A Study on the Cellular Localization of Factors Involved in Yeast Nonsense-Mediated mRNA Decay and their Mechanisms of Control on Nonsense mRNA Translation: a Dissertation

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A STUDY ON THE CELLULAR LOCALIZATION
OF FACTORS INVOLVED IN YEAST NONSENSE-
MEDIATED mRNA DECAY AND THEIR
MECHANISMS OF CONTROL ON NONSENSE
mRNA TRANSLATION

A Dissertation Presented By
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ABSTRACT

Nonsense-mediated mRNA decay (NMD) is an important mRNA surveillance mechanism conserved in eukaryotes. This thesis explores several interesting aspects of the NMD pathway. One important aspect of NMD which is presently the subject of intense controversy is the subcellular localization of NMD. In one set of experiments, the decay kinetics of the ade2-1 and pgkl nonsense mRNAs (substrates for NMD) were investigated in response to activating the NMD pathway to determine if cytoplasmic nonsense mRNAs are immune to NMD in the yeast system. The results of these studies demonstrated that activation of NMD caused rapid and immediate degradation of both the ade2-1 and the early nonsense pgkl steady state mRNA populations. The half lives of the steady state mRNA populations for both ade2-1 and pgkl (early nonsense) were shortened from >30 minutes to approximately 7 minutes. This was not observed for pgkl mRNAs that contained a late nonsense codon demonstrating that activation of NMD specifically targeted the proper substrates in these experiments. Therefore, in yeast, nonsense mRNAs residing in the cytoplasm are susceptible to NMD. While these findings are consistent with NMD occurring in the cytoplasm, they do not completely rule out the possibility of a nuclear-associated decay mechanism.

To investigate the involvement of the nucleus in NMD, the putative nuclear targeting sequence identified in Nmd2p (one of the trans-acting factors essential for NMD) was characterized. Subcellular fractionation experiments demonstrated that the majority of Nmd2p localized to the cytoplasm with a small proportion detected in the
nucleus. Specific mutations in the putative nuclear localization signal (NLS) of Nmd2p were found to have adverse effects on the protein's decay function. These effects on decay function, however, could not be attributed to a failure in nuclear localization. Therefore, the residues that comprise the putative NLS of Nmd2p are important for decay function but do not appear to be required for targeting the protein to the nucleus. These results are in accordance with the findings above which implicate the cytoplasm as an important cellular compartment for NMD.

This thesis then investigates the regulatory roles of the trans-acting factors involved in NMD (Upf1p, Nmd2p, and Upf3p) using a novel quantitative assay for translational suppression, based on a nonsense allele of the CAN1 gene (can1-100). Deletion of UPFL, NMD2, or UPF3 stabilized the can1-100 transcript and promoted can1-100 nonsense suppression. Changes in mRNA levels were not the basis of suppression, however, since deletion of DCP1 or XRN1 or high-copy can1-100 expression in wild-type cells caused mRNA stabilization similar to that obtained in upf/nmd cells but did not result in comparable suppression. can1-100 suppression was highest in cells harboring a deletion of UPFL, and overexpression of UPFL in cells with individual or multiple upf/nmd mutations lowered the level of nonsense suppression without affecting the abundance of the can1-100 mRNA. These findings indicate that Nmd2p and Upf3p regulate Upf1p activity and that Upf1p plays a critical role in promoting termination fidelity that is independent of its role in regulating mRNA decay.
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CHAPTER 1

INTRODUCTION

It has long been established that messenger RNA degradation represents an important step in modulating gene expression. Because the cellular steady-state abundance of an mRNA is dependent on the rates of its synthesis and decay, modulating the mRNA decay rate directly impacts transcript levels that ultimately influence the protein population produced in the cell. As a result, factors that influence mRNA turnover represent one of the many critical mechanisms cells utilize to regulate their gene expression.

* cis-acting sequences affect mRNA stability *

mRNA stability is an essential component affecting the overall abundance of cellular transcripts. The stability of individual transcripts can vary immensely. mRNA half-lives in yeast, for example, range from as short 1 minute to as long as 60 minutes while in mammals, the variability is even greater, ranging from 15 minutes to 10 hours or more (Caponigro and Parker, 1996; Jacobson and Peltz, 1996). Although their decay rates are normally constant, there are a number of determinants and stimuli that can affect individual mRNA half-lives.

