proximity to a properly formed 3' UTR (Hilleren and Parker 1999; Jacobson and Peltz 2000). In the first model, an NMD substrate is recognized as aberrant due to its inability to form a normal RNP structure. In this model a surveillance complex, that includes the *UPF/NMD* factors, forms as the result of a translation termination event (Figure 2). This complex is believed to recognize the presence of the DSE, and its associated binding factors, and that this recognition stimulates NMD. Upon translocation to the cytoplasm, the mRNA begins to be translated as the ribosome begins translation at the intiator codon and transitions into translation elongation. Eventually, the elongating ribosome reaches a termination codon. Once the ribosome is paused at a termination codon, it is bound by the termination factors eRF1 and eRF3, as well as by the termination regulator Upf1p. Upon completion of the termination event, Upf1p is hypothesized to interact with Upf2p/Nmd2p and Upf3p, forming the "surveillance complex." This complex is proposed to advance downstream of the termination codon looking for mRNP abnormalaties by virtue of Upf1p's ATPase/ helicase activities. Recognition of an abnormal mRNP structure by this complex is proposed to trigger rapid

decapping of the target transcript, and thereby stimulate its subsequent 5'→3' degradation (Czaplinski et al., 1999; Gonzalez et al., 2000; Jacobson and Peltz 2000; Gonzalez et al., 2001).

Although this model is attractive, several lines of experimental evidence suggest that it might not be a plausible mechanism of action for the NMD pathway. One potential flaw of this model is how the "surveillance complex" would be able to recognize a well-characterized class of NMD substrates, specifically transcripts with unusually long 3' UTRs. Studies of these transcripts have not been successful in identifying sequences that resemble the loosely conserved DSE consensus sequence. Presumably, lack of the DSE would preclude binding by the DSE associated factor Hrp1p, thereby preventing these mRNAs from being NMD substrates. Although it is conceivable that DSE-like sequences may reside in the abnormally long 3'UTRs of these mRNAs allowing decay to occur, additional lines of experimental evidence suggest that NMD does not occur via the model detailed above. Characterization of the *UPF/NMD* factors has found that these proteins are not present at similar levels (Atkins et al., 1997; Maderazo et al., 2000), contradicting previous reports suggesting that these factors form a complex to mediate NMD (He et al., 1997). In fact, recent studies directed at understanding the role of the *UPF1/NMD* factors in regulating nonsense supression have found that the *UPF1*is a central regulator of translational fidelity, whose activity is modulated by the activities of Upf2p/Nmd2p, and Upf3p (Maderazo et al., 2000).

The second model also considers the influence of RNP structure in the function of the NMD pathway. In this model, sequences downstream of certain premature

termination codons are hypothesized to function as a defective or "faux" UTR (Figure 2). This model implies that proper termination is a result of specific interaction between the translation apparatus and components of the 3' UTR of a transcript (Bonetti et al., 1995; Hilleren and Parker 1999; Jacobson and Peltz 2000). Proper completion of the termination cycle is hypothesized to stabilize the mRNA by stimulating translation by allowing proper interaction between the 3'UTR and the 5' cap structure and their associated factors to occur at a high efficiency. Improper termination at a "faux" UTR could result in inefficient ribosome release and improper release could negatively affect these interactions, thereby triggering decapping. In this model, Upf1p would function in concert with eRF1 and eRF3 to mediate all termination events, possibly using its helicase and RNA binding activities to promote ribosome release. By extension, this model would suggest that Upf1p has a general role in translation termination in the cell that is not limited to the process of NMD. Such a role is supported by recent experiments by He and Jacobson (2001) demonstrating that Upf1p regulates the decapping and exonucleoltyic degradation of wild-type and NMD substrates, suggesting that Upf1p functions in the metabolism of all mRNAs.

Despite the fact that both models provide solid hypotheses detailing the role of the *UPF/NMD* factors in NMD, I prefer the latter model. One strength of the "faux" UTR model is its ability to account for the degradation of all classes of NMD substrates known to date. Additionally, this model is able to plausibly explain how the *UPF/NMD* factors function not only in NMD, but also in nonsense suppression and translation termination.

Future research possiblities

Before one can completely rule out the surveillance complex model outlined above, several questions must be addressed. Further experiments must be performed to better characterize the potential role of Hrp1p in NMD. In their research on Hrp1p, Gonzalez and colleagues failed to convincingly prove the increased mRNA abundance that they observed in various temperature sensitive mutants of *HRP1* was a direct consequence of impaired NMD function (Gonzalez et al., 2000). The researchers also failed to demonstrate that the mini-*PGK1* and *GCN4-PGK1* nonsense substrates tested were exported to the cytoplasm and therefore available for degradation by the NMD pathway. As a consequence, one is not able to conclusively confirm that the increased mRNA abundance seen post temperature shift is due to mRNA stability effects. This problem could be remedied by *in situ* hybridzation experiments demonstrating that the nonsense-containing substrates of interest are available for decay in the cytoplasm after shift to the non-permissive temperature in these mutants.

Although I favor the "faux" UTR model outlined earlier, I also feel that additional experiments are necessary to validate this model of NMD. Specifically, experiments should be conducted to determine what *cis* and *trans*-acting factors discriminate a "faux" UTR from a normal UTR. If indeed there are differences between the host of factors associated with a "faux" and normal UTR, it must be determined if it is the presence or absence of specific factors at the "faux" UTR which mediates NMD. If a "faux" UTR is indeed able to trigger NMD future experiments need to be directed at determining how

termination regulates decapping. One hypothesis for how this regulation occurs is that Upf1p regulates ribosome release. In prokaryotes ribosome release factors (RRFs) are necessary for ribosome release, but to date there have been no characterized eukaryotic release factors (Bertram et al., 2001and references therein). After peptide release is mediated by the activity of eRF1 and eRF3, Upf1p may function to regulate the efficiency of ribosome release. Inefficient ribosome release at a nonsense-containing mRNA may result in decreased levels of translation initiation. Decreased levels of translation may then allow the cap structure to be exposed and be susceptible to the activity of the decapping enzyme.

Another intriguing possibility for how decapping may be regulated by the *UPF/NMD* factors comes from protein structure analysis of translation initiation factors. Recent research has identified an alpha-helical structure called a NIC domain, which is conserved in eIF4G, *NMD2/UPF2*, and *CBP80* (Aravind et al., 2001). It is conceivable that Upf1p is able to trigger nonsense-mediated decay of a substrate mRNA by localizing Nmd2p to the cap structure. Once Nmd2p is localized to the cap structure it may be able to effectively compete for cap binding with eIF4G via its NIC domain. Since Nmd2p does not harbor other protein domains necessary to initiate translation, binding of Nmd2p to the cap structure would reduce translation initiation, thereby disrupting the synergistic interaction between the 5' cap and 3' end structures. The disruption of this interaction may allow the cap to be removed by the activity of the decapping enzyme.

CHAPTER 2

MATERIALS AND METHODS

A. Strains, plasmids, and general methods

Yeast strains and plasmids used in this study are listed in Tables 1 and 2, respectively. Preparation of standard yeast media and methods of cell culture were as described previously (Sherman et al., 1986). Transformation of yeast was done by the high-efficiency method of Schiestl and Gietz (1989). DNA manipulations were performed according to standard techniques (Sambrook et al., 1989). All PCR amplifications were performed with Taq DNA polymerase (White et al., 1989) and confirmed, where appropriate, with DNA sequencing. DNA sequences were determined by the method of Sanger et al. (1977). Overlapping fragments of the *NMD3* gene were subcloned in Bluescript and sequenced by annealing of oligonucleotide primers specific to the T3 or T7 promoter regions of the plasmid or by use of oligonucleotide primers that annealed within the subcloned inserts. Plasmid DNAs were propagated in *E. coli* strain DH5 . All designations of ribosomal protein names followed the recently revised nomenclature of Mager et al. (1997). Computer searches for protein:protein homologies utilized the NCBI BLAST program (Altschul et al., 1997). Sequence alignment was generated using the pileup feature of the Wisconsin Package Version 9.1 of the GCG sequence analysis program.

B. Construction of galactose-inducible NMD plasmids

Galactose-inducible *NMD3* constructs were made using standard molecular biology techniques, and are described in detail in Table 2. All galactose-inducible *UPF1* constructs were made by ligating a 3.6kb *EcoR*I-*Sal*I fragment from pMA424 vectors containing wild-type *UPF1*, or mutated alleles of *UPF1* depicted in Figure 3, to pRS426 (Christianson et al., 1992) containing the *GAL1* promoter (664 bp fragment immediately upstream of the initiation codon, generated by PCR) cut with the same enzymes. The galactose-inducible *NMD2* plasmid was constructed by ligating a 3.7kb *Xba*I-*Sal*I fragment cut from the pRS315-NMD2 plasmid (He et al., 1997) to pMW29 (Zieler et al., 1995) cut with the same enzymes. The galactose-inducible *UPF3* plasmid was constructed by ligating a 1.7kb *Nco*I-*Sal*I fragment cut from the pRS316-HA-*UPF3* plasmid (He et al., 1997) to pRS314 (Sikorski and Hieter, 1989) containing the *GAL1* promoter, cut with the same enzymes. The latter plasmid was obtained by restriction digest of the pRS314-GALp-HA-*NMD3* plasmid (Belk et al., 1999).

C. RNA extraction and northern blot analysis

RNA used for analysis of cytoplasmic mRNAs was isolated by the hot phenol method as described previously (Herrick et al., 1990). Aliquots (20 μg) of each RNA sample were analyzed by northern blotting, using radiolabeled probes prepared by random priming as described above. For isolation of RNA from polysome fractions, the method as described by Benard *et al.* (1998) was used. Total RNA used for analysis of

nuclear pre-rRNAs was isolated by the glass bead/phenol method (Ulery et al., 1991) and then analyzed by northern blotting, using the oligonucleotide hybridization conditions of Peltz et al. (1993a). mRNA decay rates, expressed as half-lives ($t_{1/2}$), were determined by counting the blots with a BioRad Molecular Imager, normalization of the data such that time zero after a 10 h shift to galactose equaled 100%, and plotting the data with respect to time on semi-log axes. Probes for the *CYH2*, *RP51a*, *TCM1*, and *STE2* transcripts have been described previously (He et al., 1993; Herrick et al., 1990). Additional probes used in these studies included, *CAN1* mRNA was detected with a probe made from a 1.0 kbp EcoRI-Sal1 fragment of YEplac195-*CAN1* (Maderazo et al., 2000, *ADE2* (a 2 kb *Bgl*II fragment from an *xrn1*::*ADE2* disruption plasmid generously provided by Feng He), *PGK1* (oligonucleotide 1 from Peltz et al., 1993), and *SCR1* (a 400-bp fragment amplified from yeast genomic DNA using oligonucleotides SCR1-1 [5'-AGGCTGTAATGGCTTTCTG GTGGGATGGGA-3'] and SCR1-2 [5'-GATATGTGCTATCCCGGCCGCCTCCATCA C-3']).

D. Protein gels, western blots, and antibodies

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described by Sambrook et al (1989). Gels were electroblotted to Immobilon-P membranes (Millipore) under conditions recommended by the manufacturer. The binding conditions used for antibodies were as described by Harlow and Lane (1988). Detection was enhanced by chemiluminescence with either the ECL or ECL(+) kits from Amersham Corp. Antibodies used included:, polyclonal affinity purified anti-Upf1p and Nmd2p antibodies (Belk et al., 1999; Maderazo et al., 2000) and the monoclonal

antihemagglutinin (HA) antibody, 12CA5, (from Boehringer Mannheim Biochemicals) for detection of Upf1p, Nmd2p, and HA-epitope tagged Upf3p, respectively.

E. Galactose induction

Yeast strains containing galactose inducible *UPF/NMD* gene constructs were grown in SC –uracil +raffinose media to mid-log phase (OD_{600}=0.5). Strains containing the inducible *UPF/NMD* gene constructs and either of the *pgk1* nonsense alleles were grown in SC –uracil -leucine +raffinose media (to maintain selection for the *GAL-UPF/NMD* plasmid and the plasmid harboring the *pgk1* allele, respectively) to mid log phase (OD_{600}=0.5). Galactose was then added to a final concentration of 2%. Culture aliquots for RNA and protein isolations were taken at 10 min. intervals for 40 min.

F. Polyribosome analysis

Cytoplasmic extracts were prepared as described previously (Mangus and Jacobson, 1999). The extracts were fractionated at 4°C on 15-50% or 7-47% sucrose gradients buffered with 50 mM Tris-acetate, pH 7.4, 50 mM NH_4Cl, 12 mM $MgCl_2$, and 1 mM dithiothreitol (DTT). A Beckman SW41 rotor, centrifuged at 45,000 r.p.m. for 150 min, was used for the 15-50% gradients and an SW27 rotor, centrifuged at 27,000 r.p.m. for 300 min, was used for the 7-47% gradients. All gradients were harvested from the bottom and the distribution of RNA was analyzed by continuous monitoring of A_{254}. Polyribosome fractions were analyzed as described previously (Mangus and Jacobson,

1999). Relative stoichiometry of ribosomal subunits was determined by comparisons of the areas under the respective peaks.

G. Measurement of amino acid incorporation

Cells were grown in 200 ml of SC -ura,-met,+raffinose medium at 30°C to an OD_{600} of 0.1, harvested by centrifugation, resuspended in 20 ml of fresh medium, and shaken for 10 min at 30°C. Galactose (4 ml of 20% solution) was then added to a final concentration of 2% galactose per culture. Triplicate 1 ml aliquots were removed at the indicated times and incubated with a mixture of 5 μCi of ^{35}S-translabel (ICN; 70% methionine and ~15% cysteine) and 5 μl of 50 mM unlabeled methionine for 10 min at 30 C. Incorporation of the radiolabeled amino acids was monitored by trichloroacetic acid (TCA) precipitation. TCA (5 ml of a 5% solution) was added to each aliquot, followed by heating at 90°C for 20 min and subsequent incubation on ice. The precipitates were collected on GF/C filters, which were washed with 2x25 ml of 5% TCA and 25 ml of ethanol, dried under a heat lamp, and counted by scintillation spectrometry. Each experiment was repeated at least three times.

H. Toeprinting Protocol

Buffer A: 30 mM HEPES (pH 7.6 with KOH), 100 mM KOAc (pH 7.0), 2mM MgOAc (pH 7.0 with KOH). Stored at 4°C

PMSF stock (0.1M, 100x): 5g PMSF in 287 ml 100% 2-propanol, stored at room temperature. Add to buffers immediately prior to use.

Common buffer (40X): 400 mM HEPES pH 7.6, 40 mM DTT (added fresh), stored at room temperature

Variable buffer (10X): 34 mM MgOAc, 2.3 M KOAc, stored at room temperature

Translation reaction components: 10 mM ATP, 2.5 mM GTP, 250mM Creatine Phosphate, stored at –20°C or below

Reaction buffer (5x): 250 mM Tris-HCl (pH 8.3 at room temperature), 375 mM KCl, 50 mM $MgCl_2$

Cycloheximide: 10 mg/ml in water, stored at –20°C.

Loading buffer: 0.05% Bromophenol blue, 0.05% Xylene cyanol FF, 20 mM EDTA (pH 8.0), 91% Formamide

Annealing Solution: 1.25 µl water, 2.0 µl 5X reaction buffer, 1.0 µl 0.1 M DTT, 1.0 µl 2.5 mM dNTPs, 0.25 µl RNasin (40U/µl)

Growth of S. cerevisiae cells

Yeast cells, e.g., strain YAS 1874 (*MAT***a** *MAK10::URA3 PEP4::HIS3 prb1 prc1 ade2 leu2 trp1 his3 ura3*) are streaked onto a YPD plate and incubated at 30°C for 36-48h. One colony is selected and re-streaked on a YPD plate and incubated at 30°C for 36-48h. A single colony from the second plate is used to inoculate a 100 ml YPD culture. The starter culture is grown for 17 hours at 30°C (200 rpm, gyrating shaker). A 1.2l YPD culture is grown using 3X 400 ml YPD in 2l Erlenmeyer flasks at an initial OD_{600}=0.03-0.06 and grown at 30°C (200 rpm, gyratory shaker) until the cultures reach OD_{600}=1.5 (approximately 8 hours).

Cells are collected by centrifugation in 6 GSA bottles. For the first wash, cell pellets are resuspended in 15 ml buffer A + 8.5% mannitol and added to a single preweighed GSA bottle. The suspension is centrifuged for 5 min. at 3,000 rpm, and the resulting supernatant discarded. The cell pellet is resuspended in 10 ml buffer A+8.5% mannitol and centrifuged for 5 min at 3,000 rpm, and the supernatant discarded. The remaining cell pellet is then washed with 10 ml buffer A+8.5% mannitol and centrifuged

for 5 min at 3,000 rpm. Following centrifugation, the supernatant is discarded and the cell pellet resuspended in 10 ml buffer A+8.5% mannitol, and centrifuged for 5 min at 4,000 rpm. The supernatant resulting from this spin is discarded, and the wet weight of the cell pellet determined. The cells are resuspended in 1.5 ml of buffer A+8.5% mannitol + 0.5mM PMSF per gram of wet cell weight.

Resuspended cells (5-6 g) are combined with 6X wet cell weight of cold glass beads in a 50 ml Corning screw-cap polypropylene centrifuge tube. The cells are lysed in a cold room by manual shaking for five 1-min periods with 1 min cooling on ice between shaking-periods. Shaking is performed at a rate of 2 cycles per sec over a 50 cm hand path. The resulting cell lysate is centrifuged at 4°C for 2 min at 2,000 rpm (the GSA rotor used above is satisfactory for this relatively low speed spin). The supernatant is then transferred to a 50 ml centrifuge tube using a Pasteur pipette (Nalgene tube; compatible with SS-34 rotor), and centrifuged for 6 min at 18,000 rpm. The supernatant is removed and transferred to a 15-ml Corning tube, taking care to avoid the lipids at the top and cell debris at the bottom of the tube.

Liquid N_2 grinding protocol for S. cerevisiae lysis (based on the protocol of Otero et al (1998)

A 1.5-l YPD culture is grown at 30°C overnight to a final OD_{600}=3.0-3.5. Cultures are harvested in 0.5-l bottles and centrifuged for 15 min. at 5,000 rpm. The resulting supernatant is discarded and the cell pellets resuspended using 100-ml of buffer A. After all pellets are resuspended, the volume of the suspension is brought up to 175-ml. The

resulting suspension is then centrifuged for 5 min. at 5,000 rpm and the supernatant discarded. The resulting cell pellet is resuspended in 25-ml of buffer A, brought to a total volume of 50-ml, transferred to pre-weighed centrifuge tubes, and centrifuged at 5,000 rpm for 5 min. The supernatant is discarded and the wet weight of the resulting cell pellet determined. The cell pellet is resuspended in 1/10 the volume of the wet weight of the pellet using buffer A, and 50 μl of PMSF is added to the suspension. The resulting cell suspension is dripped into liquid N_2 to generate frozen cell pellets. The frozen cell pellets are transferred to plastic tubes for storage at -80°C until the time of cell breakage.

To lyse cells, a ceramic mortar and pestle are first pre-chilled at -80°C. A small amount of liquid N_2 is then added to the mortar. The frozen yeast pellets are added to the liquid N_2, and the remaining volume of the mortar is filled with liquid N_2. The pellets are crushed using slight pressure and a circular motion. Once most of the liquid N_2 has evaporated, the mortar is refilled with liquid N_2 and the grinding process repeated using a greater amount of pressure, crushing the pellets into a fine powder. The resulting powder is transferred into an ultracentrifuge tube and allowed to thaw on ice, typically for 2-3 hours. The thawed broken cells are centrifuged at 10,000 rpm for 10 min., and the resulting supernatant transferred to pre-chilled Nalgene 16x75 mm ultracentrifuge tubes and centrifuged at 18,000 rpm for 15 min. in a Beckman Ti50 ultracentrifuge rotor. The supernatant is transferred to a fresh 16x75 mm tube and spun for an additional 15 min. at 18,000 rpm. The resulting supernatant is removed with a Pasteur pipette, taking care to avoid both the lipid layer at the top and any pellet that had formed at the bottom of the centrifuge tube.

Column Chromatography (in a cold room or a cold box)

Sephadex G-25 superfine (Sigma; 50ml of suspension) is poured into a 2.5cm X 20cm column and equilibrated with 50 ml buffer A+0.5 mM PMSF. After equilibration, the sample (4-5 ml) is loaded onto the column that is then washed with buffer A+0.5 mM PMSF. Column fractions (0.5 ml) are collected in microfuge tubes. Peak fractions (which appear slightly opaque) typically elute approximately 25 min. after loading the sample. The A_{260} of each fraction is determined after diluting 10 µl of sample into 990 µl of water. All fractions with a diluted A_{260} of 0.9 or higher are pooled, aliquoted into microcentrifuge tubes, and quick frozen in liquid N_2 for storage at –80°C.

Columns can be reused after washing with buffer A.

Nuclease treatment

Immediately prior to assembling the translation reaction mixtures, combine 200 µl extract, 2.0 µl 100 mM $CaCl_2$, and 0.4 µl Micrococcal nuclease (25U/µl). Incubate at 21–25˚C for 10 min. Add 3.0 µl of 170 mM EGTA to stop nuclease reaction, and place on ice. Alternatively, nuclease-treatment can be accomplished immediately after pooling the peak fractions obtained by chromatography and prior to aliquoting and freezing the extracts by appropriately scaling-up the nuclease-treatment procedure.

Translation reaction mix

For a 20X *S. cerevisiae* reaction mix, combine: 33.6 µl Translation reaction components, 2.4 µl Creatine phosphokinase (7.5U/µl), 10.0 µl Common Buffer (40x), 40.0 µl Variable Buffer (10x), 4.0 µl 1 mM Amino Acids, 2.0 µl RNasin (40u/µl), (5 µl 40 mM Arg.--only if studying the *CPA1* transcript; otherwise substitute 20.0 µl of water), 68.0 µl water. If all reactions are to contain the same mRNA, then 40.0 µl mRNA (60 ng/µl) is added at this point. Combine 10 µl of this mix with 10 µl of nuclease-treated cell extract, and incubate at 25 °C for 10, 20, or 30 min. We routinely proceed immediately to the toeprint procedure. Alternatively, the translation reactions can be terminated by transferring tubes to liquid N_2. We recommend that, if freezing the reactions is desired, the suitability for the specific application be assessed by comparing fresh- and frozen translation reactions. *Note:* Premature termination toeprint reactions are pre-incubated for 0,10, 20, or 30 min. prior to addition of cycloheximide (at least 100µg/ml).

Toeprint Reaction Protocol

1. Prepare Annealing Solution and aliquot 5.5 µl to 0.6 ml Eppendorf tubes for toeprint reactions. Leave on ice.
2. Prepare translation reactions as outlined above.
3. Add 3 µl of Translation Reaction to the 5.5 µl annealing solution on ice.

4. Incubate the Translation Reaction-Annealing Solution mixture at 55°C for 2 min. For reasons that remain to be understood completely, this step is critical for the visualization of ribosomes by toeprinting.

5. Add 1.0 µl of 0.1 µM labeled primer and incubate at 37°C for 5 min.

6. Add 0.5 µl reverse transcriptase (50 units) and incubate at 37°C for 30 min.

7. Stop the reaction by adding an equal volume of phenol:chloroform. Flick tube gently to mix. Centrifuge to separate phases. Add extracted aqueous phase to an equal volume of loading buffer.

8. Heat samples at 80–85°C for 5 min. and load on a 6% urea-polyacrylamide gel (Pre-run gel at 110 W for 45 min.).

9. Electrophorese samples at 65 W until the bromophenol blue dye runs off the gel. If using shark's-tooth combs to load samples, load toeprint reactions into every other lane, putting loading buffer in the blank lanes. It may be desirable to adjust acrylamide concentrations and/or running-times to optimize the resolution of products in different size-ranges.