Through the use of chimeric mRNAs, sequence elements have been identified that can influence mRNA decay rate. These chimeric mRNAs are usually composed of
portions of transcripts that degrade at different rates (i.e. the chimera contains portions of a stable mRNA and portions of an unstable mRNA). Consequently, the half-life of the resulting chimeric transcript is interpreted to identify the important sequence elements affecting mRNA turnover. From this type of analysis it is conceivable that two types of sequence elements that affect mRNA turnover exist: those that promote rapid mRNA decay and those that stabilize transcripts.

The first class of specific sequences found to affect mRNA turnover are those that destabilize mRNAs, termed instability elements. A number of experiments have demonstrated that these cis-acting elements are localized not only in the coding region but also in the 5' and 3' untranslated regions (UTRs) of transcripts indicating that translation elongation through these elements is not an absolute requirement for them to stimulate decay (Parker and Jacobson, 1990; Heaton et al., 1992; Herrick and Jacobson, 1992; Muhrad and Parker, 1992; Caponigro et al., 1993; Pierrat et al., 1993). Currently, the mechanisms by which these instability elements act to promote destabilization is not known. It is a likely possibility that the mechanism triggering transcript degradation is dependent upon the location of that particular sequence element. For example, due to their proximity to the 5'-end of the mRNA, those elements residing in the 5'-UTR may influence the rate of decapping or they may directly affect the efficiency of translation initiation which, in turn, affects the efficiency with which the transcript is decapped and further degraded (Caponigro and Parker, 1996).

The instability elements in coding regions may involve a different mechanism that requires either translation elongation or parts of the translation machinery to stimulate
decay. Indeed, this link to translation was shown for the MATα1 instability element since translation elongation through the element was required for its activity (Parker and Jacobson, 1990). Furthermore, the observation that this element starts with a stretch of rare codons suggested that the mechanism for stimulating decay in this case may involve either ribosomal pausing and/or slowing of translation elongation (Parker and Jacobson, 1990; Caponigro et al., 1993).

It is clear that sequences in the 3'-UTR also dictate changes in mRNA turnover. While no real consensus sequence exists for this type of instability element, extensive study of 3'-UTRs, especially that of MFA2, has demonstrated that most of these elements consist of a repeated, AU-rich motif (Muhlrad and Parker, 1992; Zubiaga et al., 1995). Furthermore, the destabilizing activity of these 3'-sequences has been shown to be tolerant to a number of deletion and point mutations, suggesting redundancy of function (Muhlrad and Parker, 1992). While the exact mechanism of mRNA destabilization mediated by elements residing in the 3'-UTR is not known, studies have shown that these sequences cause enhanced rates of deadenylation and decapping which subsequently promote degradation (Muhlrad and Parker, 1992). By the nature of their location at the 3'-end, it is postulated that these sequences provide multiple binding sites for factors that stimulate deadenylation (Caponigro and Parker, 1996). Support for the existence of such factors includes studies in mammalian systems that identify a factor which specifically binds to AU-rich sequences in the c-myc mRNA and also stimulates its rapid decay in vitro (Brewer, 1991).
In spite of the fact that the bulk of evidence has predominantly identified instability sequences, there are implications that stabilizer elements also exist. Because it has been well characterized as a stable transcript, most of the evidence for stabilizer sequences are derived from studies investigating elements within the PGK1 transcript. In fact, chimeric mRNAs generated by placing instability elements into PGK1 were found to be ineffective unless most (82%) of the PGK1 coding region has been removed (Heaton et al., 1992). This implies that elements in the PGK1 mRNA have the ability to negate the destabilizing effects of the instability elements. Furthermore, specific sequences from the coding region of the PGK1 transcript could partially neutralize the destabilizing effects of a premature nonsense codon when placed upstream of the faulty terminator (Peltz et al., 1993). The observation that this stabilizing sequence is normally located in the PGK1 coding region coupled with finding that this element functioned as a stabilizer when placed upstream of the premature nonsense codon suggest that ribosomal traversal through this element is necessary for its activity.

Cumulatively, these findings indicate that cis-acting sequences are an influential determinant of mRNA stability. Because these elements are not targeted to a particular portion of the transcript, their activity may be linked to multiple processes involving translation initiation, elongation, and deadenylation. The presence of these sequences provide possible targets for factors that are associated with these processes which consequently trigger activation of a general mRNA decay pathway.
A general mRNA decay pathway

Although mRNAs degrade at different rates, it is unlikely that a specific degradation pathway exists for each particular mRNA. Rather, most mRNAs are degraded by a common decay pathway (Figure 1) and the variability in mRNA half-lives is dependent upon the efficiency with which a particular mRNA is directed to this degradation pathway.