Table 1. Strains

Strain	Genotype	Source
HFY91	*MAT **a**/ MAT α NMD3/nmd3 ade2-1/ade2-1 his3-11,15/his3-11,16 leu2-3,11/leu2-3,112 ura3-52/ura3-52 trp1-1/trp1-1*	
HFY103 (W303)	*MAT **a**/ MAT α ade2-1/ade2-1 his3-11,15/his3-11,16 leu2-3,11/leu2-3,112 ura3-52/ura3-52 trp1-1/trp1-1*	He and Jacobson, 1995
JBY010	*MAT **a** ade2-1 his3-11,16 leu2-3,112 ira3-52 trp1-1* [pRS316 *GAL-NMD3FL*]	This study
JBY012	*MAT **a** ade2-1 his3-11,16 leu2-3,112 ura3-52/ura3-52 trp1-1* [pRS316 *GAL-nmd3Δ100*]	This study
JBY014	*MAT **a** ade2-1 his3-11,16 leu2-3,112 ura3-52/ura3-52 trp1-1* [pRS316 *GAL-nmd3Δ200*]	This study
YRP582	*MAT **a** rpb1-1 ura3-52 leu2-2,112*	Decker and Parker, 1993
YRP582-FL	*MAT **a** rpb1-1 ura3-52 leu2-2,112* [pRS316 *GAL-NMD3FL*]	This study
YRP582-Δ100	*MAT **a** rpb1-1 ura3-52 leu2-2,112* pRS316 *GAL-nmd3Δ100*]	This study
HF1200	*MAT **a** ade2-1 his3-11,16 leu2-3,112 ura3-52 trp1-1*	He and Jacobson, 1995, 1997
HFY870	*MAT **a** ade2-1 his3-11,16 leu2-3,112 ura3-52 trp1-1 upf1::HIS 3*	
HFY1300	*MAT **a** ade2-1 his3-11,16 leu2-3,112 ura3-52 trp1-1 nmd2::HIS3*	
HFY861	*MAT **a** ade2-1 his3-11,16 leu2-3,112 ura3-52 trp1-1 upf3::HIS3*	
HFY1067	*MAT **a** ade2-1 his3-11,16 leu2-3,112 ura3-52 trp1-1 dcp1::HIS3*	
HFY1081	*MAT **a** ade2-1 his3-11,16 leu2-3,112 ura3-52 trp1-1 xrn1::ADE2*	

Table 2. Plasmids

Plasmid	Relevent yeast sequences	Source
pRS316 3XHA-*NMD3*	0.5-kb NotI-NcoI PCR fragment containing the *NMD3* promoter region, NcoI-EcoRI fragment containing a triple hemaglutinin epitope tag, ~2.0-kb EcoRI-SalI fragment containing the *NMD3* coding region, 3' UTR and a small portion of the pBSKS+ polylinker	This Study
pRS316 *GAL1-NMD3FL*	Contains a 0.6-kb XbaI-HindIII *GAL1* promoter fragment and an ~3.0-kb HindIII-HindIII fragment of *NMD3* containing the complete coding region (5' HindIII site introduced by PCR at the –7 position)	This study
pRS316 *GAL1-nmd3Δ100*	pRS316 *GAL-NMD3FL* containing a 0.4-kb BamHI-MluI fragment containing a stop codon at the 3' end, replaces the ~0.80-kb *NMD3* 3' coding sequence (unique Mlu I site engineered immediately upstream of the true termination codon)	This study
pRS316 *GAL1-nmd3Δ200*	pRS316 *GAL1-NMD3FL* containing a unique MluI site immediately upstream of the true termination codon. Replaces the ~0.8-kb 3' coding sequence, resulting in the removal of the C-terminal 600bp of the *NMD3* coding sequence	This study

CHAPTER 3

NONSENSE-CONTAINING mRNAs THAT ACCUMULATE IN THE ABSENCE OF A FUNCTIONAL NMD PATHWAY ARE RAPIDLY DESTABILIZED UPON ITS RESTITUTION

Introduction

Intricate mechanisms that safeguard against errors in gene expression exist in all eukaryotes (Chin and Pyle, 1995; Freist et al., 1996; Gottesman et al., 1997; He et al., 1993; Jeon et al., 1996; Yarus 1992). The phenomenon of nonsense-mediated mRNA decay (NMD) exemplifies one such mechanism, eliminating mRNAs containing premature nonsense codons within their protein coding regions and thus minimizing the synthesis of truncated polypeptides (He et al., 1993; Jacobson and Peltz, 1996; Maquat, 1995; Peltz et al., 1993a,b; Pulak and Anderson, 1993). The process of NMD has been studied extensively in *Saccharomyces cerevisiae*, where rapid degradation of nonsense-containing mRNAs involves recognition of a premature translation termination codon, deadenylation-independent decapping, and subsequent 5'—>3' exonucleolytic digestion of the remainder of the mRNA (Beelman et al., 1996, Hagan et al., 1995; Hsu and Stevens, 1993; LaGrandeur and Parker, 1998; Muhlrad et al., 1994). In addition to the decapping enzyme Dcp1p, and the exonuclease Xrn1p, three additional *trans*-acting factors are essential for NMD in yeast: Upf1p, Nmd2p/Upf2p, and Upf3p (Cui et al., 1995; He and Jacobson, 1995; He et al., 1997; Lee and Culbertson, 1995; Leeds et al., 1991,1992). Consistent with their roles in the response to aberrant translation, all three of the latter *UPF/NMD* proteins have been shown to localize predominantly to the

cytoplasm and to associate with polyribosomes (Atkin et al., 1995,1997; Mangus and Jacobson, 1999; Peltz et al., 1993; Shirley et al., 1998). These observations indicated that yeast NMD occurred in the cytoplasm and was linked to translation, conclusions consistent with other results showing that: a) drugs or mutations that inhibit translation also abrogate NMD (Losson and Lacroute, 1979; Welch and Jacobson, 1999; Zhang et al., 1997); b) nonsense-containing polysomal mRNAs stabilized in cycloheximide-treated cells reinitiate NMD as soon as the drug is withdrawn (Zhang et al., 1997); and c) a dominant-negative form of Nmd2p/Upf2p inhibits decay only when localized to the cytoplasm (He and Jacobson, 1995).

In mammalian cells it is still controversial as to whether NMD is limited to the cytoplasm. Nonsense-containing derivatives of mammalian β-globin, HEXA mRNA and adenine phosphoribosyltransferase (APRT) mRNAs, as well as glutathione peroxidase 1 (GPx1) mRNA, have been shown to decay in the cytoplasm (Maquat et al 1981; Rajavel and Neufeld, 2001; Kessler and Chasin, 1996; Moriarty et al., 1997, 1998). APRT mRNA, however, can also be degraded in the nucleus, as are globin mRNAs expressed in non-erythroid cells (Kessler and Chasin 1996; Kugler et al., 1995; Zhang et al., 1998a,b).

In contrast to these examples of cytoplasmic NMD, an increasing amount of evidence in mammalian cells supports a nonsense decay mechanism that does not occur in the cytoplasm, and which does not appear to affect all mRNAs that contain a premature nonsense codon. For example, reductions in the abundance of nonsense-containing human triosephosphate isomerase (TPI) mRNA are found in both the nuclear and cytoplasmic fractions, suggesting that the decay process occurs in association with

the nucleus (Cheng et al., 1990, 1993; Daar and Maquat, 1988). Additionally, those nonsense-containing TPI mRNAs that are exported into the cytoplasm appear to escape degradation since they are found to be as stable as wild-type TPI mRNA (Cheng et al., 1993). These findings suggest that recognition of premature nonsense codons in at least some mammalian mRNAs occurs solely in the nucleus, or during export from the nucleus, and that those mRNAs that escape to the cytoplasm become immune to degradation by the NMD pathway.

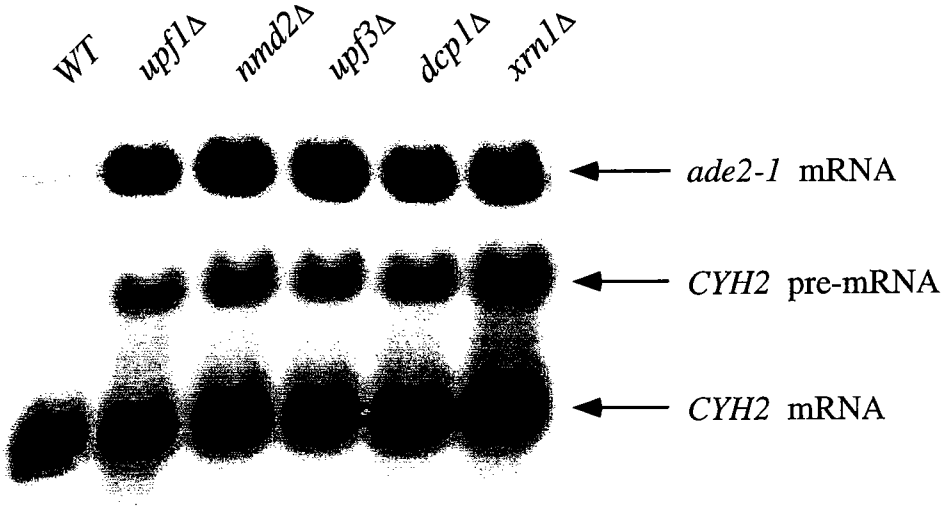
To determine if yeast cytoplasmic nonsense-containing mRNAs can become immune to rapid turnover, we examined the decay kinetics of two NMD substrate mRNAs in response to repressing or activating the NMD pathway. Both the *ade2-1* and the *pgk1*-UAG-2 mRNA nonsense-containing mRNAs were stabilized by repressing the pathway, and activation of NMD caused rapid and immediate degradation of each transcript. These findings demonstrate that nonsense-containing mRNAs residing in the cytoplasm of yeast cells are potentially susceptible to NMD at each round of translation.

Results

The *ade2-1* Transcript is a Substrate For Nonsense-mediated mRNA Decay.

To address the stability of cytoplasmic nonsense-containing mRNAs, we first took advantage of an allele of the *ADE2* gene, *ade2-1*. Earlier studies showed that the *ade2-1* mutation could be suppressed in yeast strains containing an ochre tRNA suppressor (Stotz and Linder, 1990; Sasnauskas et al., 1987), suggesting that the *ade2-1* allele was attributable to a nonsense (UAA) mutation and that the *ade2-1* mRNA was likely to be a substrate for NMD. To test the latter possibility, single deletions of *UPF1*, *NMD2*, or *UPF3* were constructed in yeast strains that harbored the *ade2-1* allele and the effects of these mutations on the abundance of the *ade2-1* transcript were examined. Northern analyses of mRNA steady-state levels demonstrated that mutations in genes regulating stability of nonsense-containing transcripts affected the *ade2-1* transcript in precisely the same manner that they affected a well characterized NMD substrate, the *CYH2* pre-mRNA (He et al., 1993; Figure 3). The *ade2-1* mRNA was approximately seven-fold more abundant in *upf/nmd* mutant cells as compared to the isogenic *UPF/NMD* (*WT*) strain (Figure 3). Likewise, deletion of genes encoding general factors involved in mRNA decay (i.e., *DCP1* and *XRN1*) also promoted a seven-fold increase in *ade2-1* transcript abundance (Figure 3). These differences in mRNA abundance were consistent with the respective differences in the decay rates of the *ade2-1* mRNA in

Figure 3. The *ade2-1* transcript is a substrate for NMD. Total RNA isolated from yeast strains with the indicated *UPF/NMD* geneotypes was analyzed by northern blotting with DNA probes that detected the *ade2-1* and *CYH2* transcripts. WT, wildtype. yeast strains used in this experiment were: HFY 1200, HFY870, HFY1300, HFY861, HFY1067 and HFY1081

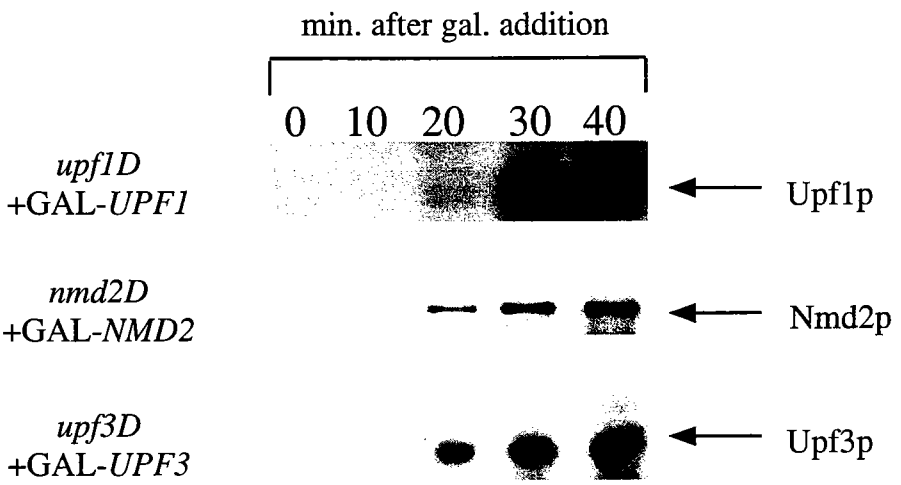

WT
upf1Δ
nmd2Δ
upf3Δ
dcp1Δ
xrn1Δ
ade2-1 mRNA
CYH2 pre-mRNA
CYH2 mRNA

UPF/NMD and *upf/nmd* mutant cells. The half-life of the *ade2-1* mRNA was found to be less than 5 min. in the *UPF/NMD* strain and approximately 35 min. in *upf/nmd* cells, suggesting that the wild-type gene, *ADE2*, encodes a relatively stable mRNA (data not shown). These results indicate that the *ade2-1* mRNA requires Upf1p, Nmd2p, Upf3p, Dcp1p, and Xrn1p for its degradation and is, therefore, a typical substrate for NMD.

Galactose-Inducible Expression of *UPF1*, *NMD2*, and *UPF3*.

To assess the stability of *ade2-1* transcripts that had avoided degradation by the NMD pathway, we sought a mechanism to regulate the activity of the pathway. To accomplish this, the *UPF1*, *NMD2*, and *UPF3* genes were cloned into either single- or high-copy plasmids containing the inducible *GAL1* promoter and the resulting plasmids were transformed into the respective *UPF/NMD* deletion strains. Each of the resulting strains contained a galactose-regulated *UPF/NMD* gene. As shown in Figure 4, Upf1p, Nmd2p, and Upf3p are not detectable in the respective regulated strains prior to galactose induction, but these proteins accumulate substantially post-induction. Quantitation of each of the western blots shown in Figure 4, and others, indicated that: a) Upf1p, Nmd2p, and Upf3p all begin to accumulate approximately 12-14 min. after galactose addition and b) by 20 min. after galactose addition, the cellular levels of each of the induced proteins are comparable to those present in the isogenic *UPF/NMD* strains (data not shown). From these data, we conclude that use of these constructs allows for inducible expression of *UPF1*, *NMD2*, and *UPF3*.

Figure 4. Galactose inducible expression of *UPF1*, *NMD2*, and *UPF3*. The *upf1Δ*, *nmd2Δ*, and *upf3Δ* strains harboring the appropriate galactose-inducible *NMD* gene constructs were grown in SC –uracil, raffinose liquid media to mid log phase (OD_{600}=0.5). Galactose was then added to a final concentration of 2% and aliquots were taken at 10 minute intervals for protein isolation. Isolated protein samples were then analyzed by western blotting. Yeast strains used in this experiment were: HFY870, HFY1300, and HFY861


min. after gal. addition
0
10
20
30
40
upf1D
+GAL-UPF1
Upf1p
nmd2D
+GAL-NMD2
Nmd2p
upf3D
+GAL-UPF3
Upf3p

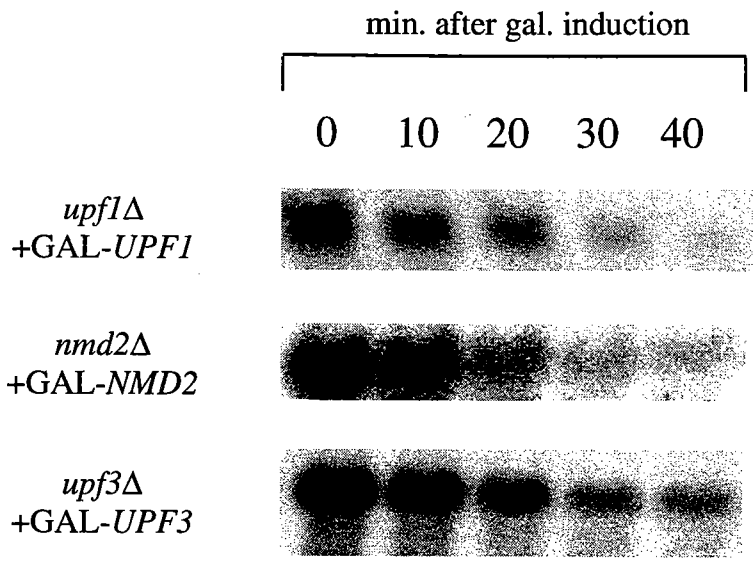
The *ade2-1* Transcript is Rapidly Degraded Upon Activation of NMD.

The availability of the strains described above makes it possible to determine the stability of *ade2-1* transcripts before and after activation of the NMD pathway. Under conditions where NMD is inactive, these nonsense-containing mRNAs accumulate in the cytoplasm and are relatively stable ($t_{1/2}$=35 min.; data not shown). Upon activation of the NMD pathway, the fate of these mRNAs can be monitored by simply measuring their relative abundance over time, leading to a determination of the decay kinetics of the steady-state *ade2-1* mRNA population. If the accumulated *ade2-1* transcripts are susceptible to NMD, then activation of this decay pathway should result in their rapid degradation. If the *ade2-1* transcripts are immune to NMD, then activation of the decay pathway should have no effect on the stability of these mRNAs. The overall *ade2-1* mRNA population would then consist of newly synthesized mRNAs that are rapidly degraded and the stable cytoplasmic transcripts that had accumulated prior to activation of NMD. Under these circumstances, the expected decay rate of the steady-state mRNA population would initially be slow (approximating that of the stabilized *ade2-1* transcripts), and then would approach a half-life approximating the average of the two populations ($t_{1/2}$=20 min.). Only after substantial dilution with newly synthesized mRNA would the population begin to reflect a more rapid decay rate.

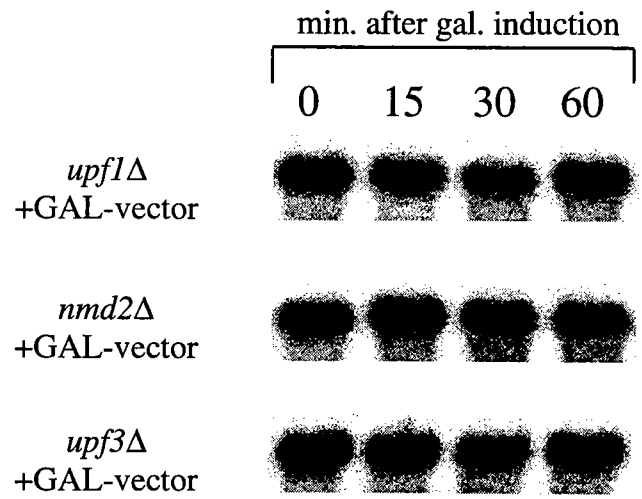
These possibilities were evaluated by northern blot analyses of yeast strains expressing regulatable *UPF1*, *NMD2*, or *UPF3*. These experiments demonstrate that, as expression of *UPF1*, *NMD2*, or *UPF3* increases (Figure 4), the abundance of the *ade2-1* mRNA decreases (Figure 5A). Subsequent to the time at which the *UPF/NMD* proteins

Figure 5. The *ade2-1* transcript is rapidly degraded upon activation of NMD. (A) Activation of NMD causes rapid degradation of the *ade2-1* mRNA. Total RNA isolated from yeast strains with the indicated *UPF/NMD* genotypes, harboring the appropriate galactose-inducible NMD gene construct (GAL-*UPF1*, GAL-*NMD2*, GAL-*UPF3*) was analyzed by northern blotting with DNA probes that detected the ade2-1 transcript. **(B)** The addition of galactose does not destabilize ade2-1 mRNA. Total RNA isolated from yeast strains with the indicated *UPF/NMD* genotypes harboring only the vector plasimd was analyzed by nortern blotting with DNA probes that detected the *ade2-1* transcript. Yeast strains used in this experiment were: HFY871, HFY1300, and HFY861

A.


min. after gal. induction
0
10
20
30
40
upf1Δ
+GAL-UPF1
nmd2Δ
+GAL-NMD2
upf3Δ
+GAL-UPF3

B.


min. after gal. induction
0
15
30
60
upf1Δ
+GAL-vector
nmd2Δ
+GAL-vector
upf3Δ
+GAL-vector

begin to accumulate (12-14 min. post-induction; see above), the *ade2-1* mRNA disappears with a half-life of approximately 7 min. in all three strains. By 30 min. after galactose induction of any of the three *UPF/NMD* genes, approximately 20% of the *ade2-1* mRNA population remains and, by 40 min., the abundance of the *ade2-1* mRNA returns to the low levels characteristic of a *UPF/NMD* (wild-type) strain. These experiments show that induction of Upf1p, Nmd2p, or Upf3p restores NMD and results in immediate destabilization of the entire *ade2-1* mRNA population, i.e., the *ade2-1* mRNA molecules present in the cell prior to galactose induction are not immune to degradation by NMD.

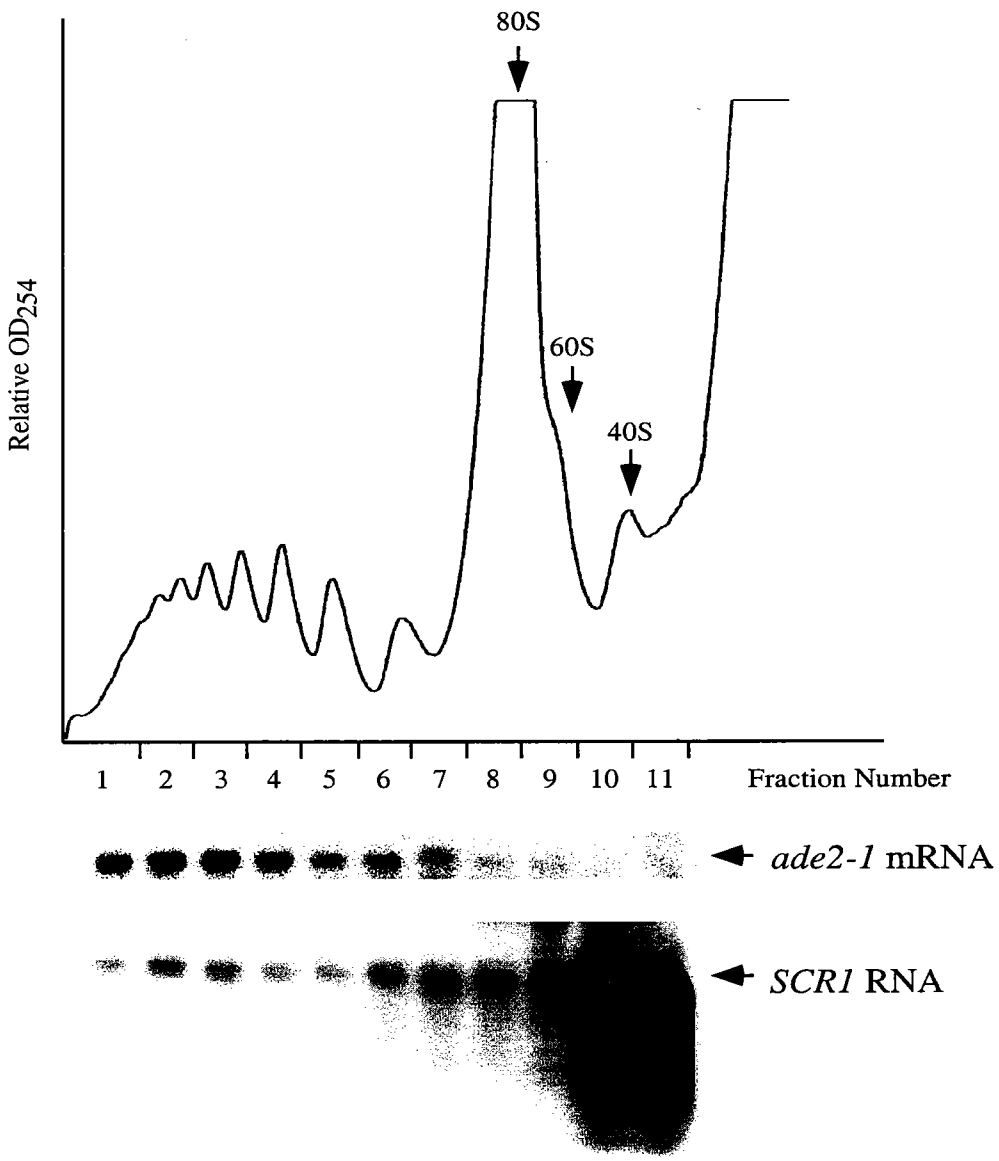
To ensure that addition of galactose, by itself, does not result in destabilization of the *ade2-1* mRNA, the galactose induction experiment was repeated in *upf1Δ, nmd2Δ,* and *upf3Δ* strains transformed with an empty *GAL1* vector. Northern analyses of RNA isolated from these strains demonstrate that the *ade2-1* transcript remains stable throughout the course of this control experiment (Figure 5B).

Degradation of the *ade2-1* mRNA Population Occurs on Polyribosomes.

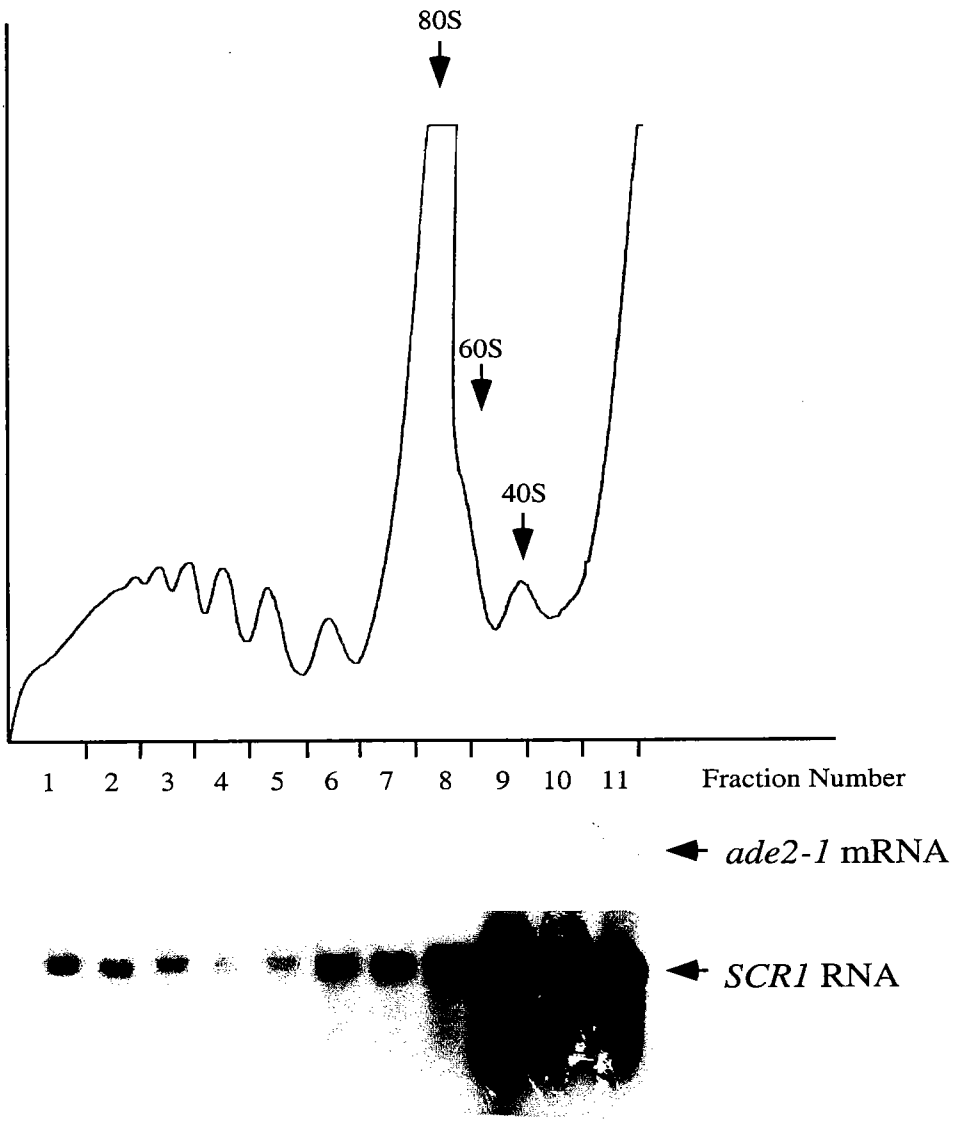
To confirm that the *ade2-1* mRNA accumulated in *upf/nmd* cells is cytoplasmic, and that its eventual decay occurs on polyribosomes (Zhang et al., 1997), the association of the *ade2-1* mRNA population with ribosomes was investigated under conditions where NMD was either inactive or active. Cytoplasmic extracts were prepared from a strain containing galactose-inducible *UPF1*, both prior to galactose-induction and 30 min. post

Figure 6. Degradation of the *ade2-1* mRNA population occurs on polyribosomes. (A) The *ade2-1* mRNA is detected in the polysome fractions before galactose induction. Total RNA isolated from polyribosome fractions collected before the addition of galactose was analyzed by northern blotting with DNA probes that detected the *ade2-1* mRNA and the *SCR1* RNA (the latter to serve as a control to ensure that RNA was isolated from the polyribosome fractions). **(B)** The *ade2-1* mRNA is no longer detected in the polyribosome fractions upon activation of NMD. Total RNA isolated from polyribosome fractions collected 30 minutes after the addition of galactose was analyzed by northern blotting as described above. The results depicted in this figure were obtained from the *upf1Δ* strain (HFY870), harboring the galactose-inducible *UPF1* construct.

A.


80S
60S
40S
Relative OD254
1 2 3 4 5 6 7 8 9 10 11
Fraction Number
ade2-1 mRNA
SCR1 RNA

B.


80S
60S
40S
1 2 3 4 5 6 7 8 9 10 11
Fraction Number
ade2-1 mRNA
SCR1 RNA

induction, and then resolved on sucrose gradients. Fractions collected from these gradients were analyzed by northern blotting. Under circumstances when NMD is inactive, the *ade2-1* mRNA was found to cosediment predominantly with the polyribosome fractions (Fig. 6A, fractions 1-7), suggesting that these transcripts are associated with actively translating ribosomes. The association of these transcripts with an average of 4-5 ribosomes is consistent with premature translational termination within a large mRNA (2.2 kB; 51, 52). Upon restoration of NMD, the *ade2-1* mRNA is rapidly degraded (Figure 5) and is no longer detected in the polyribosome fractions (Fig. 6B). As a control for these experiments, the northern blots of Figure 6A and B were also probed for the *SCR1* RNA. The latter blots demonstrate that the quality and quantity of RNA isolated from the two sets of gradients (0' and 30' post-galactose induction) was similar (Figure 6A and B).

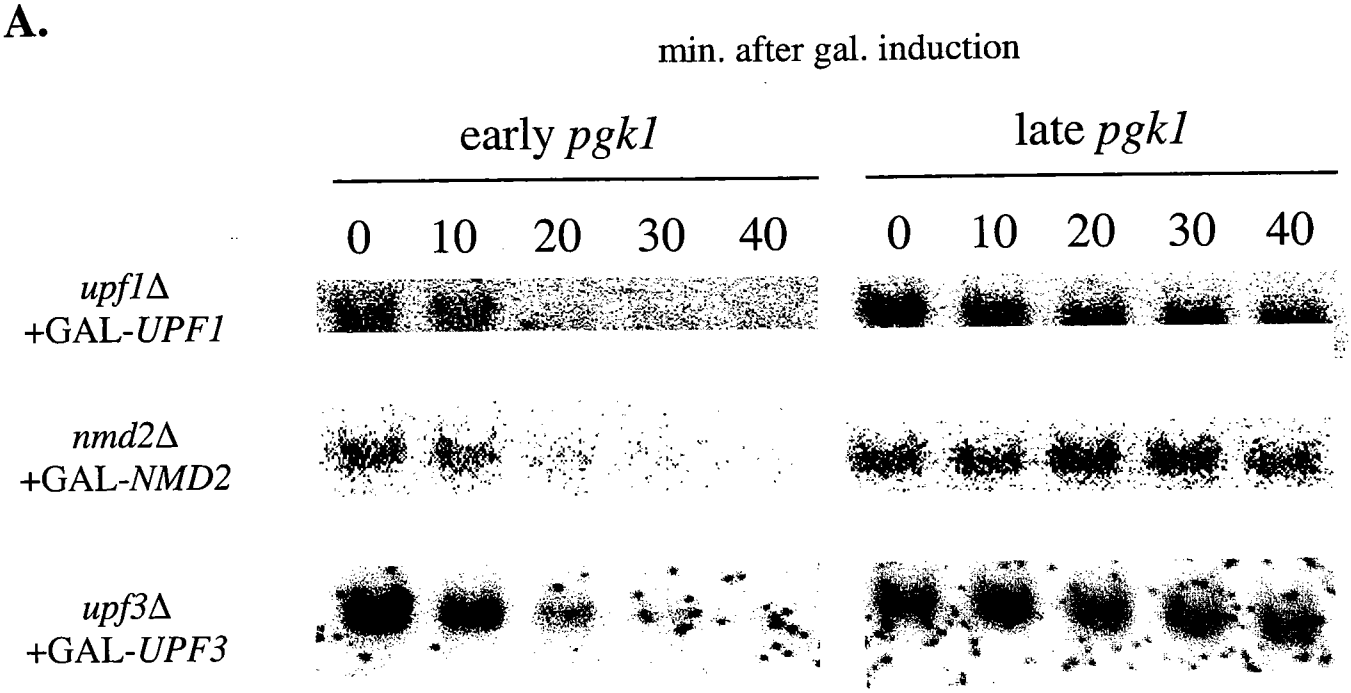
Results virtually identical to those of Figs. 6A and B were obtained using the galactose-regulated *NMD2* and *UPF3* constructs (data not shown). Taken together, these findings indicate that the *ade2-1* mRNA that accumulates when NMD is inactive associates with cytoplasmic ribosomes and that this mRNA disappears from the polyribosomal fraction when its degradation by the NMD pathway is activated.

Activation of NMD Triggers Rapid Decay of *PGK1* Transcripts With Early But Not Late Nonsense Codons .

To substantiate our findings with the *ade2-1* mRNA, we investigated the effect that restoration of NMD had on the decay kinetics of another nonsense-containing

Figure 7. Early nonsense *pgk1* mRNA degrades rapidly upon activation of NMD. (A) Activation of NMD results in degradation of the early nonsense-containing *pgk1* transcript, but does not destabilize the late nonsense-containing *pgk1* mRNA. Total RNA isolated from yeast strains with the indicated *UPF/NMD* genotypes, harboring the corresponding galactose-inducible NMD gene constructs was analyzed by northern blotting with DNA probes that detected the *pgk1* transcript. **(B)** The addition of galactose does not destabilize the early nonsense-containing *pgk1* mRNA. Total RNA isolated from yeast strains with the indicated *UPF1/NMD* genotypes harboring only a vector control plasmid, was analzed by northern blotting as described above. Yeast strains used in this experiment were: HFY870, HFY1300, and HFY861

A.


min. after gal. induction
early *pgk1*
late *pgk1*
0 10 20 30 40
0 10 20 30 40
*upf1*Δ
+GAL-*UPF1*
*nmd2*Δ
+GAL-*NMD2*
*upf3*Δ
+GAL-*UPF3*

B.

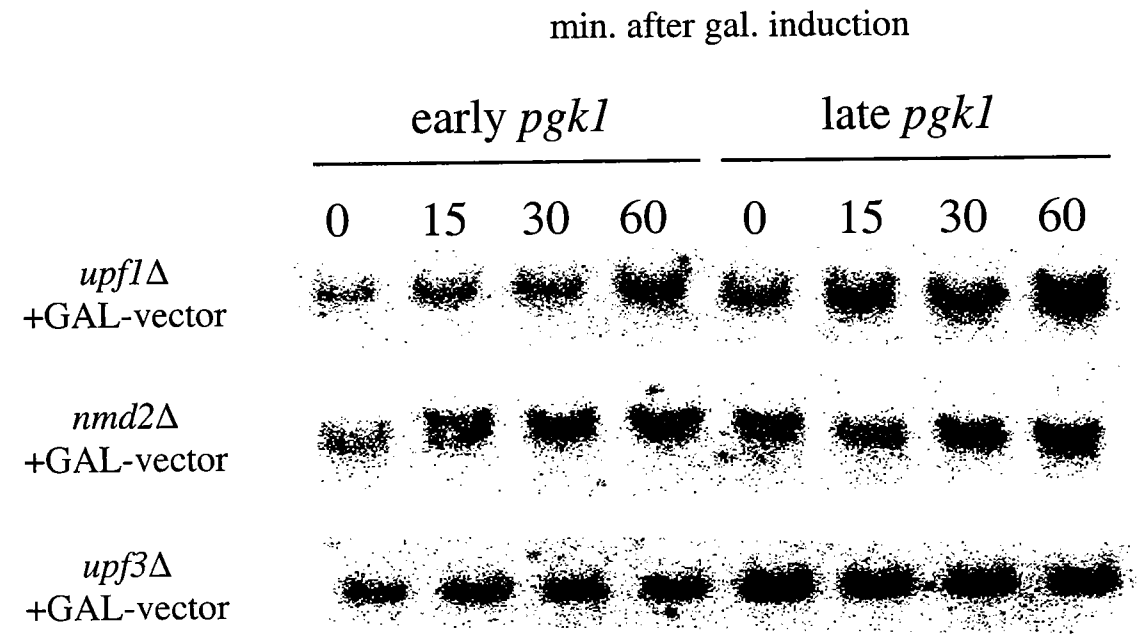
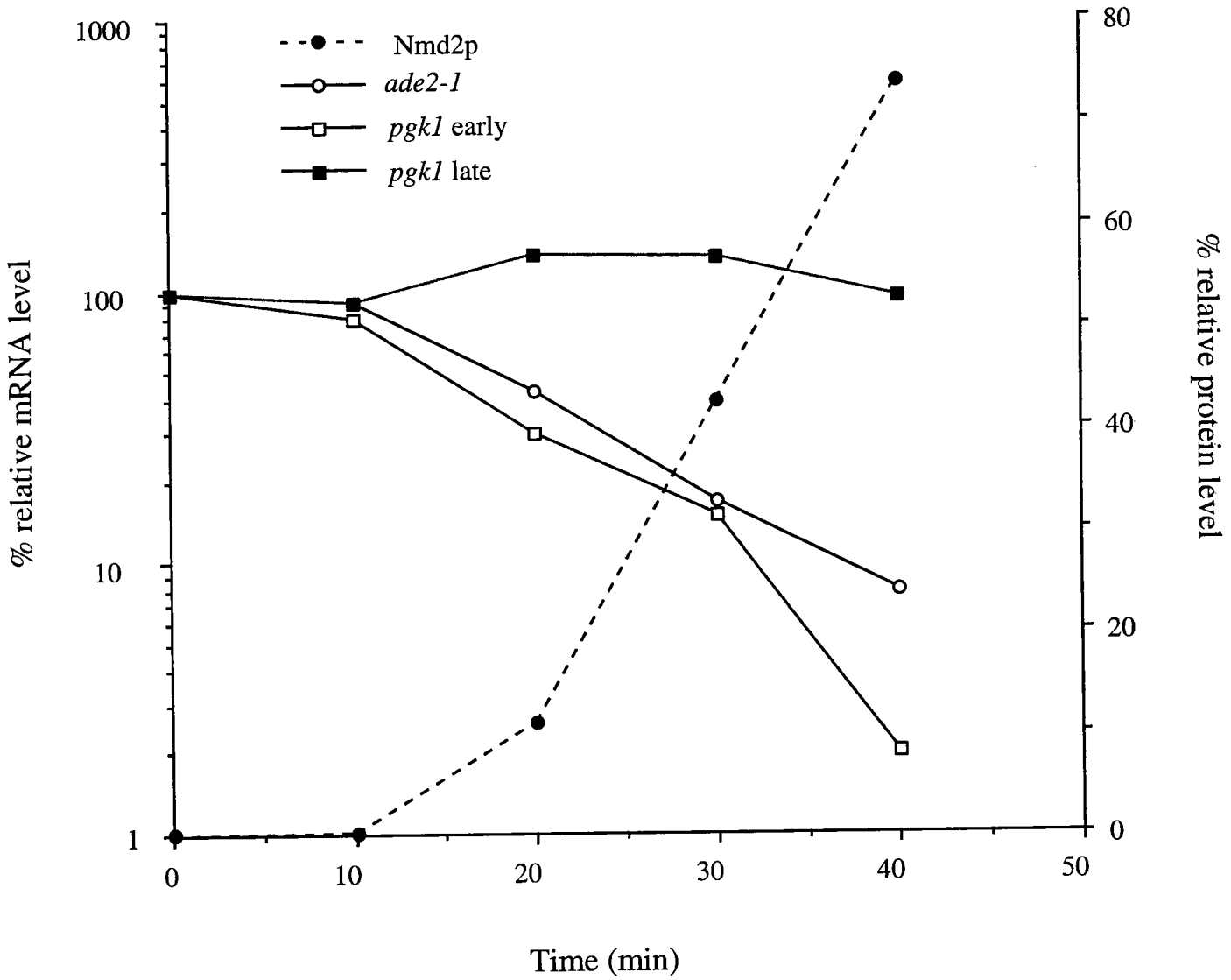

min. after gal. induction
early *pgk1*
late *pgk1*
0 15 30 60
0 15 30 60
*upf1*Δ
+GAL-vector
*nmd2*Δ
+GAL-vector
*upf3*Δ
+GAL-vector

Figure 8. Activation of NMD results in the rapid decay of substrate transcripts. Graphical depiction of the relative levels of *ade2-1* (○), early nonsense pgk1 (❑), and late nonsense *pgk1* (■) mRNAs with the relative level of induced protein, Nmd2p (●), upon activation of NMD (by addition of galactose). The data for construction of this graph was quantitated from the northern blots of Figure 5A (*ade2-1*) and Figure 7A (early and late *pgk1*) and from the western blot of Figure 4.


1000
100
10
1
% relative mRNA level
Nmd2p
ade2-1
pgk1 early
pgk1 late
80
60
40
20
0
% relative protein level
0
10
20
30
40
50
Time (min)

transcript. The *PGK1* mRNA is normally very stable, having a half-life of approximately 60 min. (Peltz et al., 1993), but a derivative with a nonsense mutation at codon 22 (*pgk1*-UAG-2) is extremely unstable ($t_{1/2}$=6 min.; Peltz et al., 1993a,b). Inactivation of the NMD pathway (by mutations in *UPF1*, *NMD2*, or *UPF3*) restore the stability of this nonsense-containing mRNA ($t_{1/2}$=60 min), confirming that it is a substrate for NMD (Peltz et al., 1993a,b). The large differences in the half-lives of this transcript in the active and inactive states of NMD make it ideal for an investigation of the possible existence of mRNA immunity to rapid decay. Fig. 7A demonstrates that induction of *UPF1*, *NMD2*, or *UPF3* (in the respective deletion strains) resulted in rapid disappearance of the *pgk1*-UAG-2 mRNA (see "early *pgk*1"). The decay kinetics for the steady-state population of this mRNA were comparable to those of the *ade2-1* mRNA, such that: a) it disappeared with a half-life of approximately 7 min., after a lag for induction of the pathway, and b) by 30 min. post-induction, most (85%) of the mRNA was degraded (Figure 7A and 8). These results support previous findings that this transcript is a substrate for NMD and indicate that restoration of the NMD pathway causes its rapid and immediate degradation.

Destabilization of mRNAs by premature nonsense codons is a position-dependent phenomenon wherein mRNAs with nonsense codons occurring in the last 20-30% of the coding region retain their wild-type decay rates (Jacobson and Peltz, 1996; Peltz et al., 1993; Hagan et al., 1995; Mangus and Jacobson, 1999). As an additional means to determine whether the post-induction disappearance of the *pgk1*-UAG-2 and *ade2-1* mRNAs was a direct consequence of restoration of NMD, we repeated the NMD induction experiments in cells harboring a *pgk1* allele with a nonsense mutation at codon

385 (*pgk1*-UAG-7). This mutation does not affect the stability of the encoded mRNA ($t_{1/2}$>60 min.) and does not render it a substrate for NMD (Peltz et al., 1993). As such, the *pgk1*-UAG-7 transcript serves as an ideal control to test whether galactose induction of the *UPF/NMD* genes results in selective degradation of *bona fide* NMD substrates. Figure 7A shows that galactose induction of *UPF1*, *NMD2*, or *UPF3* does not affect the abundance of the *pgk1*-UAG-7 mRNA (see "late *pgk1*"). This result demonstrates that the decay pathway activated by induction of the *UPF/NMD* genes remains specific for proper substrate mRNAs and reiterates the finding that the *pgk1*-UAG-7 mRNA is not a substrate for NMD.

Control experiments were also conducted to ensure that destabilization of the *pgk1*-UAG-2 mRNA subsequent to restoration of NMD was not due to an effect of galactose addition. Fig. 7B shows that *upf1Δ, nmd2 Δ,* and *upf3 Δ* strains containing either the early or late *pgk1* nonsense alleles and an empty *GAL1*-vector do not alter the stability of either the early or late *pgk1* nonsense mRNAs in response to galactose addition to the growth media. Therefore, it is activation of the NMD pathway and not simply the addition of galactose, which causes destabilization of the *pgk1*-UAG-2 mRNA.

DISCUSSION

What comprises a substrate for nonsense-mediated decay of yeast mRNAs?

Yeast mRNAs containing premature translation termination codons are rapidly degraded via the NMD pathway when several criteria are met. The termination codon in question must occur within the first two-thirds to three quarters of the mRNA coding region and be 5' proximal to an essential sequence element (the downstream element, or DSE; Peltz et al., 1993a,b; Zhang et al., 1995). Moreover, the nonsense-containing mRNA needs to be translated (Losson and Lacroute, 1979; Gozalbo and Hohmann, 1990), and several factors essential to the NMD process need to be present and functional. (Leeds et al., 1992; He and Jacobson, 1995; Cui et al., 1995; Lee and Culbertson, 1995). The nonsense codon that promotes mRNA destabilization can occur within a conventional coding region or be derived from an upstream open reading frame (Cui et al., 1995), present within an unprocessed intron (He et al., 1993), recognized only during out-of-frame translation that occurs as a consequence of leaky scanning (Welch and Jacobson, 1999), or be the normal termination codon in an mRNA with an extended 3'-UTR (Muhlrad and Parker, 1999). Since NMD has been shown to occur without prior shortening of the mRNA poly(A) tail (Muhlrad and Parker, 1994), it has been suggested that the decay-initiating event can occur very early in the functional lifetime of the

mRNA (Jacobson and Peltz, 1996). At issue, however, is whether an mRNA qualifies as an NMD substrate at any time during its cellular "life cycle."