The general pathway for mRNA turnover of most wild-type mRNAs initiates with shortening of the 3'–poly(A) tail to a specified length, termed deadenylation. There are several experimental findings that implicate deadenylation as the initial nucleolytic event in mRNA decay. Perhaps the most convincing were transcriptional pulse-chase experiments investigating the decay of both an unstable and stable mRNA (MFα2 and PGK1, respectively) demonstrating that the body of both mRNAs remained intact until the poly(A) tail was shortened (Decker and Parker, 1993). Once the poly(A) tail reached a length between 10 and 15 nucleotides, decay from the 5'-end of the mRNAs ensued (Decker and Parker, 1993).

After poly(A) shortening, transcripts are cleaved one or two nucleotides from their 5'-ends leading to the removal of the 5'-cap structure. The enzyme responsible for cap removal was identified as Dcp1p. Evidence that implicates Dcp1p as the decapping enzyme include studies demonstrating that this factor is capable of removing the 5'–caps of synthetic RNAs (Stevens, 1988; Beelman et al., 1996; LaGrandeur and Parker, 1998) and, moreover, deletion of DCP1 results in the accumulation of capped transcripts (Hatfield et al., 1996).
The deadenylated and decapped mRNAs are then degraded in a 5' to 3' direction by exonucleolytic digestion. This conclusion is derived from various studies investigating decay intermediates trapped by the insertion of a poly(G) tract, which creates a stable secondary structure in the mRNA that prevents exonucleolytic decay. The results of these experiments showed that the size of the decay intermediates containing the poly(G) tract was consistent with degradation originating from the 5'-end. For example, insertion of the poly(G) tract in the 3'-end of the mRNA exhibited shorter decay intermediates than those that harbored the poly(G) tract closer to the 5'-end (Decker and Parker, 1993; Muhlrad et al., 1994). Characterization of the factor responsible for this 5' to 3' degradation identified Xrn1p as the principal 5' to 3' exoribonuclease involved in general turnover of mRNA (Hsu and Stevens, 1993; Muhlrad et al., 1994). Characterization of Xrn1p demonstrated that this nuclease rapidly degraded mRNAs that lacked a 5'-cap and deletion of XRN1 was found to globally stabilize uncapped mRNAs (Hsu and Stevens, 1993; Muhlrad et al., 1994).

Because the initial nucleolytic event observed in the degradation of most wild-type mRNAs involves shortening of the poly(A) tail, it has been hypothesized that the status of the poly(A) tail influences the stability of the mRNP which ultimately contributes to translation efficiency. This is supported by numerous observations demonstrating that active translation coincides with lengthening of the poly(A) tail, while shorter tail lengths are indicative of translation repression (Bachvarova, 1992; Wormington, 1993). The link between poly(A) status and translation implies that the 3' end of the transcript is able to influence events occurring at the 5'-end. This notion has
been widely accepted as many studies have provided supporting evidence that factors binding to the 3'-end of the mRNA do indeed interact with factors involved in translation initiation (Sachs and Davis, 1989; Munroe and Jacobson, 1990a; Munroe and Jacobson, 1990b). The result of this 5'-3' interaction forms a structure, commonly referred to as a "closed loop" (Jacobson, 1996), that is believed to characterize a stable, fully functional mRNP. Events that result in perturbation of this structure are thought to make the 5'-end of the mRNA more susceptible to nucleolytic attack by Dcp1p, subsequently leading to rapid mRNA decay (Figure 1).

While this pathway appears to be the predominant mechanism by which most transcripts are degraded, other mechanisms of mRNA decay also exist. Among these is a pathway that initiates decay of specific substrates without prior deadenylation. This degradation pathway, termed nonsense-mediated mRNA decay, targets a subset of mRNAs which include those transcripts harboring premature nonsense codons (see below). The existence of this alternate decay pathway provides the cell with additional points of regulation to control mRNA half-lives.

**Mechanisms of nonsense-mediated mRNA decay**

Like most of the processes in the cell, quality-control mechanisms have evolved that recognize and correct errors that threaten the fidelity of gene expression. One such mechanism that monitors the integrity of mRNA and its subsequent translational competency in eukayotic cells is the nonsense-mediated mRNA decay (NMD) pathway which selectively targets and degrades aberrant transcripts containing premature
termination codons. Because of this function, this degradation pathway plays a direct role in selecting the transcripts that are acceptable for translation which, in turn, ultimately dictate the cellular repertoire of proteins.