One model, derived from data in mammalian cells, suggests that spatial relationships reflect temporal relationships, i.e., that the apparent nuclear proximity of NMD and the deposition of factors essential for NMD during pre-mRNA splicing must reflect a decay process that occurs during an early round of translation, or not at all (Nagy and Maquat, 1998). Recent experiments studying yeast NMD carried out by Gonzalez et al (2000) led them to suggest that NMD in yeast may also occur during the initial or early rounds of translation. Gonzalez et al (2000) found that the RNA binding protein Hrp1p, is able to associate with the *PGK1* DSE, and that this association appears to promote NMD, as disruption of *HRP1* or mutation of the region responsible for DSE binding stabilized two artificial NMD substrates assayed (Gonzalez et al., 2000). Given previous studies which demonstrated a role for Hrp1p in RNA end formation and mRNA transport (Henry et al., 1996; Kessler et al., 1997; Minvielle-Sebastia et al., 1998), Gonzalez et al (2000) proposed a model that suggests NMD occurs during the initial rounds of translation. In this model Hrp1p is proposed to bind the *PGK1* DSE during 3' end formation, or export. The mRNA/Hrp1p complex is then exported to the cytoplasm. Once the mRNA/Hrp1p complex reaches the cytoplasm it would then be actively translated. In a wild-type message any bound Hrp1p would be liberated as a consequence of translation, while Hrp1p bound to a nonsense-containing message would not be removed, as premature termination would occur upstream Hrp1p's binding site (the DSE). Gonzalez et al (2000) suggest that retention of Hrp1p by the nonsense containing mRNA results in the generation of an abnormal mRNP structure that targets the mRNA

for degradation.

In the models described above, mRNAs are thus capable of being degraded only during their initial rounds of translation, after which they acquire immunity to NMD. The data presented here demonstrate that, at least in yeast, NMD is not limited to early rounds of mRNA translation, but rather, can occur at any time during an mRNA's life cycle. This implies that either there are no NMD-essential factors that are shed during translation, or that factors that are shed can reassociate with an mRNA while it remains in the cytoplasm.

The continual availability of substrates for the yeast NMD apparatus implies that decay occurs in the cytoplasm

The processes that mediate normal and nonsense-mediated mRNA decay have been characterized in various eukaryotic systems. While study of NMD in numerous systems has provided insight into the mechanism and function of this degradation pathway, NMD has been most extensively characterized in the yeast, *Saccharomyces cerevisiae*. In yeast, a large body of evidence indicates that NMD occurs in the cytoplasm. A primary piece of evidence supporting cytoplasmic decay, is the fact that destabilization of nonsense-containing mRNAs requires their translation, indicating that the mere presence of a premature nonsense codon within an mRNA is not sufficient to promote its degradation by the NMD pathway. Normal decay can be restored to a nonsense-containing mRNA if a nonsense suppressing tRNA is co-expressed in the same cells (Losson and Lacroute, 1979; Gozalbo and Hohmann, 1990) or if the initiator AUG

is deleted from the transcript. NMD is also sensitive to drugs that alter cellular translation levels. This is supported by the observation that substrates of the NMD pathway are stabilized in cells that have been treated with drugs that interfere with translation initiation and elongation, but rapidly destabilized upon removal of these drugs (Herrick et al., 1990; Peltz et al., 1992; Zhang et al., 1997). Another piece of evidence indicating that decay in yeast occurs in the cytoplasm, is the observation that the NMD factors are predominantly cytoplasmic. In addition, sucrose density gradient analysis has found that the NMD factors co-sediment with polyribosomes, confirming their association with the translation apparatus (Atkin et al., 1995,1997; Mangus and Jacobson, 1999). Further evidence that NMD in yeast is a cytoplasmic event stems from experiments that found mutation of the *UPF/NMD* genes lead to the stabilization of nonsense-containing mRNAs, but also resulted in a nonsense suppression phenotype (Leeds et al., 1992; Weng et al., 1996a,b; Maderazo et al., 2000). An important role for the *UPF/NMD* factors in the modulation of translation termination is also indicated by experiments showing that yeast (and human) Upf1p interact with the polypeptide release factors Sup35p (eRF3) and Sup45p (eRF1) (Czaplinski et al., 1999). Collectively these results imply that recognition and degradation of yeast substrates of the NMD pathway occur in the cytoplasm. Many of these observations have been confirmed by experimental analysis in higher eukaryotes, including mammalian systems, suggesting that the NMD pathway is a highly conserved degradation pathway.

To address the observation that some mammalian nonsense-containing mRNAs appear to evade mRNA degradation after nuclear export, we devised a series of experiments to determine if yeast nonsense containing substrates were also capable of

evading NMD. To this end, we created a series of inducible NMD strains. Specifically, a collection of plasmids harboring one of the *UPF/NMD* genes under the control of the inducible *GAL1* promoter, were transformed into their respective deletion strains. In effect these manipulations allowed us to modulate the activity of the NMD system, simply by altering media composition.

In separate experiments, we analyzed the decay kinetics of *ADE2* and *PGK1* nonsense-containing mRNAs when expression of *UPF1, NMD2*, or *UPF3* was repressed, or subsequently induced with galactose. Use of these particular mRNAs was important because, when stabilized, they have relatively long half-lives (>30 min). This was critical, since a lag time of 12-14 minutes occurred before the measurable quantities of the NMD factors were detected. If less stable transcripts were utilized (i.e., transcripts with half-lives >15 min), we would be unable to discern if the effects measured were a result of degradation via the NMD pathway, or the result of degradation by the deadenylation dependent pathway. By using the *PGK1* and *ADE2* nonsense alleles, we were able to measure how quickly these mRNAs disappeared as the NMD pathway was induced, taking into account the rate of *UPF/NMD* factor accumulation and that of new mRNA synthesis. Induction of any of the *UPF/NMD* proteins, in their respective deletion strains, resulted in the rapid and immediate degradation of the *ade2-1* and *pgk1* substrate transcripts. By the 40 min. time point, the steady state levels of both substrate mRNAs were decreased to levels observed in a wild-type *UPF/NMD* strain (Fig. 5 & 7). It is therefore unlikely that a stable mRNA population exists, since the half-life of this population would be greater that 30 min. Additionally, induction of the *UPF/NMD* proteins actually occurs ~12-14 min. after galactose addition (as determined from Figure

4 and Figure 8). Therefore, the abundance of the *ade2-1* and *pgk1* substrate transcripts was actually reduced to wild-type levels in less than 30 min. Moreover, the half-lives of both the *ade2-1* and *pgk1* substrates at steady state were calculated to be approximately 7 min. (as determined by the slopes in Fig. 8). Therefore, if two populations of the same mRNA, degrading at different rates were present, (newly synthesized, nuclear associated mRNAs, $t_{1/2}$=5min, and cytoplasmic mRNAs, $t_{1/2}$=35-60 min.) in order for the total mRNA population to show a 7 min. half life, the newly synthesized population would have to comprise at least 85% of the total mRNA. The observation, that yeast NMD substrates are unable to escape degradation by NMD, is also supported by sucrose sedimentation analysis we conducted with varioius *PGK1* alleles. In strains where NMD was inactivated, both a *PGK1*-late (subject to normal decay) and *PGK1*-early (subject to NMD) were found to co-sediment with polyribosomes, indicating that these mRNAs are actively translated and stable. However, sucrose density gradient analysis, conducted 30 minutes after induction of NMD, found that only the PGK1-late nonsense allele was detected on polysomes. This suggests that induction of the NMD pathway leads to the rapid degradation of the PGK1-early transcript, degradation can occur in association with the translation apparatus, and that NMD pathway can recognize and degrade a substrate mRNA at any point in its lifespan. In addition, if the half-lives determined in our experiments were due to the contribution of two separate subpopulations of mRNA (i.e., stable pre-induction mRNA and unstable post-induction mRNA), we should have detected the presence of *ade2-1* mRNA on northern blots performed on mRNA isolated sucrose density gradients after 30 minutes of galactose induction.

Interestingly, our findings appear to correlate well with recent studies of the

mammalian GPx1 mRNA. It has been found that cytoplasmic nonsense-mediated decay of GPx1 mRNA is not restricted to newly synthesized transcripts, demonstrating that NMD is able to act on the steady state mRNA population. This observation suggests that there is not a strict requirement for recognition of a nonsense-substrate during nuclear export, and that decay may occur after a mRNA has been completely exported to the cytoplasm (Sun et al., 2000).

The faux UTR model

The two predominant (and related) models for the mechanism of NMD recognize that no direct link between Dcp1p and the *UPF/NMD* factors has been found and thus postulate that decay is a consequence of events occurring during or after translation termination. In one model, decay occurs in response to recognition of a sequence element by a scanning complex of *UPF/NMD* factors (Ruiz-Eschevarria et al., 1996; Czaplinski et al., 1998,1999) and, in the other, decay is triggered by the failure to terminate adjacent to a properly configured 3' UTR (Bonnetti et al., 1994; Hilleren and Parker, 1999) regarding the degradation of NMD substrates, as disruption of the NMD pathway results in disruption of translational fidelity. Readthrough of the premature translation termination codon would allow the ribosome to translate through the DSE, allowing it to displace any Hrp1p bound at the DSE. However, our results could be explained by the surveillance complex model if one assumes that nuclear marking factors such as Hrp1p are able to rebind cytoplasmic mRNAs once they have been displaced by the translation apparatus.

Like the surveillance complex model, the faux UTR model also considers the

influence of RNP structure on the proper function of the NMD pathway. In this model, sequences downstream of a premature termination codon are hypothesized to function as a defective or "faux" UTR. This model implies that proper termination is a result of a specific interaction between the translation apparatus and components of the 3' UTR of a transcript (Bonetti et al., 1995; Hilleren and Parker, 1999; Jacobson and Peltz, 2000). Proper completion of the termination cycle is believed to stabilize the message by stimulating translation by allowing proper interaction between the 3' UTR and the 5' cap structure and their associated factors to occur at a high efficiency. Improper termination at a "faux" UTR could result in inefficient ribosome release, and improper release could negatively affect these interactions, thereby triggering decapping. In this model, Upf1p would function in conjunction with eRF1 and eRF3 to mediate all termination events, possibly using its helicase and RNA binding activities to promote ribosome release. The faux UTR model is also able account for our observation that yeast mRNAs may be degraded by the NMD pathway at any point during their lifecycle. Since the faux UTR model does not depend on the nuclear marking of an mRNA as a requirement for degradation. The faux UTR model can also readily accommodate the results of studies that show that Upf1p has a more general role in translation termination that is not limited to NMD. Experiments conducted by He and Jacobson (2001) found that Upf1p regulates the decapping and exonucleolytic degradation of both wild-type and NMD substrates, suggesting that Upf1p functions in the metabolism of all mRNAs.

CHAPTER 4

OVEREXPRESSION OF SELECTED *UPF1* ALLELES ALTERS CELL VIABILITY, NONSENSE-MEDIATED mRNA DECAY, AND TRANSLATION

INTRODUCTION

Several studies indicate that, in yeast, there is an intimate link between mRNA translation and recognition of NMD substrates, including experiments showing that: 1) recognition of a premature nonsense codon requires translation, since drugs or mutations that inhibit translation stabilize NMD substrates (Herrick et al., 1990; Peltz et al., 1992; Zhang et al., 1997), 2) NMD factors co-fractionate with polyribosomes on sucrose gradients, suggesting that decay occurs in association with the translation apparatus (He et al., 1993; Atkin et al., 1995, 1997; Mangus and Jacobson 1999), 3) nonsense-containing mRNAs degrade in association with polyribosomes (Zhang et al., 1997), 4) NMD factors interact with translation termination factors eRF1 and eRF3 (Czaplinski et al., 1999; Weng et al., 2001), and 5) Deletion of *UPF1*,*UPF2/NMD2*, or *UPF3*, results in increased levels of nonsense suppression (Leeds et al., 1992; Weng et al., 1996a,b; Maderazo et al., 2000).

Despite the large body of evidence suggesting that the processes of NMD and translation are tightly linked, little evidence has been found to suggest that disruption of NMD function in yeast can alter cell viability. Deletion of *UPF1*, *UPF2/NMD2*, or

UPF3 abrogates NMD, but does not alter cell growth (Leeds et al., 1992; He and Jacobson 1995; Lee and Culbertson 1995). This observation is surprising, since the *UPF/NMD* factors have been shown to interact with components of the translation apparatus, as well as serve a regulatory role in the decapping and exonucleoltyic degradation of both wild-type and nonsense-containing transcripts (Atkin et al., 1995, 1997; Mangus and Jacobson, 1999; Czaplinski et al., 1999; He and Jacobson 2001).

Since the *UPF/NMD* factors play a critical role in several cellular processes, I decided to investigate whether overexpression of dominant-negative mutants of Upf1p, would be capable of inhibiting cell viability. The production of dominant alleles of *UPF1* is not without precedent, as the initial characterizations of *UPF1* yielded several dominant *UPF1* mutants. Experients conducted by Leeds and colleagues resulted in the production of multiple *UPF1* mutations (Leeds et al., 1992), including seven independent mutants of *UPF1* in which a single amino acid change was able to confer a dominant-negative phenotype. Several of these mutants were shown to influce both the efficiency of nonsense suppression and NMD in a dosage dependent mannner (Leeds et al., 1992). Although these dominant *UPF1* mutants were able to influence both nonsense suppression and NMD, none of the mutants analyzed appreciably altered cell viability.

Additional mutational studies of *UPF1* have been conducted by Weng et al., (1996a,b), who took advantage of previous studies, indicating that *UPF1* is a member of RNA/DNA helicase superfamily I (Altamura et al., 1992; Koonin 1992; Leeds et al., 1991; Leeds et al., 1992). Since members of this family of helicases share a series of highly conserved motifs, Weng and colleagues generated amino acid substitutions within

these conserved regions, and assayed their effects on nonsense suppression and NMD. These studies found that a point mutation within the *UPF1* ATP binding domain was able to affect both nonsense suppression and NMD activity, but a mutation in the ATP hydrolysis domain was only able to alter NMD activity, suggesting that Upf1p's function in these two processes can be seperated (Weng et al., 1996a,b). However, these experiments did not detect an effect on cell viability with any of the mutants tested (Weng et al., 1996b).

Although these studies were unable to detect effects on viability, several key observations were made in these studies. It was established that the region between amino acid residues 533 and 842 is critical for Upf1p function, since mutations within this region were able to alter nonsense suppression and/or NMD (Leeds et al., 1992; Weng et al., 1996b). Furthermore, Weng et al were also able to confirm Upf1p's NTPase, RNA binding, and helicase activities using biochemical techniques (Weng et al., 1996a,b). An additional interesting observation was that the effects of various mutants of Upf1p were enhanced in a dosage dependent manner, such that an increase in the ratio of mutant Upf1p to wild-type Upf1p resulted in stronger nonsense suppression and inhibition of NMD (Leeds et al., 1992; Weng et al., 1996b).

Careful analysis of these results, led me to believe that *UPF1* mutants capable of affecting cell viability may already exist. Indeed, it seemed likely that the reason effects on viability had not been observed were a result of insufficient production of the mutant proteins. Therefore, I set out to test this hypothesis by studying the consequences of overexpressing previously characterized point mutants, using high copy vectors with

UPF1 gene expression driven by the *GAL1* promoter. The *GAL1* promoter was selected because it is a tightly regulated inducible promoter that is able to cause high-level expression of genes under its control.

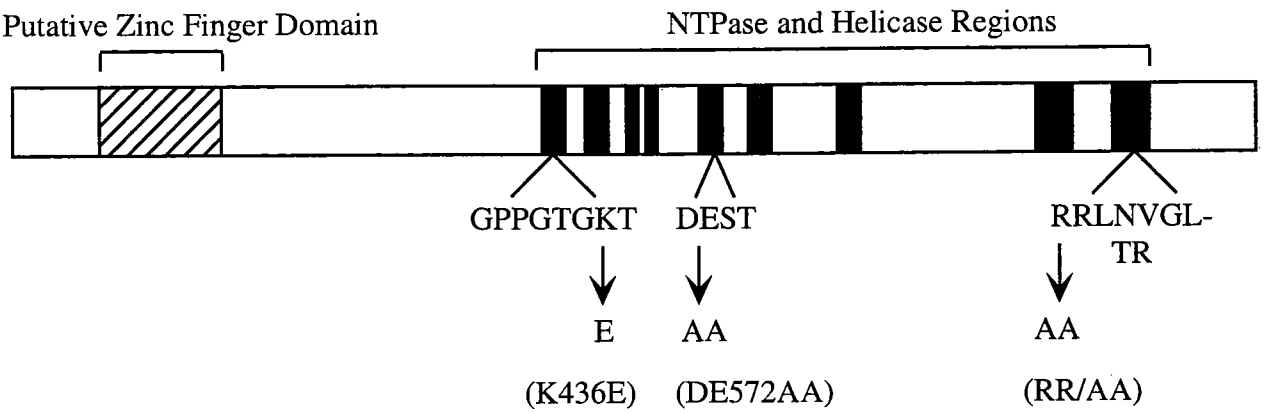
RESULTS

To determine if overexpression of point mutants in *UPF1* would exert dominant-negative effects on NMD or other cellular processes, I sub-cloned various alleles of *UPF1*adjacent to the *GAL1* promoter in a high-copy vector. The constructs utilized are depicted in Figure 9A. The resulting plasmids were analyzed and their structures confirmed by restriction mapping and they were then transformed into the yeast strain HFY114. Strains harboring these high-copy plasmids were grown in SC –ura raffinose media and the expression of the constructs was induced by the addition of galactose. SDS-polyacrylamide gel electrophoresis and subsequent western blotting performed with Upf1p polyclonal antibody revealed that all constructs were inducible by galactose, and expressed at similar levels (see Figure 9B).

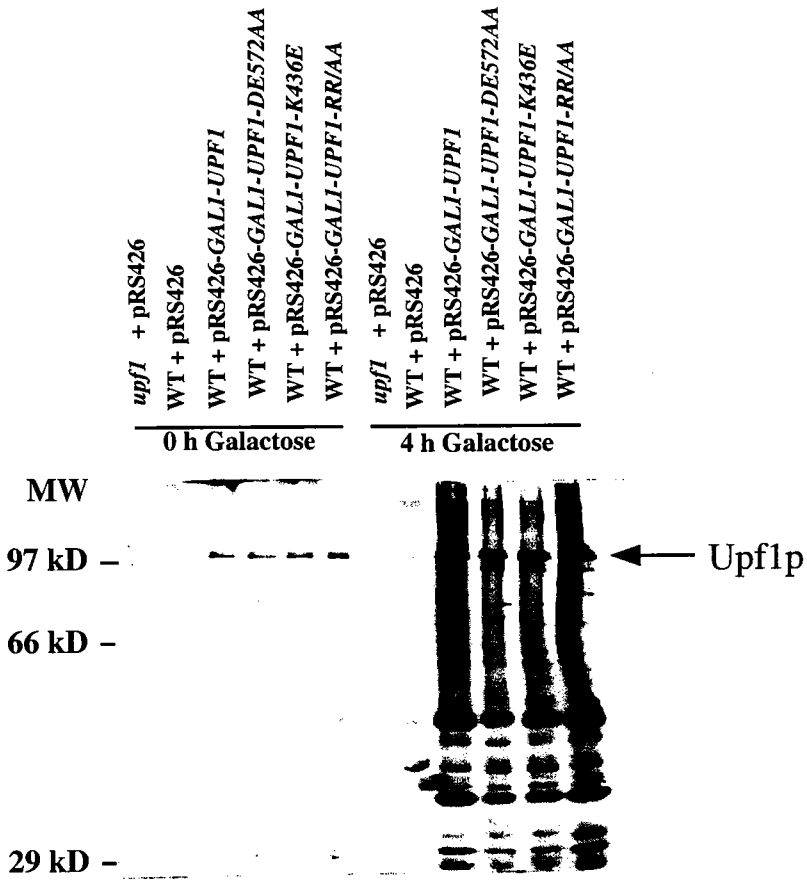
Given dominant-negative proteins are known to be potent inhibitors of the macromolecular complexes in which they function (Eisenger et al., 1997; Belk et al., 1999), and Upf1p has been shown to interact with cellular translation factors (Czaplinski et al., 1999). I sought to determine if overexpression of any *UPF1* alleles would exert a dominant-negative effect on the viability of yeast cells. Strains harboring the various overexpressed *UPF1* alleles, as well as an empty vector control, were assayed for their ability to grow on glucose or galactose. All cells assayed grew to similar levels on

Figure 9. Overexpression of *UPF1* alleles in wild-type cells (A) Schematic of the *UPF1* coding region. Mutations studied and functional domains are indicated. **(B)** *UPF1* alleles are overexpressed upon galactose induction. The constructs shown in **A** and the empty vector pRS426 were transformed into HFY114, which contains a wild-type copy of the *UPF1* gene. Individual transformants were selected, grown in SC –ura raffinose liquid medium, and subsequently induced with galactose. The equivalent of 1ml of cells at an OD_{600} 0.2 was collected at 0h and 4h for protein analysis. Aliquots of whole cell extracts were loaded onto SDS-PAGE gels, immunoblotted, and probed with polyclonal Upf1p antibody.

A.


Putative Zinc Finger Domain
NTPase and Helicase Regions
GPPGTGKT
DEST
RRLNVGL-
TR
E
AA
AA
(K436E)
(DE572AA)
(RR/AA)

B.


upf1 + pRS426
WT + pRS426
WT + pRS426-GAL1-UPF1
WT + pRS426-GAL1-UPF1-DE572AA
WT + pRS426-GAL1-UPF1-K436E
WT + pRS426-GAL1-UPF1-RR/AA
upf1 + pRS426
WT + pRS426
WT + pRS426-GAL1-UPF1
WT + pRS426-GAL1-UPF1-DE572AA
WT + pRS426-GAL1-UPF1-K436E
WT + pRS426-GAL1-UPF1-RR/AA
0 h Galactose
4 h Galactose
MW
97 kD –
66 kD –
29 kD –
Upf1p

glucose(Figure 10). However cells plated on galactose exhibited differences in growth phenotypes. Cells over-expressing wild-type *UPF1*, or *upf1*-RR/AA grew at slightly reduced rates in galactose when compared to the strain harboring the empty vector control. However, the growth of strains over-expressing the *upf1*-DE572AA and *upf1*-K436E alleles was dramatically reduced (Figure 10) relative to the control strains. These results suggested that over-expression of *UPF1* alleles interfered with the function of an essential cellular process.

Growth curves were performed to confirm the results observed in the plate assay. Cells were grown in SC –ura raffinose media to early log phase, diluted to standardized cell density with SC –ura raffinose media, and then induced by addition of galactose. Cell growth, analyzed at various time points after addition of galactose, closely paralleled the results obtained in the plate assay. Strains overexpressing the *UPF1*-WT or *upf1*-RR/AA constructs doubled at slightly slower rates than the empty vector control (3.5h compared to 3 h; see Figure 11), while strains overexpressing the *upf1*-DE5762AA or *upf1*-K436E constructs exhibited a stronger inhibition of growth when compared to the empty vector control strain (4h compared to 3h; see Figure 11).

Since Upf1p is an essential factor of the NMD pathway, I then determined whether overexpression of the various *UPF1* alleles in a wild-type background might affect NMD function. Cells harboring the different alleles, as well as a *upf1*Δ strain with an empty vector, were grown to early log phase in SC –ura raffinose and cell samples were collected for RNA isolation. Expression of the *UPF1* alleles was then induced by

Figure 10. Alleles of *UPF1* exert dominant-negative growth phenotypes when overexpressed. The constructs shown in Figure 10**A** cloned into the vector pRS426 and the empty vector pRS426 were transformed into HFY114, which contains a wild-type copy of the *UPF1* gene. Individual transformants were selected and then diluted serially onto SC –ura plates with either glucose or galactose as the sole carbon source.

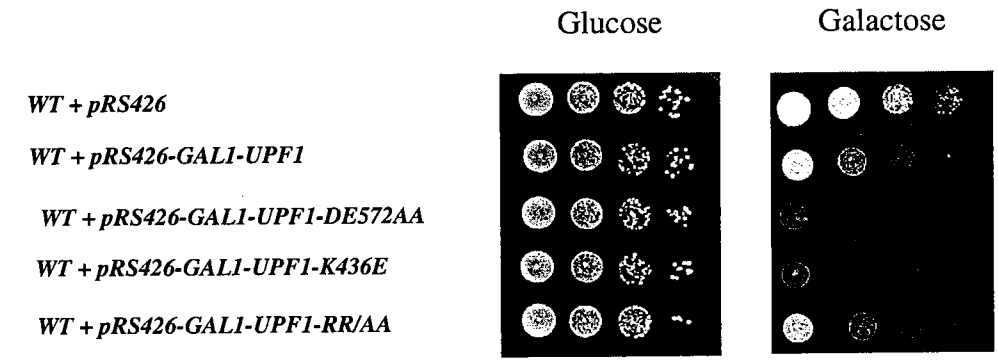
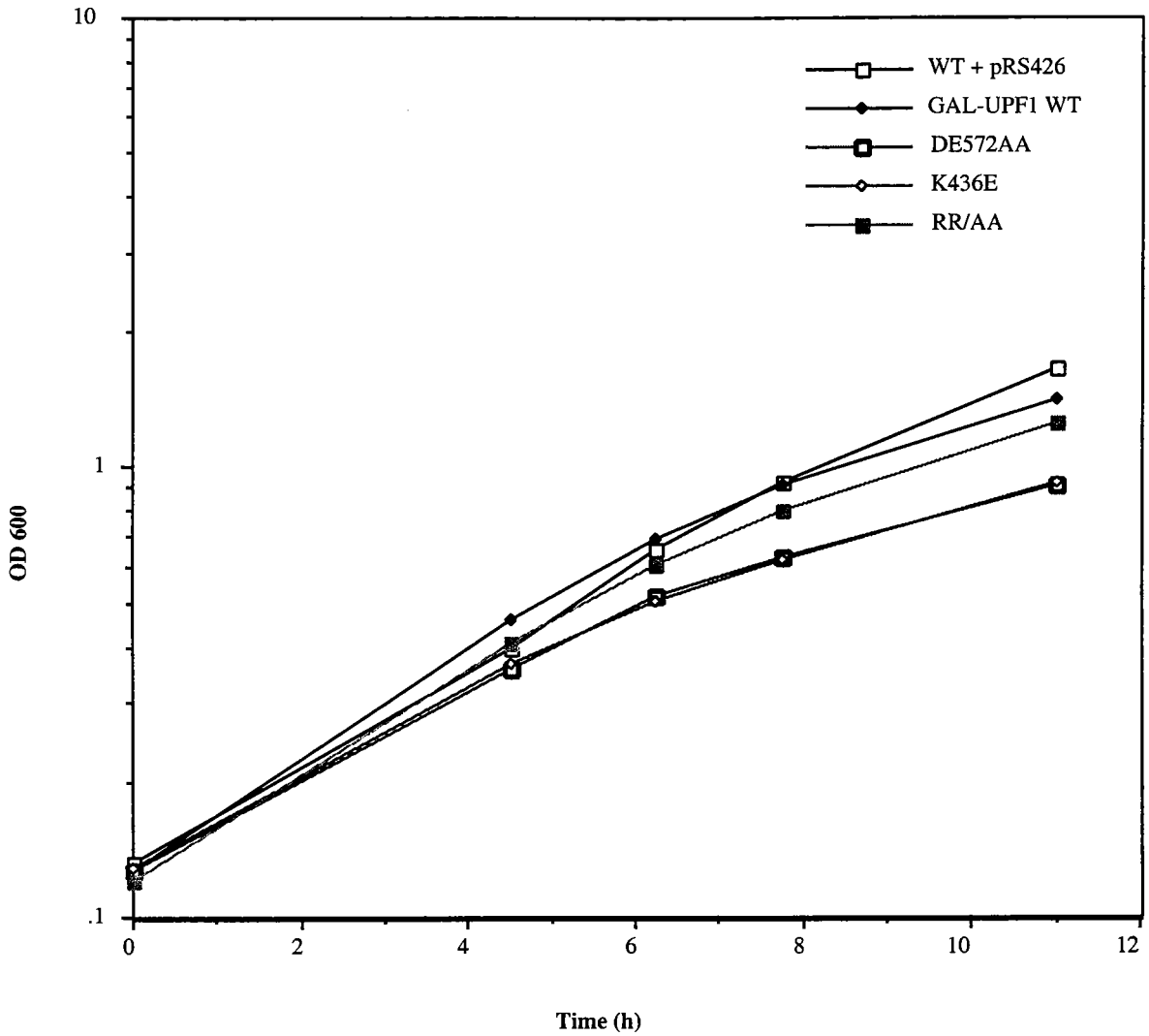

Glucose
Galactose
WT + pRS426
WT + pRS426-GAL1-UPF1
WT + pRS426-GAL1-UPF1-DE572AA
WT + pRS426-GAL1-UPF1-K436E
WT + pRS426-GAL1-UPF1-RR/AA

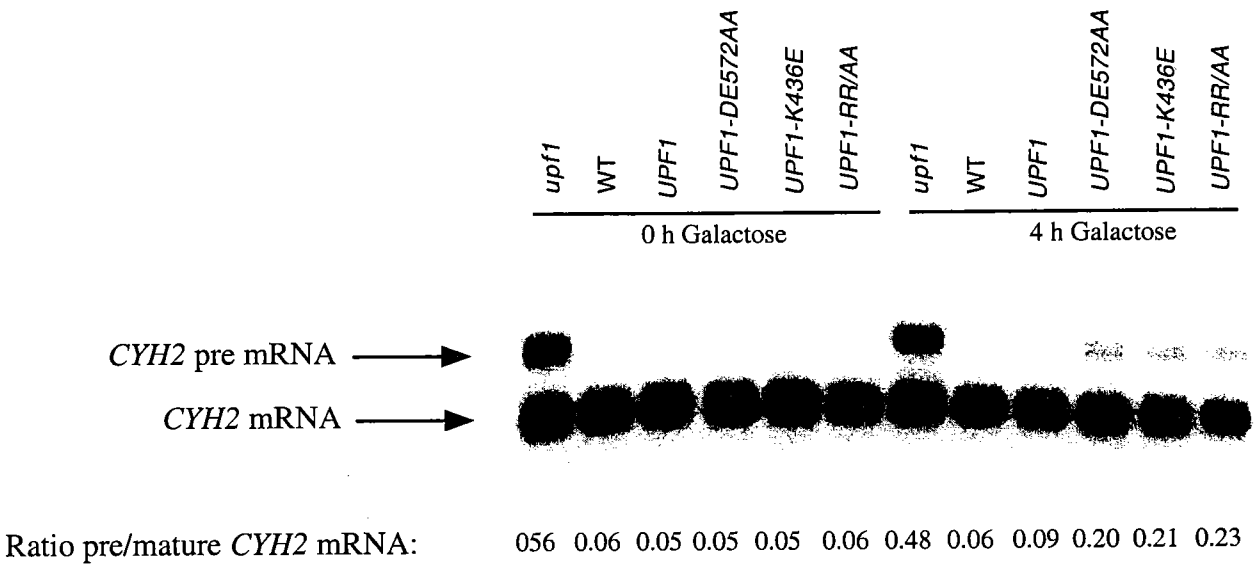
Figure 11. Overexpression of *UPF1* alleles results in dominant-negative growth phenotypes. The constructs shown in Figure 10A cloned into pRS426, and the empty vector pRS426 were transformed into HFY114, which contains a wild-type copy of the *UPF1* gene. Individual transformants were selected, grown in SC –ura raffinose medium to an OD_{600} of 0.15 and subsequently induced with galactose. Cell density, as OD_{600} ,was measured at various time points after galactose induction.


10
1
.1
OD 600
0
2
4
6
8
10
12
Time (h)
WT + pRS426
GAL-UPF1 WT
DE572AA
K436E
RR/AA

addition of galactose and additional cell samples for RNA isolation were taken after 4h of galactose induction. The 4h time point was selected since growth rate experiments had shown that mutant alleles of Upf1p were able to exert cellular effects by this time point. Cytoplasmic RNA, isolated from the cell samples, was subsequently analyzed by northern blotting to determine the relative levels of the *CYH2* mRNA and pre-mRNA. These transcripts were chosen for analysis because the *CYH2* pre-mRNA is an endogenous substrate of the NMD pathway, and the abundance of the *CYH2* pre-mRNA is a direct indicator of the degree of inhibition of the pathway (He et al., 1993; He and Jacobson 1995). Northern analysis of samples taken prior to galactose induction showed that the presence of the constructs in non-inducing conditions had no detectable effect on the NMD pathway (Figure 12, see lanes labeled 0h galactose). However, overexpression of some forms of *UPF1* did interfere with the function of the NMD pathway. Overexpression of wild-type *UPF1* had no effect on otherwise wild-type cells, but overexpression of *upf1*-DE572AA, *upf1*-K436E, or *upf1*-RR/AA resulted in a 4-fold stabilization of *CYH2* pre-mRNA when compared to the wild-type empty vector control (Figure 12, see lanes labeled galactose). The mutant *UPF1* alleles were able to interfere with NMD function, but were not able to completely inactivate NMD since they were not able to inhibit its function to the levels detected in the *upf1*Δ control strain (Figure 12).

Given the well-established links between translation and the nonsense-mediated mRNA decay pathway, I conducted experiments to determine if the inhibition of growth observed upon over-expression of alleles of *UPF1* resulted from perturbation of the translation apparatus. Since the experiments of Figures 9-12 had shown that the *upf1*-

Figure 12. Overexpression of *UPF1* alleles results in dominant-negative inhibition of NMD. The constructs shown in Figure 10**A,** cloned into pRS426, and the empty vector pRS426 were transformed into HFY114, which contains a wild-type copy of the *UPF1* gene. Individual transformants were selected, grown in SC –ura raffinose medium, subsequently induced with galactose. Samples were collected for RNA isolation and northern blot analysis at 0h and 4 h post galactose induction. The blots were hybridized with a radiolabeled *CYH2* probe.

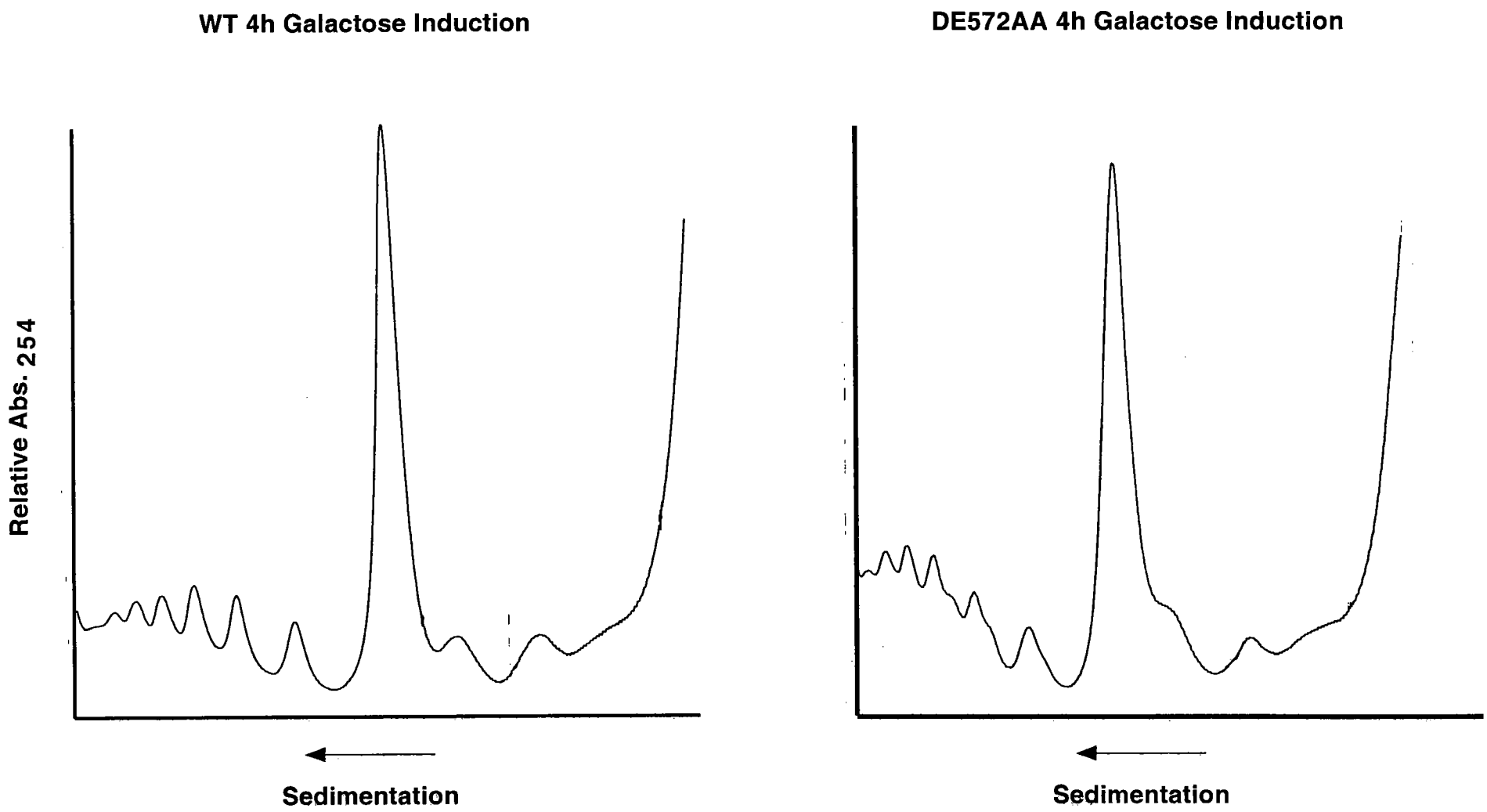


Ratio pre/mature *CYH2* mRNA: 056 0.06 0.05 0.05 0.05 0.06 0.48 0.06 0.09 0.20 0.21 0.23

DE572AA and *upf1*-K436E displayed identical phenotypes, I focused on the characterization of the DE572AA mutant for the remainder of my studies. Sucrose-gradient analysis was used to determine whether overexpression of the DE572AA allele caused any specific alterations in cellular polyribosome profiles. Cells containing the *upf1*-DE572AA allele were grown in SC –ura raffinose and subsequently induced for 4 h in galactose-containing medium. Figure 13 shows that cells harboring the empty vector contol displayed a wild-type polyribosome profile after 4 h of galactose induction, whereas cells over-expressing the *upf1*-DE572AA allele exhibited an aberrant polyribosome profile. These analyses also indicated irregularities in the polyribosome peaks profile of the *upf1*-DE572AA strain, i.e., the peaks were not sharp and discrete like those observed with the control strain (Figure 13). These irregularities are reminiscent of half-mer polyribosme peaks, with the exception that the discontinuities detected with the *upf1*-DE572AA mutant are found on the opposite side of the polyribosome peak from that of ahalf-mer polyribosome profiles (Hesler et al., 1981).

Knowing that half-mer polyribosome profiles are often indicative of ribosome-joining defects (Hesler et al., 1981; Fried et al., 1985; Rotenberg et al., 1988; Moritz et al., 1991), and that Upf1p has been shown to interact with translation termination factors (Czaplinksi et al., 1999), I hypothesized that this aberrant peak might be the result of a defect in termination, and that the discontinuities I detected might be due to the presence of a 60S ribosomal subunit being present on a transcript without a 40S partner. This situation might result if expression of the DE572AA mutant interfered with the ability of termination factors to efficiently uncouple the 80S subunit. To determine if this might be

Figure 13. Overexpression of the *UPF1*-DE572 allele results in abnormal polyribosome profiles. Cytoplasmic extracts were prepared from cells after 4 h of galactose induction. Extracts were fractionated on 7-47% sucrose gradients. The A_{254} traces of the ribosome profiles are shown, with the peaks of the 40S subunits, 60S subunits, and 80S monosomes labeled as such.


WT 4h Galactose Induction
DE572AA 4h Galactose Induction
Relative Abs. 254
Sedimentation
Sedimentation

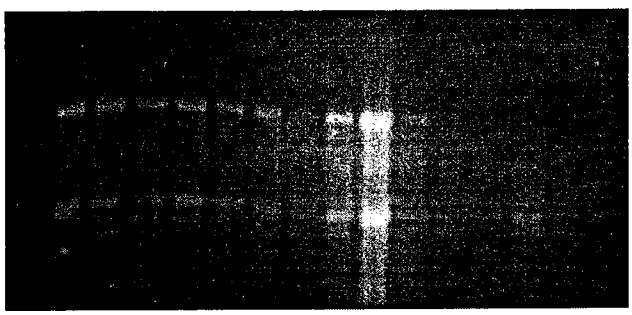
the case, RNA was isolated from the polyribosome fractions, and subjected to agarose gel electrophoresis. The resulting gel was stained with ethidium bromide to detect the presence of the ribosomal mRNA. If the irregularities detected in the polyribosome profile were due to the retention of the 60S without its 40S partner, a change in the change in the abundance of 25S ribosomal mRNA would be expected. Ethidium staining of the DE572AA and wild-type control revealed no significant differences in the abundance of this ribosomal subunit RNA (Figure 14).

Since wild-type Upf1p interacts with the peptidyl release factors (Czaplinski et al., 1999), and this interaction is hypothesized to mediate events at translation termination, I performed a series of co-overexpression experiments, aimed at determining if overexpression of the peptidly release factors could relieve the dominant-negative phenotype observed when the DE572AA mutant is overexpressed. To accomplish this, I co-transformed *GAL1* regulated wild-type or upf1-DE572AA contructs with a high-copy plasmid that both overexpressed eRF1 and eRF3 (Bidou et al., 2000). Overexpression of the peptidyl release factors was unable to alleviate the dominant negative growth phenotype observed with overexpression of the DE572AA mutant in three independent experiments (data not shown).

As a further test of the hypothesis that overexpression of the DE572AA mutant results in a defect in ribosome release, I attempted to perform primer extension inhibition (toeprint) assays on wild-type and DE572AA strains that were induced with galactose for 4 h. To perform a toeprint assay, it is necessary to create *in vitro* translation extracts from the cells of interest. Exogenous mRNA is then added to the extracts, translation of

Figure 14. Overexpression of the *UPF1*-DE572 allele does not alter ribosomal RNA profiles significantly. RNA was isolated from the fractions collected from the sucrose gradients in Figure 14. Samples were then loaded onto a 1% denaturing agarose gel and assayed for rRNA abundance by ethidium bromide staining.

pRS426



pRS426-*GAL1*-*UPF1*-DE572AA

this mRNA is allowed to proceed for a given time period, and the reaction is then terminated by addition of cycloheximide (Wang et al., 1999). The addition of the elongation inhibitor cycloheximide (Vazquez, 1979) is used to stop the progression of translating ribosomes. Primer extension is then performed on the mRNA in the translation reactions, i.e., without additional purification. The cDNA products of these reactionis are visualized by polyacrylamide gel electrophoresis. Extension by reverse transcriptases can be blocked by secondary structures within an RNA, and by ribosomes and their associated factors that are trapped on the mRNA by the assay conditions. This allows the monitoring of the position, and relative frequency, of a ribosome at a specific region on a given transcript. Using this assay, I sought to determine whether overexpression of the DE572AA mutant altered ribosome position or frequency at termination codons.

I attempted to generate functional translation reactions by growing cells in such a way that post-galactose induction, the optical density of the cultures was at the optimum recommended for preparation of toeprint-competent extracts, i.e., OD_{600} 2.5-3.0 (Wang et al., 1999). Unfortunately, I was unable to obtain functional translation extracts when growing the mutant and wild-type cells to the recommended cell density in SC –ura raffinose media. Knowing that the growth rate of the cell has significant effects on the abundance of translation factors, I generated translation extracts from cells that were in a more active growth stage (OD_{600} 1.5.). This resulted in the generation of highly active translation extracts, as determined by their ability to translate the yeast *CAN1* mRNA fused in frame to a luciferase reporter. Toeprint reactions with these extracts were able to

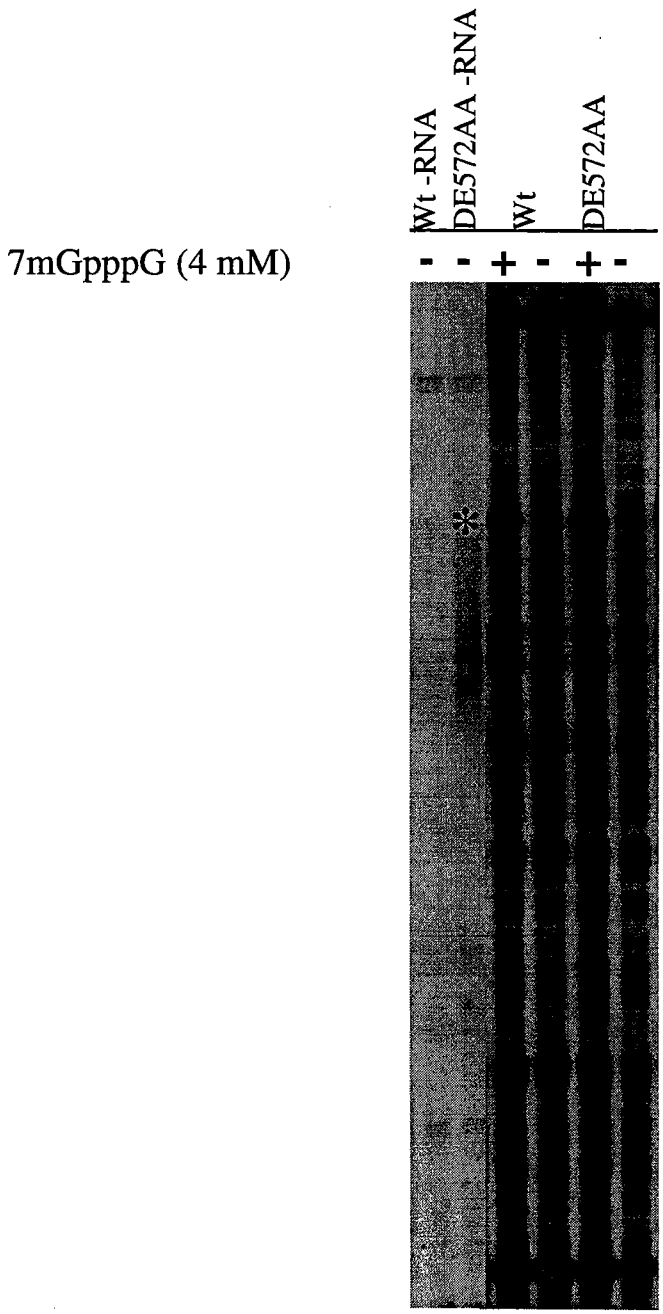
recognize ribosomes localized to the initiator codon of a CAN-LUC-UAA mRNA containing a pre-mature termination codon, but were unable to detect a toeprint at the premature termination codon in this mRNA (Figure 15A).

To test whether I could detect a CAN-LUC-UAA toeprint, I performed toeprint experiments using a wild-type strain, grown in rich media to the recommended OD_{600} of 2.5-3.0. Toeprinting reactions performed with extract generated from these cells were able to detect both initiation and termination toeprints (Figure 15B). These experiments indicated that the assay was functional and that the growth rate of the cells had a significant effect on the ability to detect translation termination toeprints.