A. NMD in *Saccharomyces cerevisiae*

The process of NMD has been most comprehensively studied in the yeast *Saccharomyces cerevisiae*, largely due to the ease of genetic manipulation and analyses (Losson and Lacroute, 1979; Leeds et al., 1991; Leeds et al., 1992; Peltz et al., 1994; Caponigro and Parker, 1996; Jacobson and Peltz, 1996; Ruiz-Echevarria et al., 1996; Culbertson, 1999; Czaplinski et al., 1999). A defining feature of yeast NMD came from the observation that nonsense-containing mRNAs were diverted into a late step of the general decay pathway. This conclusion is evident from studies investigating the spectrum of mRNAs stabilized in a strain that lacks the gene encoding the major cellular exoribonuclease involved in the general pathway of mRNA degradation, *XRNI*. Under these conditions, both wild-type mRNAs and nonsense-containing mRNAs (including intron-containing mRNAs that are natural substrates for NMD, see below) show increased abundance (Muhlrad and Parker, 1994; Hagan et al., 1995). Furthermore, the nature of the nonsense-containing mRNAs stabilized by deletion of *XRNI* has been found to be similar to that of wild type mRNAs in that both transcript populations lack a 5' cap structure (Muhlrad and Parker, 1994; Hagan et al., 1995). These findings indicate the decay of nonsense mRNAs has at least two steps in common with the decay of wild-type mRNAs: decapping and exonucleolytic digestion by Xrn1p (Muhlrad and Parker, 1994; Hagan et
al., 1995). In essence, the main difference in the decay of these nonsense-containing mRNAs as compared to the degradation of wild-type mRNAs is in the ability of NMD to bypass deadenylation. As such, substrates for NMD undergo Dcp1p-dependent decapping and Xrn1p-mediated exonucleolytic digestion without prior shortening of their poly(A) tails (Muhlrad et al., 1994; Hagan et al., 1995; Beelman et al., 1996; Caponigro and Parker, 1996) (Figure 1).

Although NMD initiates decapping of substrate transcripts without prior deadenylation, the 3'-poly(A) tails of these mRNAs are still capable of being shortened. In fact, the poly(A) tails of mRNAs stabilized in a xrn1Δ strain have been found to undergo deadenylation eventually indicating that the natural process of poly(A) tail removal remained intact and functioned at wild type efficiency (Muhlrad and Parker, 1994; Hagan et al., 1995). Therefore, it is believed that NMD promotes 5'-attack on its substrate transcripts before the mechanisms controlling poly(A) shortening can be activated. As it is generally accepted that poly(A) shortening, itself, signals the elimination of an "old" message, this finding suggests that mRNAs are targeted and degraded by NMD at a relatively early stage in their existence, possibly concurrent with their synthesis (Jacobson and Peltz, 1996).

1. trans-acting factors essential for NMD.

In yeast, there are at least three trans-acting factors that regulate the NMD pathway. These are the factors encoded by the UPF1, NMD2, and UPF3 genes. Mutations in these genes were identified by a number of genetic screens including those that sought to
identify: mutations that caused enhancement of allosuppression, mutations that result in omnipotent suppression, mutations that affect frameshift efficiency, and suppressors of upstream initiation codons (Culbertson et al., 1980; Hampsey et al., 1991; Pinto et al., 1992; Dinman and Wickner, 1994; Cui et al., 1995; He and Jacobson, 1995; Lee and Culbertson, 1995; He et al., 1997; Welch and Jacobson, 1999). Further characterization of UPF1, NMD2, and UPF3 have shown that mutations in these genes stabilize mRNAs containing premature nonsense codons without affecting the decay rates of most wild-type mRNAs (Leeds et al., 1991; Cui et al., 1995; He and Jacobson, 1995; He et al., 1997). Therefore, the wild-type function of these gene products is to selectively target a subset population of mRNAs that include aberrant transcripts containing premature nonsense codons.