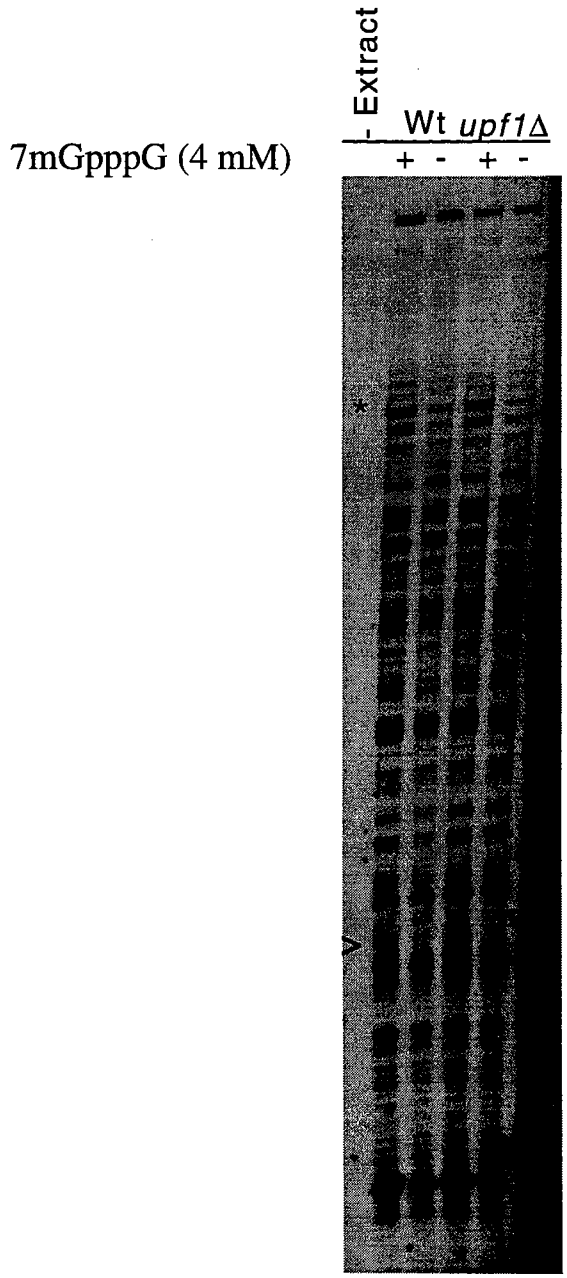
In an attempt to circumvent the need to grow cells in SC –ura raffinose medium, and thereby allow me to grow the cells in rich medium, I constructed a series of integration constructs in which expression of wild-type *UPF1* and its DE572AA mutant allele was driven by the *GAL1* promoter. These constructs were integrated into yeast strains and assayed for their ability to alter cellular growth rate when grown on galactose. Induction of either of these constructs was insufficient to alter the cell growth on glucose or galactose containing media. These experiments demonstrated the integration constructs were unable to induce the dominant-negative phenotype that I had previously observed. Collectively, these experiments, as well as those outlined above, indicated that performing successful toeprinting experiments with these strains was unlikely to occur.

Figure 15. Toeprint analyses of initiation and termination. Equal amounts (120 ng) of synthetic RNA transcripts were used to program 20-µl translation mixtures derived from *S. cerevisiae*. **(A)** *CAN-LUC-UAA* mRNA was incubated at 25 °C for 10 min in micrococcal nuclease-treated *S. cerevisiae* translation extracts from the yeast strain MBS-WT harboring either *GAL-UPF1-WT* or *GAL-UPF1-DE572AA* in SC –ura raffinose media that had subsequently induced with galactose for 4h. Reaction mixtures were supplemented with CHX after 10 min of incubation at 25 °C. AUG toeprints are indicated by a (*). **(B)** *CAN-LUC-UAA* mRNA was incubated at 25 °C for 10 min in micrococcal nuclease-treated *S. cerevisiae* translation extracts from the yeast strain MBS-WT or MBS-*upf1Δ* grown to and $O.D._{600}$ 2.0 in YPD. AUG toeprints are indicated by the presence of the (*), termination toeprints are indicated by a (>).

A.



B.



DISCUSSION

Overexpression of mutated alleles of *UPF1*, result in cellular growth defects

To examine the consequences of overexpression of mutations in *UPF1*, I constructed galactose-inducible, high-copy plasmids that carried various mutations in *UPF1*. Overexpression of particular alleles of *UPF1* resulted in significant alterations in cellular growth in both plate and liquid culture assays. Specifically, overexpression of *UPF1* mutants harboring mutations in the ATP binding and ATP hyrdroylis motifs were able alter cellular growth rates (see Figure 11), with long-term expression resulting in significant effects on cell viability (see Figure 10). The specific phenotype observed from overexpression of these alleles was not directly attributable to differences in the cellular abundance of the various mutant proteins, as all the mutants analyzed expressed Upf1p to comparable levels, as determined by western blotting (Figure 9B).

Overexpression of mutant forms of Upf1p that are deficient in ATP binding, ATP hydrolysis, or RNA binding affect NMD

Since wild-type Upf1p is known to function in the process of nonsense-mediated mRNA decay, I examined the consequences of overexpressing alleles of Upf1p in an otherwise wild-type cell background (Leeds et al., 1991,1992). Overexpression of alleles harboring mutations in the ATP binding, ATP hydrolysis, or RNA binding domains all altered the efficiency of NMD, as detected by a 4-fold change in *CYH2* pre-mRNA abundance (Figure 12). These results have interesting repercussions when viewed in the light of the cell growth experiments. The fact that all three mutants alter NMD function to a similar degree, but only the ATP binding and hydrolysis mutants affect cell viability indicates that the observed effects on viablity are not exerted via the NMD pathway.

Overexpression of *upf1*-DE572AA may affect translation

Given the well-documented link between NMD and translation (Jacobson and Peltz, 1996,2000), I analyzed sucrose density gradients of cell extracts generated from cells overexpressing wild-type *UPF1* and cells overexpressing *upf1*-DE572AA, to determine if the growth defects previously detected were a result of a *upf1*-DE572AA effect on translation. Overexpression of the ATP hydrolysis mutant in galactose caused irregularities its polyribosome profiles, when compared to cells overexpressing wild-type *UPF1* (Figure 13). These changes in the polyribosome profiles suggest that overexpression of the ATP hydrolysis mutant, directly or indirectly, alters the proper function of translation.

Further analysis of this apparent affect on translation by biochemical and genetic techniques was not fruitful. Analysis of ribosomal rRNA, isolated from fractions

obtained from sucrose gradient analysis of strains overexpressing wild-type or mutant *UPF1*, did not detect any significant change in the abundance or character of rRNAs (see Figure 14). Attempts at characterizing this defect by primer extension inhibition were also unsuccessful, given technical contraints of the assay. Furthermore, overexpression of the peptidly release factors eRF1 and eRF3, which are known Upf1p interactors, was unsuccessfull in relieving the dominant-negative growth defect observed in strains overexpressing the DE572AA allele.

Impact of *UPF1* overexpression analysis on current theories of its function

Previous studies characterizing the biological and biochemical activity of mutations in *UPF1* yielded many interesting results. These studies found that mutations within highly conserved motifs of *UPF1*, allowed one to separate Upf1p's function in NMD and termination codon recognition. Specifically, mRNA stability and suppression analysis found that mutation of the ATP hydrolysis region of Upf1p (ie. upf1-DE572AA), resulted in a protein that was able to properly recognize premature terminations, but was unable to function in NMD. These results suggested that ATP binding was a critical event in nonsense-codon recognition, but ATP hydrolysis is functionally dispensible for this event (Weng et al., 1996b).

The conclusions of Weng et al. (1996a,b), are called into question by my overexpression studies with their *UPF1* mutants. As mentioned earlier, overexpression of the ATP binding/hydrolysis mutants, or the RNA binding mutant, results in similar

effects on NMD. This observation correlates well with the previous studies conducted by Weng and colleagues (1996a,b). The cellular growth defects, and nonsense-mRNA stability levels observed upon overexpression of *upf1*-K436E and *upf1*-DE572AA were identical. These results suggested that it might be prudent to revisit Weng and co-workers' interpretations of their nonsense-suppression data. Specifically, nonsense-suppression was measured by the ability of strains harboring nonsense mutations in *LEU2* and *TYR7* to grow on plates lacking these essential amino acids. Theoretically, if nonsense-suppression were occurring in these strains, cells would be able to read-through the premature termination codon and produce some functional full-length protein which would allow the synthesis of the critical amino acid. The studies of Weng et al (1996a,b) found that the DE572AA mutant expressed on a low-copy centromeric vector resulted in suppression of nonsense mutations in *leu2* and *tyr7* when grown on plates lacking these amino acids, but not when expressed on a high-copy episomal plasmid (Weng et al., 1996a). The authors suggest that the DE572AA mutant is only a modestly defective version of the wild-type protein and that high-level expression must result in translational fidelity identical to that of wild-type Upf1p. This observation is challenged by my overexpression studies with the DE572AA mutant. As previously mentioned, galactose-induced overexpression of the DE572AA mutant resulted in severe growth defect in an otherwise wild-type strain. Since Weng and co-workers assayed translational fidelity by the inability of a strain to grow, they could not distinguish proper function in translation fidelity from a growth defect caused by overexpression of a dominant allele of Upf1p. To clarify this discrepancy, it will be necessary to perform these translational fidelity

assays in a manner that determines nonsense suppression by a loss of function similar to that developed by Maderazo et al (2000). Unfortunately, this assay is not readily adaptable to the analysis of the overexpression mutants that I have characterized since overexpression of the DE572AA allele results in significant growth effects, therefore it would be difficult to determine whether growth effects observed by this assay are due to overexpression of the DE572AA allele or due to alteration of translational fidelity. Further characterization of the function of these mutants in translational fidelity will require an assay that does not utilize cell viablity as an indictor for tranaslational fidelity.

An additional area of future research should involve further characterization of the overexpression phenotypes of the *upf1*-K436E and *upf1*-DE572AA mutants. Since the translational defects detected were subtle, but the growth defects measured in these strains were significant, a high-copy suppressor screen for genes capable of restoring wild-type growth to these strains seems a likely course of action. Analysis of genes obtained in such studies could provide insight into the mechanism of dominant-negative growth inhibition, and shed further light on the cellular role of wild-type Upf1p. Although the *upf1*-K436 and *upf1*-DE572AA mutants have been phenotypically similar to this point, such a screen in tandem may reveal subtle differences in their defects.

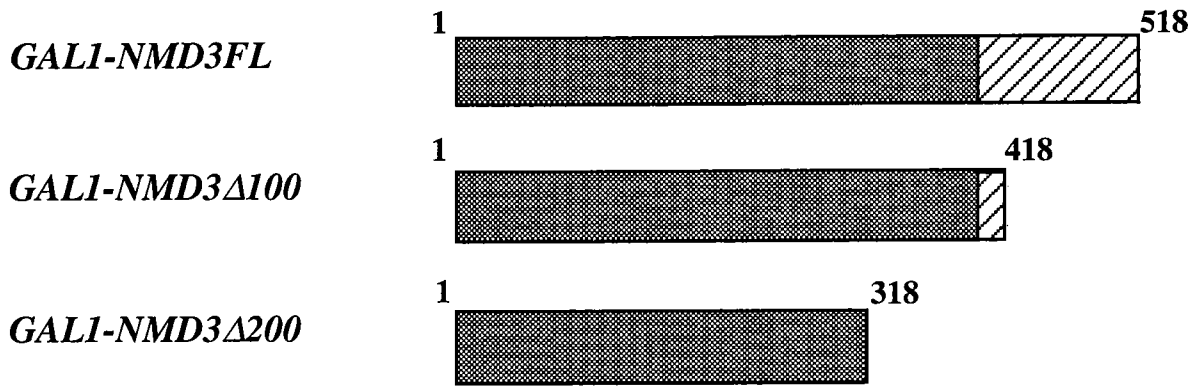
and Jacobson, 1995; Eisinger et al., 1997), we examined the consequences of overexpression of *NMD3* constructs that lacked the C-terminal Upf1p-interacting domain. A unique restriction site was inserted immediately downstream of the *NMD3* stop codon and used to generate C-terminal truncations, which resulted in the deletion of 100 or 200 amino acids from Nmd3p. The resulting constructs, as well as full-length (FL) *NMD3*, were cloned into pRS316 under the control of the inducible *GAL1* promoter (Figure 16A) and transformed into W303 haploid cells, and assayed for the ability to grow on media containing glucose or galactose. On galactose-containing medium, cells harboring the pRS316-*GAL-nmd3Δ100* plasmid were unable to grow, whereas strains harboring the pRS316-*GAL-NMD3FL,* the pRS316-*GAL-nmd3Δ200*, or the pRS316 control plasmid were viable (Figure 16B). Since all four strains grew with comparable efficiency on glucose-containing medium, and since all strains contain an intact endogenous *NMD3* gene, high level expression of the *nmd3* allele lacking 100 C-terminal codons must inhibit cell growth in a dominant-negative manner. The absence of a dominant-negative phenotype in cells expressing the pRS316-*GAL-nmd3Δ200* construct suggests that the Nmd3p domain responsible for growth inhibition resides within amino acids 318-418 or that the presence of this segment of the protein allows the formation of a specific inhbitory structure.

The dominant-negative effect of Nmd3p 100 expression is targeted to mRNA translation

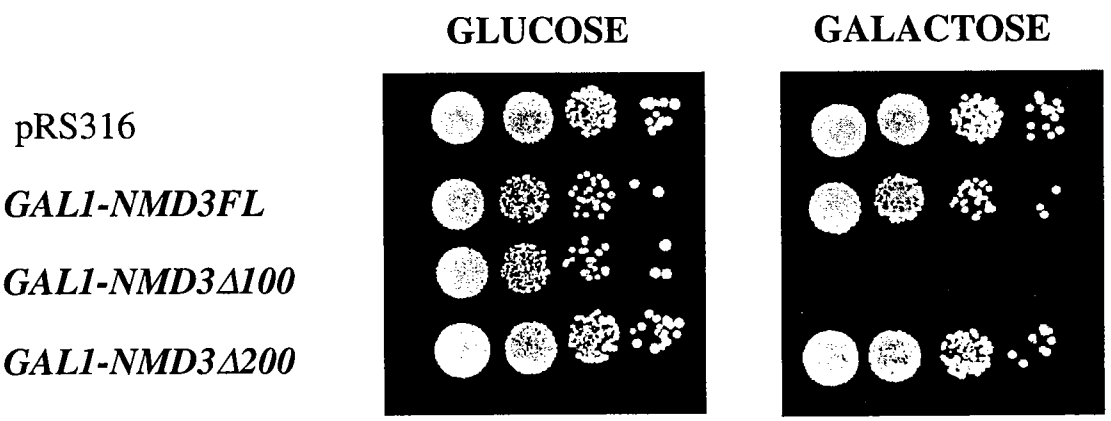
Given the well-documented links between nonsense-mediated mRNA decay and translation (Jacobson and Peltz, 1996,2000), we investigated whether the lethal

Figure 16. A truncation allele of the *NMD3* gene exhibits a dominant-negative growth phenotype. **(A)** Schematic of *NMD3* coding region segments placed under the control of the *GAL1* promoter and cloned into pRS316. The hatched area indicates the Upf1p interacting region of Nmd3p, defined by its recovery in a two-hybrid screen. **(B)** Induction of the *nmd3Δ100* allele inhibits cell growth. The constructs shown in **A** and the empty vector pRS316 were transformed into HFY121, which contains a wild-type copy of the *NMD3* gene. Individual transformants were selected and then serially diluted onto SC –ura plates with either glucose or galactose as the sole carbon source.

A.

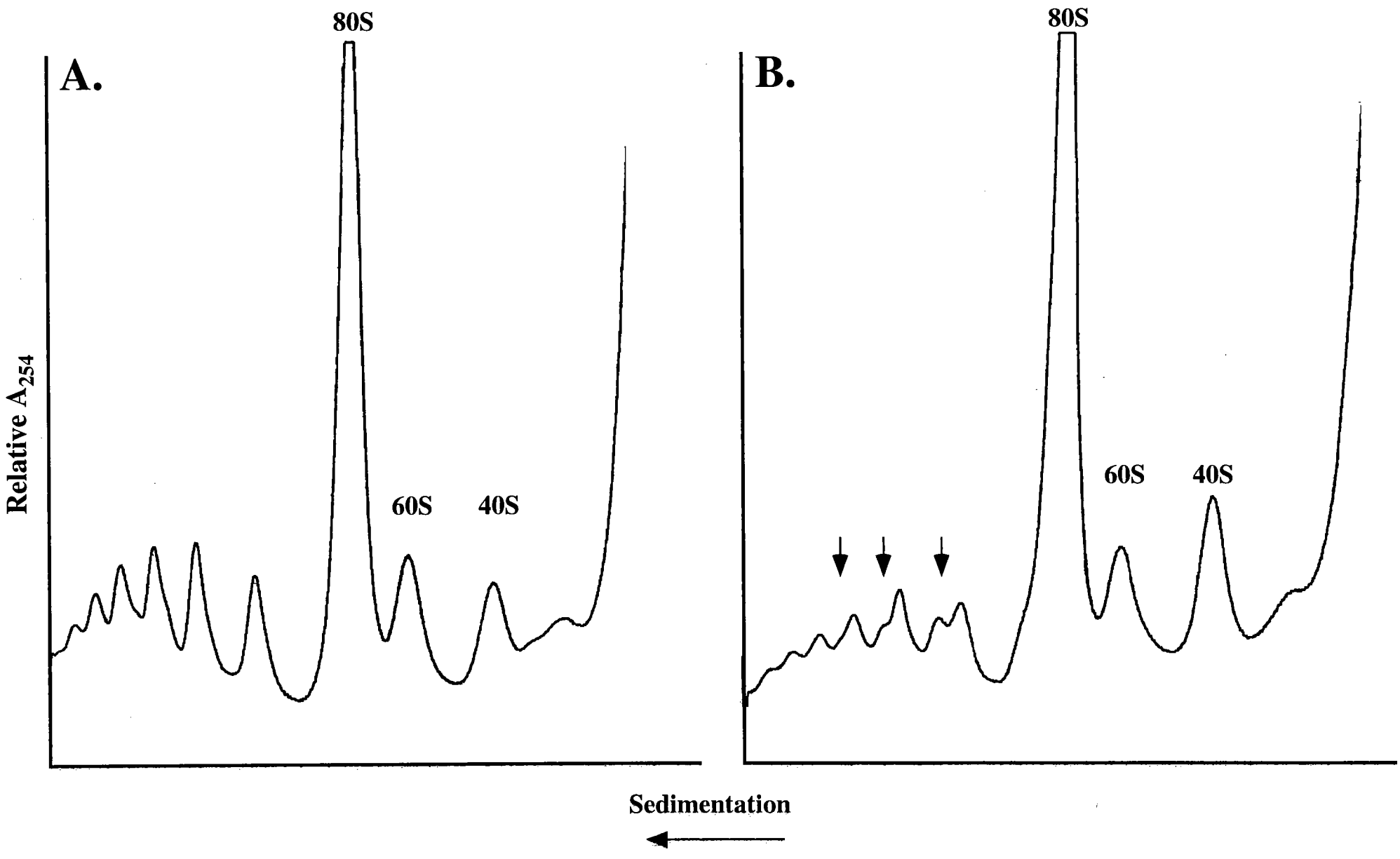

GAL1-NMD3FL
1
518
GAL1-NMD3Δ100
1
418
GAL1-NMD3Δ200
1
318

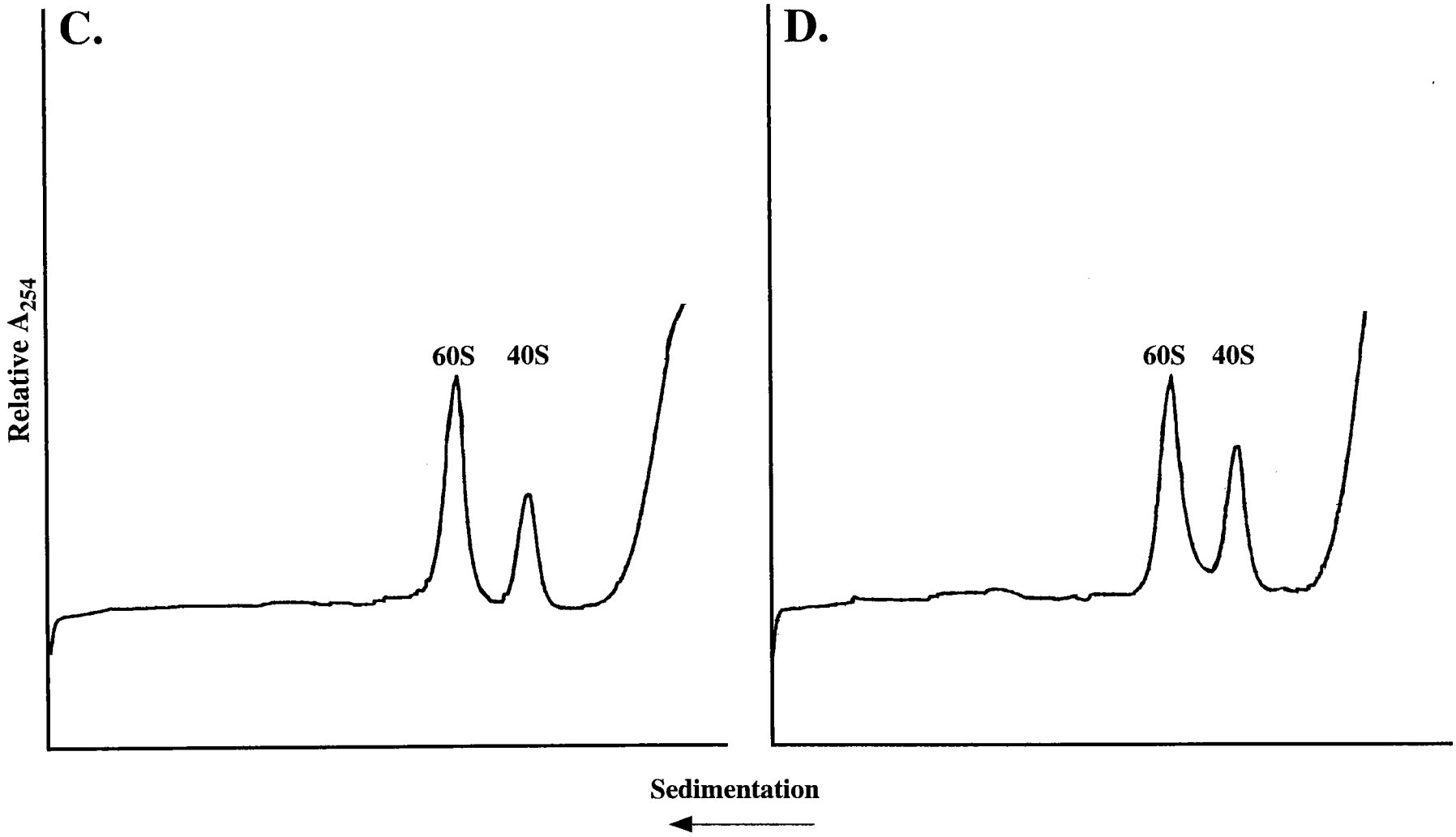
B.


GLUCOSE
GALACTOSE
pRS316
GAL1-NMD3FL
GAL1-NMD3Δ100
GAL1-NMD3Δ200

phenotype exhibited by cells expressing the pRS316-*GAL-nmd3Δ100* plasmid was a consequence of the inhibition of protein synthesis. First, sucrose gradient analysis was used to determine whether expression of truncated Nmd3p caused any specific alterations in cellular polyribosome profiles. Cells containing pRS316-*GAL-NMD3FL* and pRS316-*GAL-nmd3Δ100* were grown in medium containing raffinose as a carbon source and subsequently induced for two hours in galactose-containing medium. Cytoplasmic extracts were prepared and fractionated on sucrose gradients. Figures 17A and B show that cells harboring the plasmid expressing full-length Nmd3p displayed a wild-type polyribosome profile after two hours of galactose induction, while cells expressing the truncated protein exhibited a profile indicative of a translation defect. More specifically, polyribosome peaks from the latter cells all showed discontinuities characteristic of half-mer formation, i.e., polyribosomes which lacked stoichiometric amounts of both ribosomal subunits (Helser et al., 1981; Moritz et al., 1991; Rotenberg et al., 1988). This effect was a specific consequence of overexpression of Nmd3pΔ100 since galactose-grown cells that harbored the plasmids pRS316-*GAL-NMD3FL* or pRS316-*GAL-nmd3Δ200*, and glucose grown cells that harbored the plasmids pRS316-*GAL-NMD3FL,* pRS316-*GAL-nmd3Δ100,* or pRS316-*GAL-nmd Δ200,* all displayed wild-type polyribosome profiles (data not shown). The inhibitory activity of Nmd3pΔ100 was: a) not due to preferential stability of this form of the protein since western blotting experiments showed that the *Δ100*, *Δ200*, and *FL* forms all reached comparable levels in the same amount of time (data not shown) and b) a direct consequence of the extent of induction of Nmd3pΔ100 since polyribosome analysis of cells in which galactose

Figure 17.Induction of pRS316-*GAL-nmd3Δ100* promotes the formation of polyribosome half-mers. Cytoplasmic extracts were prepared from cells after 2 h of galactose induction. Extracts were fractionated on 7-47% sucrose gradients lacking (**A** and **B**) or containing (**C** and **D**) 0.7 M NaCl. The OD traces of the ribosome profiles are shown, with half-mer polyribosome peaks indicated by arrows and the peaks of the 40S subunits, 60S subunits, and 80S monosomes labeled as such. **A** and **C**: Cells harboring pRS316-*GAL-NMD3FL*. **B** and **D**: Cells harboring pRS316-*GAL-nmd3Δ100*.


A.
80S
60S
40S
B.
80S
60S
40S
Relative A_{254}
Sedimentation

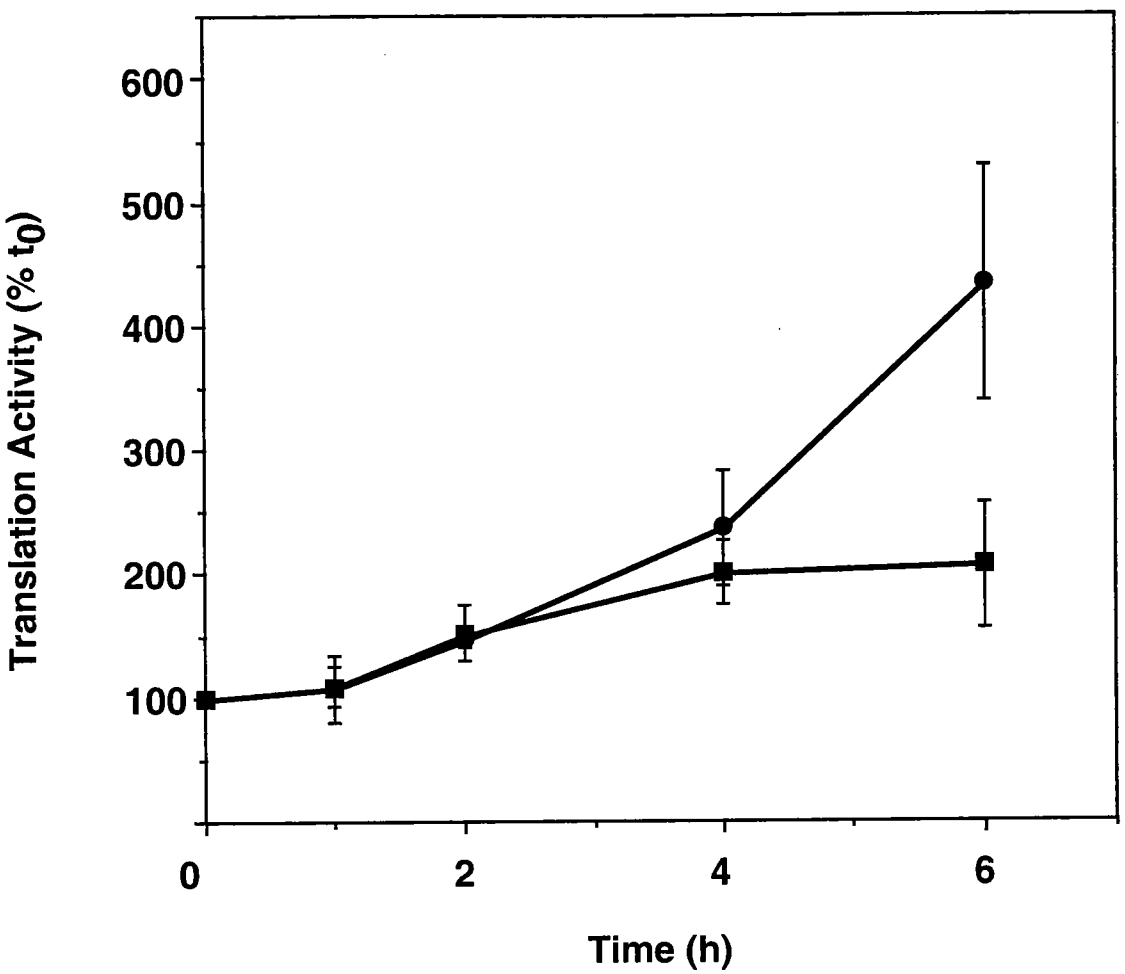

C.
D.
Relative A_{254}
60S
40S
60S
40S
Sedimentation

induction of pRS316-*GAL-nmd3 100* was continued to 4 or 6 h exhibited higher levels of half-mers (data not shown).

To confirm the lack of ribosome subunit stoichiomtery detected when Nmd3pΔ100 was overexpressed, we used high salt sucrose gradients to evaluate the relative levels of 60S and 40S subunits. Wild-type and truncated Nmd3p were induced as in Figures 17A and B, but extracts and sucrose gradients contained 0.7M NaCl to disrupt polyribosomes and monosomes into individual ribosomal subunits. Analysis of the relative amounts of 60S to 40S ribosomal subunits revealed a shift from a ratio of 2.15 in the strain expressing pRS316-*GAL-NMD3FL* to a ratio of 1.59 in the strain expressing pRS316-*GAL-nmd3Δ100* (Figs. 17C and D). This analysis thus confirmed the disruption of normal 60S/40S stoichiometry suggested by the formation of half-mers in Figure 17B.

To investigate the apparent translation defect in a more quantitative manner, we measured the ability of cells to incorporate ^{35}S-labeled amino acids after galactose induction of the full-length and *nmd3Δ100* alleles. Figure 18 shows that, for the first two hours after induction, cells expressing either construct had comparable abilities to incorporate labeled amino acids. Pulse-labeling of cells at later times indicated that the truncated Nmd3p inhibited protein synthesis, i.e., by 6 h post-induction, cells expressing pRS316-*GAL-nmd3Δ100* had only 50% of the incorporation activity of cells expressing pRS316-*GAL-NMD3FL*.

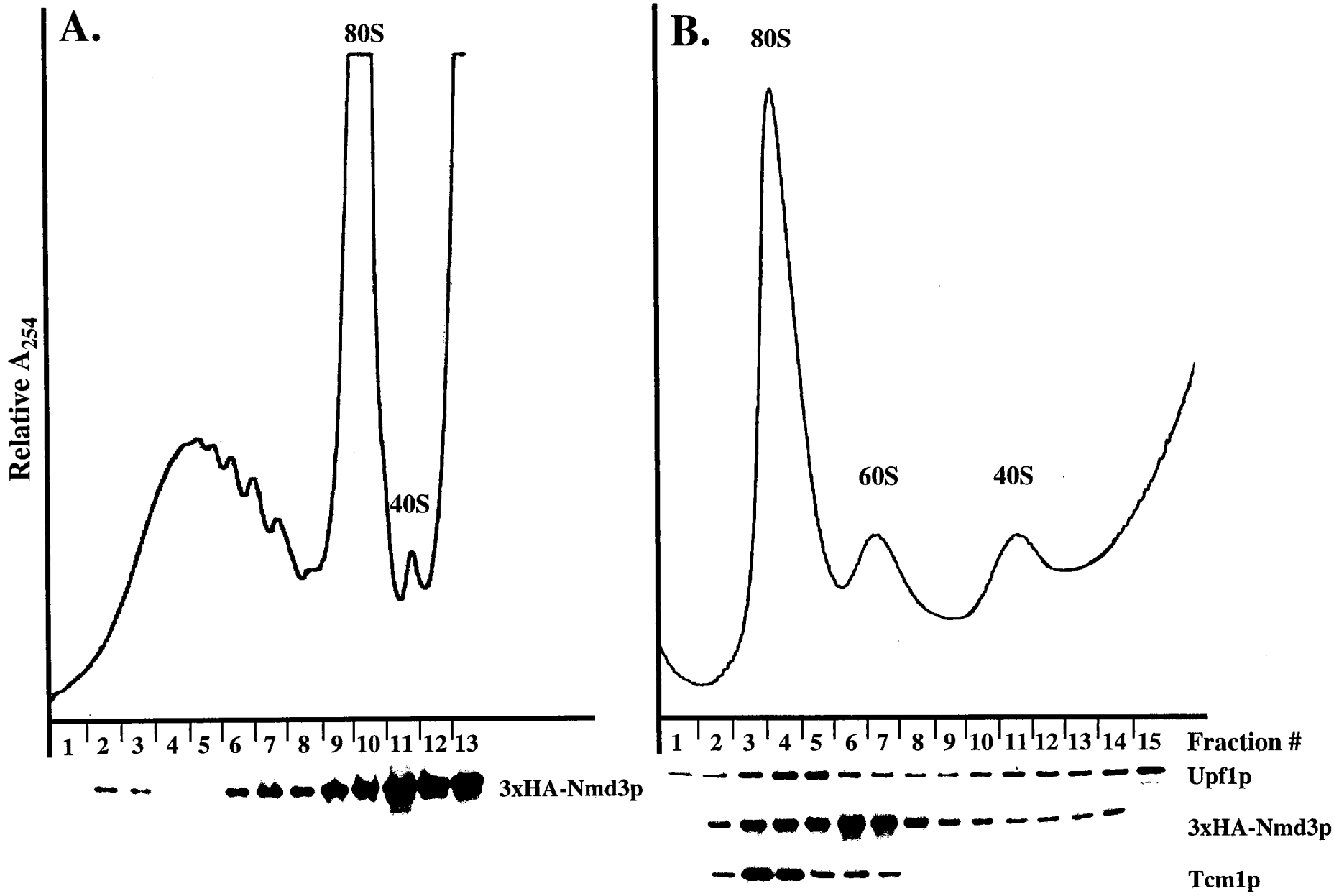
Figure 18. Amino acid incoporation in cells harboring pRS316-*GAL-NMD3FL* or pRS316-*GAL-nmd3Δ100*. Cells were subjected to galactose induction for different lengths of time, and incorporation of ^{35}S-labeled amino acids was measured as described in Chapter 2. Data are expressed as the percentage of incorporation at t_0, and are averages of three separate experiments. (●) depict cells containing pRS316-*GAL-NMD3FL* and (■) depict cells with pRS316-*GAL-nmd3Δ100*.


Translation Activity (% t0)
600
500
400
300
200
100
0
2
4
6
Time (h)

Nmd3p co-fractionates with the 60S ribosomal subunit

The experiments of Figures 17 and 18 indicated that the dominant-negative effect of truncated Nmd3p was targeted to the translation apparatus, thus suggesting that wild-type Nmd3p had a role in protein synthesis. To test this possibility further, we sought to determine whether Nmd3p was normally associated with ribosomes. We constructed a plasmid-borne, HA-epitope-tagged allele of *NMD3* (pRS316-3xHA-*NMD3*; see Table 2) that was deemed functional by virtue of its ability to restore viability to an *nmd3::his3* disruption strain (data not shown). Cytoplasmic extracts from cells harboring this allele were fractionated on sucrose gradients and western blotting was used to identify the fractions containing HA-Nmd3p. These experiments indicated that HA-Nmd3p co-fractionated with polyribosomes and with either 60S or 40S ribosomal subunits (Fig. 19A). To localize HA-Nmd3p more specifically, higher resolution sucrose gradients were utilized. These experiments demonstrated that the majority of HA-Nmd3p co-sedimented with the 60S ribosomal subunit (Fig. 19B). Confirmation that the peak designated 60S was indeed the large ribosomal subunit was obtained by simultaneously monitoring the sedimentation of Tcm1p, the large ribosomal subunit protein L3. The majority of Tcm1p was shown to co-sediment with the 80s and 60s peaks of the sucrose gradient (Fig. 19B), thus validating the 60S assignment of Nmd3p. Interestingly, the principal ribosomal peak with which Upf1p was associated in this gradient was that of the 80S monosome. This

Figure 19. Nmd3p cofractionates with the 60S ribosomal subunit. Extracts from strain JBY001, a haploid *nmd3:HIS3* disruption strain containing a plasmid-borne 3XHA-*NMD3* allele, were fractionated on sucrose gradients that were subsequently analyzed by western blotting. In each panel, the top depicts the OD profile, with sedimentation proceeding form right to left and the 80S, 60S, and 40S peaks indicated. The bottom of each panel presents the results of western blotting analyses of the gradient fractions. Panels were serially stripped and rebound with the antibodies indicated to the right of the blot. **(A)** 15-50% sucrose gradient. **(B)** 7-47% sucrose gradient.


A.
80S
40S
Relative A_{254}
1 2 3 4 5 6 7 8 9 10 11 12 13
3xHA-Nmd3p
B.
80S
60S
40S
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Fraction #
Upf1p
3xHA-Nmd3p
Tcm1p

suggests that Upf1p interaction with Nmd3p bound to the 60S subunit may be dependent on 40S/60S joining.

Overexpression of Nmd3p 100 increases the abundance and decreases the stability of a subset of ribosomal protein mRNAs

Since Nmd3p was identified as a two-hybrid interactor with Upf1p, we sought to determine whether cells expressing pRS316-*GAL-nmd3Δ100* would stabilize nonsense-containing mRNAs. RNA was isolated from cells harboring pRS316, pRS316-*GAL-nmd3Δ100*, pRS316-*GAL-nmd3Δ200*, or pRS316-*GAL-NMD3FL* at different times after galactose induction and analyzed by northern blotting for the relative levels of the *CYH2* mRNA and pre-mRNA. These transcripts were chosen for analysis because the *CYH2* pre-mRNA is an endogenous substrate of the nonsense-mediated mRNA decay pathway and its abundance is a direct indicator of the degree of inhibition of the pathway (He et al., 1993; He and Jacobson, 1995; He et al., 1996, 1997). The results of these experiments paralleled those analyzing translational inhibition, i.e., the abundance of the *CYH2* transcripts was altered only in those cells in which pRS316-*GAL-nmd3Δ100* had been induced for 2-6 h (Figure 20). In galactose-induced cells containing the other plasmids, the levels of the *CYH2* transcripts diminished versus time of induction, such that by 6 h post-induction the abundance of the *CYH2* mRNA and pre-mRNA had decreased 10-15-fold and 2-3-fold, respectively (Figure 20 and Table 3). In contrast, cells expressing pRS316-*GAL-nmd3Δ100* maintained t_0 levels of the *CYH2* mRNA and increased the level of the pre-mRNA approximately 3-fold (Figure 20 and Table 3). Analyses of *STE2*

Figure 20. Overexpression of pRS316-*GAL-nmd3Δ* increases the abundance of *CYH2* transcripts. Cells harboring pRS316 with no insert or with the indicated inserts, were grown in SC raffinose media lacking uracil to an OD_{600} of 0.4 and then shifted to media containing 2% galactose. Samples were taken at 2 h intervals for RNA isolation and northern blot analysis (using 20μg of RNA per lane). The blots were hybridized to a radiolabeled *CYH2* probe, exposed, and then sequentially stripped and reprobed with *STE2* and *SCR1* DNAs, the latter as a loading control.

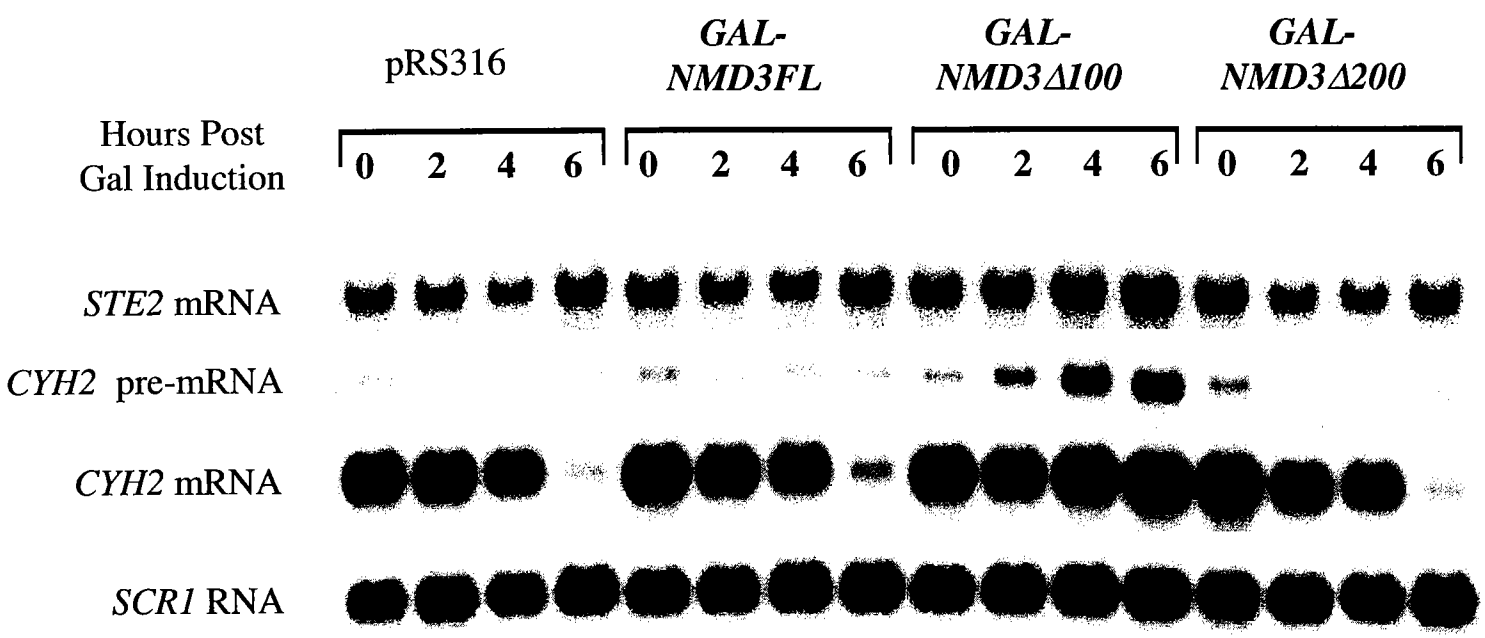

pRS316
GAL-NMD3FL
GAL-NMD3Δ100
GAL-NMD3Δ200
Hours Post Gal Induction
0 2 4 6 0 2 4 6 0 2 4 6 0 2 4 6
STE2 mRNA
CYH2 pre-mRNA
CYH2 mRNA
SCR1 RNA

Table 3. Relative levels of mRNAs after galactose induction

Plasmid/induction (h)	*CYH2*mRNA	*CYH2* pre-mRNA	*STE2* mRNA	*can1-100* mRNA	*TCM1* mRNA	*RP51a* mRNA
pRS316						
0	100	100	100	ND[a]	ND	ND
2	77	54	77	ND	ND	ND
4	56	43	68	ND	ND	ND
6	6	34	93	ND	ND	ND
pRS316-*GAL-NMD3FL*						
0	100	100	100	100	100	100
2	66	57	70	94	111	92
4	38	51	66	77	62	59
6	8	49	77	76	51	48
pRS316-*GAL-nmd3Δ100*						
0	100	100	100	100	100	100
2	64	140	99	47	55	77
4	100	274	117	53	52	104
6	99	287	128	52	52	103

Levels of all transcripts were determined by northern blotting, corrected for loading using *SCR1*, and normalized to the respective t_o values.

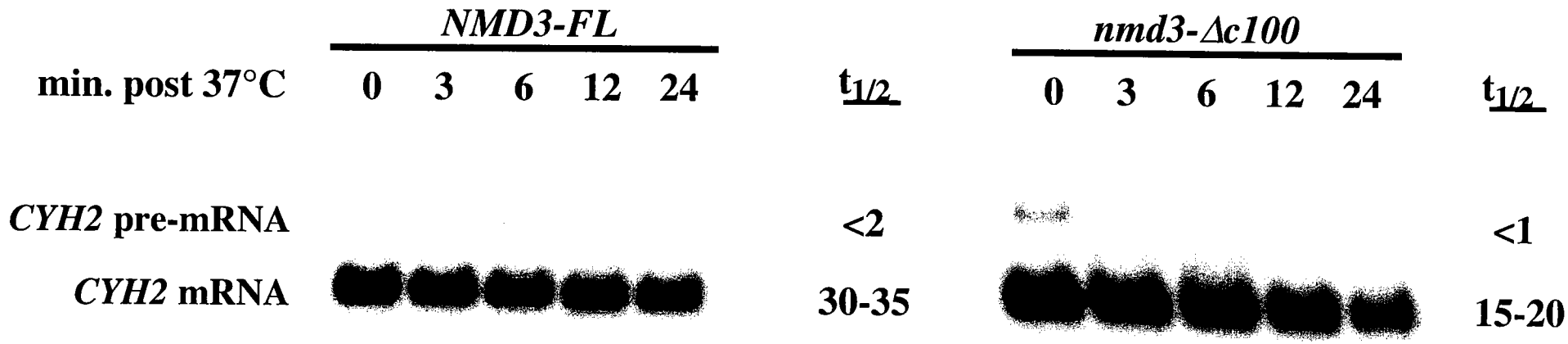
[a]ND: Value not determined

mRNA abundance in the same set of cells indicated that these effects did not apply to all classes of mRNA (Figure 20 and Table 3). To better understand the effect of expressing Nmd3pΔ100, additional mRNAs were also analyzed, with the results of these analysis found in Table 3. To test whether the increased abundance of the *CYH2* pre-mRNA was an effect on the nonsense-mediated mRNA decay pathway, or a general stabilization of a subset of normal mRNAs, we measured the abundance of the *can1-100* mRNA in cells expressing either pRS316-*GAL-NMD3FL* or pRS316-*GAL-nmd3Δ100*. The *can1-100* mRNA contains a premature UAA codon and is normally a substrate for the nonsense-mediated mRNA decay pathway; therefore, it should be stabilized if the dominant-negative effect was specifically targeted to nonsense-containing substrates (Maderazo et al., 2000). However, steady-state levels of *can1-100* mRNA were not increased in cells expressing the truncated form of *NMD3*, indicating that the increased abundance detected for *CYH2* pre-mRNA was not due to inhibition of nonsense-mediated mRNA decay (Table 3). Given that overexpression of Nmd3pΔ100 resulted in impaired translation, increased abundance of *CYH2* pre-mRNA and mRNA, and the fact Nmd3p co-sedimented with the 60S fractions on sucrose gradients, we reasoned that Nmd3pΔ100 might be affecting the stability of other ribosomal protein mRNAs. To test this possibility, we analyzed the steady-state levels of the *TCM1* and *RP51a* ribosomal protein mRNAs. In cells expressing Nmd3pΔ100, *RP51a* mRNA decreased steadily as a function of time after galactose induction such that, by 6h post-galactose induction, cells contained only 48% of their t_0 amount of the mRNA (Table 3). In contrast, cells expressing Nmd3pΔ100 maintained almost identical levels of *RP51a* mRNA at all time points after galactose induction (Table 3). Unlike the *RP51a* mRNA, the levels of the

TCM1 mRNA were similar in cells expressing either Nmd3p or Nmd3pΔ100 (Table 3), indicating that the increased abundance conferred by expression of Nmd3pΔ100 is specific only to a subset of ribosomal protein mRNAs.

The differences in *CYH2* and *RP51a* mRNA levels brought on by expression of Nmd3pΔ100 could reflect increases in the synthesis or stability of these RNAs. To distinguish between these possibilities, half-lives of the *CYH2* mRNA and pre-mRNA were determined in cells expressing either pRS316-*GAL-nmd3Δ100* or pRS316-*GAL-NMD3FL*. These plasmids were transformed into yRP582, a yeast strain harboring a temperature-sensitive allele of RNA polymerase II. Control experiments indicated that it was necessary to induce these cells with galactose for 10 h to obtain changes in mRNA abundance comparable to those obtained in the experiments of Figure 20 (data not shown). Cells treated in this way were then shifted to 37°C and mRNA decay rates determined by northern blot analysis of RNA samples isolated at different times after the temperature-shift. These experiments demonstrated that expression of pRS316-*GAL-nmd3Δ100* reduced the half-lives of the *CYH2* pre-mRNA and mRNA approximately 2-fold (Figure 21), but had no effect on the half-life of the *STE2* mRNA (data not shown). These results indicate that the elevated levels of *CYH2* transcripts were caused by changes in RNA synthesis and suggest that such increased synthesis may trigger feed-back mechanisms that regulate stability of the respective RNAs (see Discussion).