Because UPF1 was the first of the three genes shown to be involved in NMD, numerous studies have contributed to its characterization. The UPF1 gene was found to encode a 109kDa protein that contains zinc-finger domains, a nucleotide (GTP) binding site, and seven motifs characteristic of the RNA/DNA helicase superfamily I (Altamura et al., 1992; Koonin, 1992; Leeds et al., 1992). The findings from fractionation experiments and confocal microscopy indicate that the bulk of Upf1p is localized in the cytoplasmic compartment on polysomes (Peltz et al., 1994; Atkin et al., 1995). In vitro studies have shown that purified Upf1p binds indiscriminately to either DNA or RNA and that its ATPase and helicase activities are dependent upon nucleic acid binding (Czapinski et al., 1995). The function of Upf1p has been implicated in various studies. For example, genetic screens have identified specific mutant upf1 alleles that can enhance
the efficiency of ribosomal frameshifting (Cui et al., 1996) as well as separate the mRNA decay and nonsense suppression phenotypes (Weng et al., 1996). Taken together, these findings indicate that Upf1p has several functions encompassing the processes of mRNA turnover, translation, and termination.

*NMD2* encodes an acidic protein with a predicted molecular weight of 127 kDa (Cui et al., 1995; He and Jacobson, 1995). Deletion of *NMD2* exhibits a decay defect similar to that of upf1A strains (He and Jacobson, 1995). This finding, coupled with two-hybrid analysis demonstrating that Upf1p and Nmd2p are interacting proteins, imply that Upf1p and Nmd2p function in a common pathway (He et al., 1996). Analyses of the *NMD2* sequence did not reveal any extensive homologies to other polypeptides but did identify a putative nuclear localization signal (NLS, spanning residues 26-46) and a putative transmembrane domain (spanning residues 470-490). These two regions have been shown to be critical for the protein’s function in NMD since independent deletion of either region shows a decay defect identical to that obtained when the entire gene is deleted (He and Jacobson, 1995). While these domains are clearly essential for function, their specific roles have yet to be determined. The carboxy-terminal end of *NMD2* includes a domain comprised predominantly of acidic residues (He and Jacobson, 1995). A carboxy-terminal, truncated fragment that includes this acidic domain results in a dominant-negative inhibition of NMD activity when the protein is expressed at high levels and localized to the cytoplasm (He and Jacobson, 1995). This dominant-negative effect, however, is not observed when the truncated protein is targeted to the nucleus, suggesting that Nmd2p functions in the cytoplasm (He and Jacobson, 1995).
UPF3 encodes a basic 45 kD protein that does not show strong homologies to any presently characterized factors (Lee and Culbertson, 1995; He et al., 1997). Mutations in this gene cause the identical decay defect as observed in upfl and nmd2 mutant strains, identifying Upf3p as an additional effector of the NMD pathway (He et al., 1997). Structural analyses of this protein reveal putative NLS- and nuclear export signal (NES)-domains, suggesting that Upf3p may shuttle between the nucleus and the cytoplasm (Lee and Culbertson, 1995; Shirley et al., 1998).

The observation that single deletions of UPF1, NMD2, or UPF3 have similar decay phenotypes as the multiple deletions imply that all three gene products are functionally related and to act in a common pathway (He et al., 1997). This notion is strengthened by protein-protein analyses demonstrating that Upf1p, Nmd2p, and Upf3p are interacting proteins (He et al., 1997). These results suggest that Upf1p, Nmd2p, and Upf3p may function concurrently to regulate NMD.

2. NMD is a cytoplasmic, translation-dependent event.
There is a tremendous amount of evidence suggesting that yeast NMD occurs in the cytoplasm and depends on the components of the translation apparatus. For example, the trans-acting factors essential for NMD and the nonsense mRNAs, themselves, were found to be associated with polysomes (Leeds et al., 1991; He et al., 1993; Peltz et al., 1993; Atkin et al., 1995; Weng et al., 1996; Weng et al., 1996; Atkin et al., 1997; He et al., 1997; Mangus and Jacobson, 1999). Additionally, expression of a suppressor tRNA, which enhances readthrough of the premature nonsense codon, was found to stabilize
nonsense-containing mRNAs, indicating that recognition of the aberrant nonsense codon relies on the translation machinery (Losson and Lacroute, 1979; Gozalbo and Hohmann, 1990). Consistent with the idea that NMD relies on aspects of translation, inhibition of translation initiation (by mutation of the initiator AUG or hairpin structures in the 5’UTR) or translation elongation (by the use of cycloheximide) inhibited the degradation of nonsense transcripts (Jacobson and Peltz, 1996; Ruiz-Echevarria et al., 1998; Frischmeyer and Dietz, 1999). Therefore, the activity of NMD relies on recognition of premature nonsense codons as aberrant by the translational apparatus highlighting the fundamental interrelationships between the pathways of mRNA translation and mRNA decay in yeast.