Figure 21. Overexpression of pRS316-*GAL-nmd3Δ100* decreases *CYH2* transcript stability. YRP582 cells harboring pRS316-*GAL-NMD3FL* or pRS316-*GAL-nmd3Δ100* were grown in SC galactose media lacking uracil to an OD_{600} of 0.6, shifted to 37°C, and aliquots of the cultures were removed at the indicated times. RNA was isolated from each sample and analyzed by Northern blotting using a radiolabeled *CYH2* probe.


NMD3-FL
nmd3-Δc100
min. post 37°C
0 3 6 12 24
t1/2
0 3 6 12 24
t1/2
CYH2 pre-mRNA
<2
<1
CYH2 mRNA
30-35
15-20

Overexpression of Nmd3p 100 disrupts normal rRNA processing

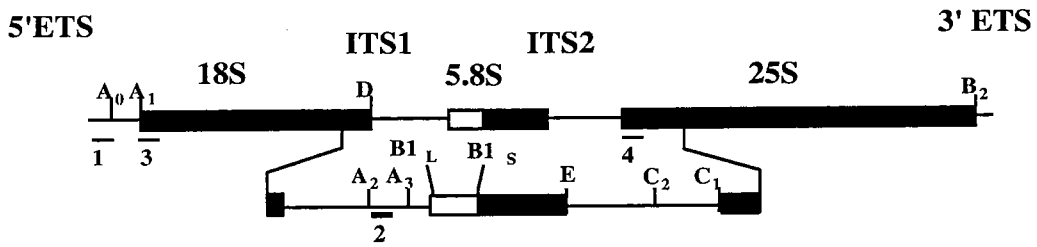
Since expression of pRS316-*GAL-nmd3Δ100* inhibited protein synthesis and altered the abundance of some ribosomal protein mRNAs, we hypothesized that these effects might be indirect conseqences of an Nmd3pΔ100 effect on rRNA processing. To determine whether overexpression of Nmd3pΔ100 affected rRNA processing, total RNA was isolated from cells harboring pRS316-*GAL-NMD3FL* or pRS316-*GAL-nmd3Δ100* at different times after galactose induction and analyzed by northern blotting.

The yeast 35S pre-rRNA is shown in Figure 22A, together with its principal processing sites and the location of sequences complementary to the oligonucleotide probes used in our analysis. Figure 22B illustrates the effects of pRS316-*GAL-nmd3Δ100* expression on the accumulation of mature 25S and 18S rRNA. At 6h post-galactose induction, a significant decrease in the abundance of mature 18S rRNA was detected in the *nmd3Δ100* strain, indicating that overexpression of Nmd3pΔ100 was indeed inhibiting normal rRNA processing. To better determine the point in the processing pathway at which Nmd3pΔ100 was acting, the membrane used in Figure 22B was stripped and then re-hybridized with a probe complementary to ITS1 (inteRNAl transcribed spacer region 1). Hybridization with the ITS1 oligonucleotide revealed that 35S pre-rRNA, as well an aberrant 24S processing intermediate, accumulate in cells expressing Nmd3pΔ100 (Fig. 22C). The 24S rRNA intermediate is known to accumulate when intial cleavages at 35S rRNA sites A_0, A_1, and D are inhibited and cleavage then occurs within ITS2, followed by 3' ---> 5' exonucleolytic digestion up to site E (Venema

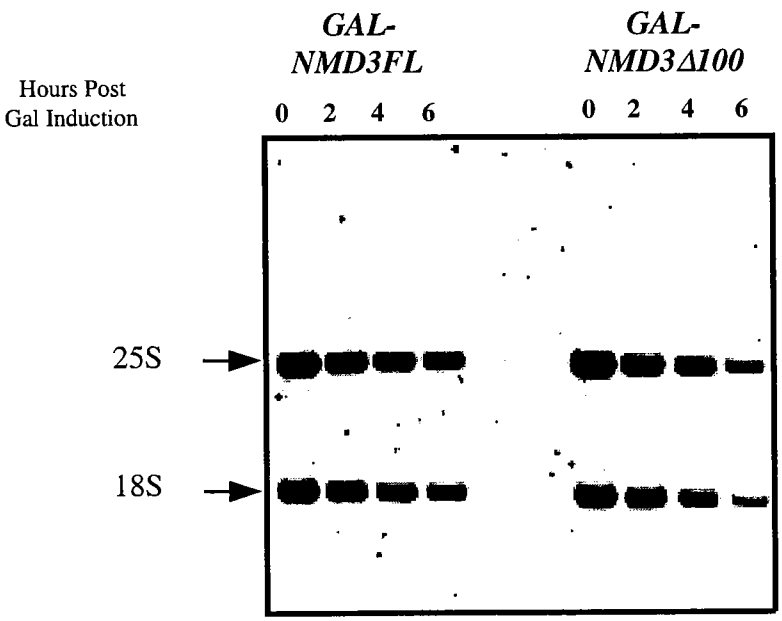
and Tollerey, 1996; Fig. 22A). To confirm that the processing intermediate detected in Figure 23C was indeed the 24S pre-rRNA, the blot was stripped and re-hybridized with a 5' ETS (exteRNAl transcribed sequence) probe that is complementary to sequences 5' to the A_0/A_1 region of the pre-rRNA (Fig. 22A). This blot showed that hybridization with either the 5' ETS or the ITS1 probe detected intermediates of the same size (Figure 22C and D), leading us to conclude that rRNA processing is altered in cells overexpressing Nmd3pΔ100.

Figure 22. Overexpression of pRS316-*GAL-nmd3Δ100* alters normal rRNA processing. **(A)** Schematic of the yeast 35S pre-rRNA and its principal processing sites and products (adapted from Venema & Tollervey, 1996). The locations of oligonucleotide probes used in this study (numbered 1-4) are indicated by black bars underneath the schematics. **(B)** Northern analysis of rRNAs. Total cellular RNA was isolated from JBY010 cells or JBY012 cells after 0-6 h of induction with galactose. Oligonucleotide 3 (5'-CATGGCTTAATCTTTGAGAC-3') and oligonucleotide 4 (5'-CTCCGCTTATTGATATGC-3'), complementary to sites within the mature 18S and 25S rRNA sequences, respectively, were used to probe the blot. **(C)** The blot analyzed in **B** was stripped and reprobed with oligonucleotide 2 (5'-TGTTACCTCTGGGCCC-3'), which hybridizes downstream of site A_2 in ITS1. **(D)** The blot analyzed in **C** was stripped and reprobed with oligonucleotide 1 (5'-TCGGGTCTCTCTGCTGC-3'), which is complementary to a portion of the 5' ETS.

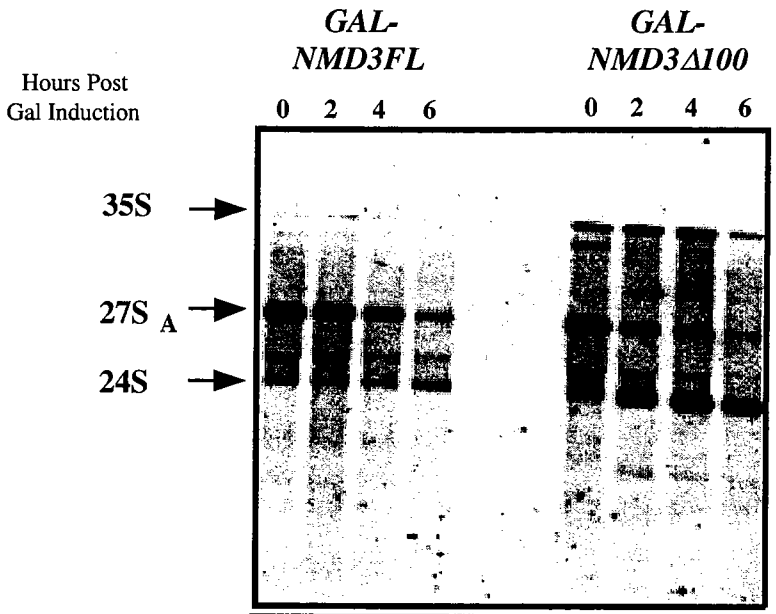
A.


5'ETS
ITS1
ITS2
3' ETS
18S
5.8S
25S
A0 A1
D
B2
1
3
B1L
B1S
4
A2 A3
E
C2
C1
2

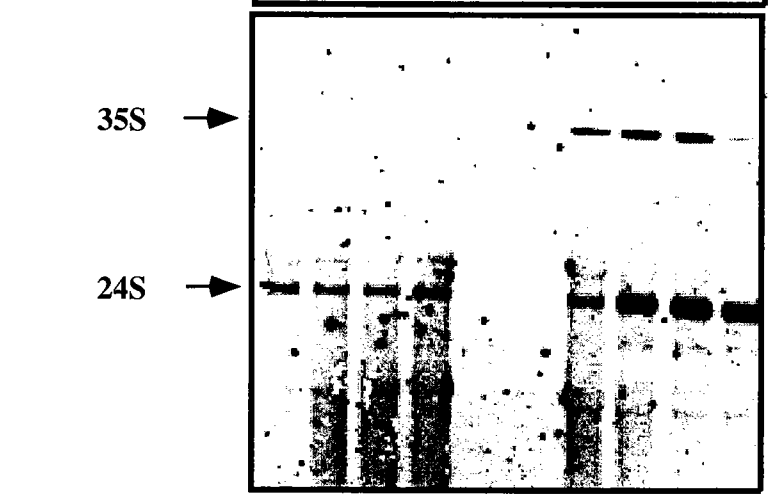
B.


GAL-NMD3FL
GAL-NMD3Δ100
Hours Post Gal Induction
0 2 4 6
0 2 4 6
25S
18S

C.


GAL-
NMD3FL
GAL-
NMD3Δ100
Hours Post
Gal Induction
0 2 4 6
0 2 4 6
35S
27S A
24S

D.


35S
24S

Discussion

Nonsense-mediated mRNA decay, the rapid turnover of mRNAs with premature translational termination codons, requires the activity of the *UPF1* gene in yeast, roundworms, and humans (Pulak and Anderson, 1993; Cali and Anderson, 1998; Leeds et al., 1991, 1992; Peltz et al., 1993a; Sun et al., 1998; Weng et al., 1996). To identify additional factors involved in yeast nonsense-mediated mRNA decay, we conducted a two-hybrid screen for Upf1p-interacting proteins (He and Jacobson, 1995). The screen identified six potential interactors, two of which, Nmd2p and Dbp2p, have been analyzed previously and shown to have important roles in the degradation of nonsense-containing mRNAs (He and Jacobson, 1995; He et al., 1996, 1997; Bond et al., 2001). In this paper, we have characterized *NMD3*, another gene identified in the screen for *UPF1*-interactors. *NMD3* is essential for viability (Fig. 16), has sequence motifs characteristic of zinc-finger proteins and ribosomal protein S13, and has well conserved homologs in *Caenorhabditis elegans, Drosophila melanogaster*, and *Homo sapiens*. The high degree of sequence conservation and the essential nature of the yeast *NMD3* gene, indicate that Nmd3p may have a vital function in numerous organisms.

To gain insight into the role of Nmd3p, we characterized a conditional *nmd3* allele. Overexpression of a truncated form of Nmd3p, lacking its C-terminal 100 amino acids and the majority of its Upf1p-interacting domain, was shown to have a dominant-negative effect on cell growth. High level expression of the *nmd3Δ100* allele also had

consequences for the protein synthesis apparatus and its function, causing an accumulation of half-mer polyribosomes (Figure 17), up to a 2-fold reduction in rates of amino acid incorporation (Figure 18), and alterations in the normal course of ribosomal RNA processing (Figure 23). These effects were not observed with overexpression of full-length Nmd3p or Nmd3p 200, suggesting that removal of amino acids 419-518 from Nmd3p creates novel interaction capabilities for the protein, which are abrogated by further removal of amino acids 319-418.

The detection of polyribosome half-mers, which are generally caused by the binding to mRNA of 40S subunits without concurrent binding of 60S subunits (Eisinger et al., 1997; Hesler et al., 1981; Moritz et al., 1991; Ohtake et al., 1995; Nelson and Winkler, 1987; van Venrooij et al., 1977), as well as the 60S subunit association of epitope-tagged Nmd3p (Figure 19), suggests that the novel interactions of truncated Nmd3p may occur with components of the 60S ribosomal subunit. Since the translation phenotypes caused by overexpression of Nmd3pΔ100 require the cell to pass through approximately two doublings before they are apparent, it is likely that newly synthesized ribosomes are preferentially affected. If truncated Nmd3p had an effect on existing ribosomal subunits, an earlier onset of the dominant-negative phenotypes would have been expected.

Further evidence for the association of Nmd3p with the 60S subunit comes from studies of the *GRC5/QSR1* gene. Eisinger et al. (1997) have shown that Grc5p/Qsr1p is an integral 60S ribosomal protein necessary for the proper joining of the 40S and 60S ribosomal subunits and that cells with a temperature-sensitive mutation in *GRC5/QSR1* display an aberrant polyribosome sedimentation profile comparable to that detected in

cells overexpressing the *nmd3Δ100* allele. In another study, we identified a *grc5/qsr1* allele in a screen of 4,000 temperature-sensitive mutants for strains that stabilized the *CYH2* pre-mRNA (Zuk and Jacobson, 1998; Zuk et al., 1999). The mutant strain stabilized inherently unstable mRNAs 2-3-fold, and both the temperature-sensitivity and mRNA decay phenotypes of this strain were suppressed by expression of a triple HA-tagged version of *NMD3* on a high copy plasmid.

The detection of *NMD3* in a two-hybrid screen in which the *UPF1* gene was used as bait raised the possibility that Nmd3p, like two other Upf1p-interactors, Nmd2p and Dbp2p, would have a role in nonsense-mediated mRNA decay. Although the level of the *CYH2* pre-mRNA increased in cells overexpressing truncated Nmd3p (Figure 21), the half-life of this transcript actually decreased in these cells (Figure 22). Moreover, the abundance of the *can1-100* nonsense-containing mRNA decreased in response to overexpression of truncated Nmd3p. Since the *CYH2* gene enodes ribosomal protein L28, these results suggested that the increased abundance of the *CYH2* pre-mRNA might be attributable to stimulation of the synthesis of components of the ribosome, rather than to an effect on a specific decay pathway.

The possibility that ribosome biogenesis might be altered in cells expressing Nmd3pΔ100 is consistent with the observed increases in the levels of two ribosomal protein mRNAs (*CYH2* mRNA and *RP51a* mRNA [encoding protein S17a]; Table 3), the increased accumulation of 35S pre-rRNA (Figure 23), and the alterations in amounts of rRNA processing intermediates (Figure 23). The increased abundance of the ribosomal protein mRNAs, as well as the decreased stability of the *CYH2* transcripts (Figure 22), suggests that the translation defect(s) brought on by overexpression of truncated Nmd3p

leads to destabilization of at least some ribosomal protein mRNAs. Work by Nam and Fried (1986) has shown that defects in 60S ribosomal subunit assembly can cause such destabilization. The enhanced abundance of some ribosomal protein mRNAs and the 35S pre-rRNA may indicate the existence of a feed-back loop that compensates for loss of ribosomes by increasing the transcription of the respective genes. The failure of the *TCM1* mRNA, encoding ribosomal protein L3, to also increase under these circumstances may be attributable differences in the factors responsible for the respective transcriptional regulatory events. In this regard, it is interesting to note that transcription of the *CYH2, RP51a,* and pre-rRNA genes is regulated by the positive acting factor Rap1p, whereas that of *TCM1* is regulated by Abf1p (Hodges et al., 1999; Mizuta et al., 1998; Moehle and Hinnebusch, 1991).

Collectively, the data presented here and elsewhere (Eisinger et al., 1997; Ho and Johnson, 1999; Zuk et al., 1999) are consistent with a role for Nmd3p in the formation, function, or maintenance of the 60S ribosomal subunit and suggest that overexpression of the *nmd3Δ100* allele may disrupt the formation of these subunits. Since Upf1p appears to be a regulator of translational termination (Czaplinski et al., 1998; Maderazo et al., 2000), this interpretation also suggests that Nmd3p may provide a link for Upf1p to the ribosome.

Addendum-

Recent advances in the study of Nmd3p cause me to re-evaluate my findings regarding the function of Nmd3p. Ho et al (2000) have used an epitope-tagged allele of Nmd3p to

immunoprecipiate free 60S ribosomal subunits, without the concurrent precipitation of 40S ribosomal subuints. This technique was then coupled with a pulse-chase labeling of ribosomal proteins to show that Nmd3p preferentially associated with nascent 60S ribosomal subunits. Additional immunoprecipation experiments in which the production of nascent ribosomal subunits was disrupted via the use of transcriptional inhibitors, or through a conditional translation initiation mutant (*prt1*), revealed that epitope-tagged Nmd3p was still able to immunoprecipitate the 60S ribosomal subunit. This result indicated that Nmd3p not only associates with nascent 60S ribosomal subunits but also interacts with cytoplasmic subunits.

Nmd3p's association with the 60S ribosomal subunit was elucidated further in independent studies performed by Ho et al.(2000) and Gadal et al. (2001). Both groups made use of a 60S ribosome export assay developed by Hurt et al.(1999). In this assay GFP is fused to a large ribosomal subunit protein (L25-GFP). Hurt and colleagues found that this fusion is incorporated into functional ribosomal subunits, and is therefore an effective tool for monitoring the location of the 60S subunit.

Ho and her collaborators generated a construct similar to that which I used in my original studies on Nmd3p, in which a contruct harboring a 100aa C-terminal truncation of Nmd3p was placed under the control of a galactose inducible promoter. This construct or a construct containing a full-length Nmd3p (as a control), were co-transformed into yeast. Expression of the Nmd3p constructs was then induced via the addition of galactose, and the localization of the L25-GFP construct was monitored by flouresent microscopy. Yeast harboring a galactose-induced wild-type copy of Nmd3p localized the L25-GFP construct in the cyctoplasm, whereas induction of the Nmd3pΔ100 led to an

accumualtion of the L25-GFP reporter in the nucleus (Ho et al., 2000). After surveying recent literature, Ho and colleagues found a motif in the C-terminal 50aa of Nmd3p that corresponded to a highly conerved leucine-rich NES. Export of proteins containing this particular NES motif is mediated through the activity of Crm1p/Xpo1p (Fornerod et al., 1997; Stade et al., 1997). Given this information, Ho et al (2000) investigated the consequences of inhibiting Crm1p/Xpo1p function, using leptomycin B in a leptomycin-sensitive yeast strain generated by Neville and Roshbash (1999). Ho et al (2000) found that export of Nmd3p is blocked after addition of leptomycin B. Since Ho et al (2000) believed that Nmd3p mediates export of the 60S subunit they assayed the localization of the L25-GFP reporter construct before and after leptomycin B addition. The L25-GFP reporter construct was found to accumulate in the nucleus after leptomycin B additon. This, coupled with the fact that the export defect of Nmd3pΔ100 can be alleviated by the addition of a heterologous NES to the truncated protein, led the authors to conclude that Nmd3p participates in the export of the 60S ribosomal subunit in a Crm1p/Xpo1p dependent manner.

Gadal et al (2001) identified a mutant allele of *NMD3* in a screen for temperature sensitive mutants of ribosomal export factors, which they termed *rix* mutants. In this screen, a bank of 900 temperature-sensitive mutants was screened for the inability to export a L25-GFP reporter. Interestingly, this screen also identified a gene which has a genetic relationship with *NMD3*, i.e., *GRC5/RPL10*, as a *rix* mutant (Gadal et al., 2001; Belk et al., 1999; Zuk et al., 1999; Karl et al., 1999). Temperature-sensitive mutations in *GRC5/RPL10* have been complemented by overexpression of HA-*NMD3* or via a dominant allele of *NMD3* (Zuk et al., 1999; Karl et al., 1999). Gadal et al (2001) found

that His-tagged Nmd3p co-purified with Rpl10p-GST on glutatione-sepharose beads, but did not purify with glutatione-sepharose beads in the presence of GSTp alone. This indicated a specific interaction between Rpl10p and Nmd3p, confirming the genetic interaction shown by Zuk et al (1999), and Karl et al (1999). Gadal and colleagues also identified two NES signals in the carboxy terminus of Nmd3p, and were also able to determine that Nmd3p shuttled between the nucleus and cytoplasm by flourescent microscopy of a GFP-tagged version of Nmd3p. Gadal et al (2001) also tested the ability of Nmd3p to shuttle as well as export the L25-GFP construct in an Xpo1p mutant. Gadal and co-workers were also able to reproduce the observation of Ho et al (2000), that export of Nmd3p, as well as that of the L25-GFP construct were dependent upon proper function of Xpo1p/Crm1p.

Taken together these recent advances in the study of Nmd3p, indicate a role for Nmd3p in the export of the 60S ribosomal subunit. This view is well supported by our earlier observations using Nmd3pΔ100. Overexpression of Nmd3pΔ100 led to a decrease in free 60 ribosomal subunits, as well as a half-mer polyribosome phenotype, indicative of a lack functional 60S ribosomal subunits. Furthermore, the transciptional increase in ribosmal RNA as well as ribosomal protein RNA which I detected suggests that the cell has a mechanism in place to "sense" the availability, or level of free ribosomal subunits and to upregulate the expression of these components when they are in rate-limiting numbers. Further study of the cellular mechanism that mediates this phenomenon is certainly warranted.

Curiously, studies by myself and others have been unable to determine Nmd3p's role, if any, in nonsense-mediated mRNA decay. Although Nmd3p has been shown to

interact with Upf1p via two-hybid assays(He et al., 1995), all mutant alleles tested have had no detectable effect on the abundance of nonsense-containing transcripts. An intriguing possibilitiy for the interaction between Nmd3p and Upf1p can be envisioned. It is possible that Nmd3p is required for the association of Upf1p with the ribosome. I initially tested this possibility by monitoring the sedimentation of Upf1p on sucrose gradients in cells which had been induced with Nmd3p or Nmd3pΔ100 for 2 hours. I was unable to detect any discernable difference between the distribution of Upf1p on these sucrose gradients. However, in light of the observation of Ho et al (2000), that GFP-Nmd3pΔ100 is retained in the nucleus, I would not predict a change in Upf1p's ability to associate with the ribosome, as Upf1p is predominantly cytoplasmic protein (Atkin et al., 1995,1997; Mangus and Jacobson, 1999) and the nuclear retention of Nmd3pΔ100 would therefore preclude any interaction with Upf1p. Nmd3p may aid in the association of Upf1p during export of the 60S from the nucleus. This role for Nmd3p in Upf1p function would favor a yeast nonsense decay model, in which NMD occurs primarily during the initial rounds of translation. A second possibility is that Nmd3p targets Upf1p to the 60S ribosomal subunit at termination. Work by Ho et al (2000) has shown that Nmd3p preferentially associates with nascent 60S ribosomal subunits, but that a significant portion of Nmd3p also is associated with free 60S subunits. The authors suggest that Nmd3p might play a role in ribosome recycling. If this were the case, Nmd3p could deliver Upf1p to a prematurely terminating ribosome so that Upf1p could help mediate termination and thereby recycling of the ribosome. Either of these possibilities are certainly plausible and merit further investigation.

Unfortunately, future study of the Upf1p/Nmd3p interaction does not promise to be easy. The Nmd3p/Upf1p interacting domain is comprised of the C-terminal 120 amino acids of Nmd3p, which also harbors two nuclear export signals and one nuclear localization signal. Therefore, any mutants that disrupt the Nmd3p/Upf1p interaction would also have to be assayed for the ability to export the 60S ribosomal subunit. It is also possible that the amino acids critical for nuclear import and export of Nmd3p are required for Upf1p interaction, as dissertation research by Alan Maderazo (2000) has shown that the residues of the putative NLS of Nmd2p are also critical for its role in NMD.

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