3. NMD is also influenced by specific cis-acting elements

Because recognition of a premature translation termination codon appears to trigger NMD, there is a question concerning what distinguishes a “normal” nonsense codon from one that promotes rapid degradation by NMD. One obvious difference that discriminates between normal and aberrant nonsense codons is the spatial positioning of that codon from either the 5' or the 3'-end of the transcript. In order to determine the effect of nonsense codon positioning on NMD, a number of studies were conducted in which a premature nonsense codon was systematically inserted into the protein-coding regions of several genes (Peltz et al., 1993a; Peltz et al., 1993b; Hagan et al., 1995; Jacobson and Peltz, 1996). The findings of such studies demonstrate that any nonsense codon inserted within the first two-thirds to three-quarters of the protein coding region is capable of
activating NMD whereas nonsense mutations located in the remaining portion of the coding region have little to no 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 observations demonstrate that only early nonsense codons activate the NMD pathway, suggesting a spatial positioning requirement for recognition of an aberrant terminator.

More detailed studies investigating the contribution of surrounding sequences to NMD found that, in addition to the premature nonsense codon, a downstream sequence was also required to activate the decay pathway. Such studies find that deletion of most of the PGK1 protein coding region downstream of an early nonsense mutation abrogated degradation by the NMD pathway (Peltz et al., 1993a&b). In an attempt to identify the destabilizing cis-acting element, portions of the deleted sequence were then reinserted into a PGK1 gene containing an early nonsense mutation, and the resulting decay phenotype was monitored (Peltz et al., 1993a&b). The results of these experiments identified a small 106 nucleotide segment (termed the DSE: downstream element) that was sufficient to activate NMD in combination with an upstream aberrant terminator (Peltz et al., 1993a&b). Furthermore, a specific spatial relationship is necessary since the DSE could not be localized more than approximately 200 base pairs downstream of the premature nonsense codon to activate NMD (Ruiz-Echevarria et al., 1998). Because these sequence elements do not promote rapid decay unless preceded by an upstream termination codon, it is believed that traversal of these instability elements by translating ribosomes renders the DSE nonfunctional. Comparable experiments identified similar
cis-acting elements in other mRNAs allowing for the formulation of a weak DSE consensus sequence (Peltz et al., 1993; Hagan et al., 1995). By scanning the yeast genome for the presence of a minimal degenerate consensus sequence, these DSE-like elements have been proposed to be present in the coding regions of most yeast mRNAs (>75%) making it conceivable that these elements are dispersed universally to assist in the regulation of NMD (Peltz et al., 1993; Hagan et al., 1995; Zhang et al., 1995).

The mechanism by which DSEs trigger mRNA decay is not known but several hypotheses are available. One suggests that the DSE is a binding site for a factor that stimulates degradation. Under normal conditions, the site is masked by elongating ribosomes and is therefore inaccessible to factor binding, or the bound factor is displaced by translocating ribosomes. Because premature translation termination inhibits traversal through the DSE, the factor is able to bind and to promote rapid decay (Zhang et al., 1995; Ruiz-Echevarria and Peltz, 1996). Support for this notion comes from the identification of a factor, Hrp1p, that specifically binds the DSE and stimulates the decay of some nonsense mRNAs (Gonzalez et al., 2000). Another possibility contends that recognition of proper termination is dependent upon particular elements residing downstream of the nonsense codon and that the DSE neither fulfills the proper termination context requirements nor includes the proper constituents that wild type 3'-UTR sequences encompass (A. Jacobson, pers. communication). As a result, the transcript is not recognized as wild-type and, by default, it is subjected to rapid decay. This model is supported by findings demonstrating that an unstable nonsense mRNA can be stabilized by physically positioning poly(A)-binding protein just 3' of the premature
nonsense codon (R. Ganeson, unpublished expts.). Furthermore, these results implicate poly(A)-binding protein as a significant factor involved in recognition of the correct termination context.

B. NMD in the mammalian system

The idea that NMD is an entirely cytoplasmic event is challenged by studies performed with mammalian cells, demonstrating that degradation of nonsense mRNAs can take place in the nucleus. Separation of the nuclear and cytoplasmic compartments by using subcellular fractionation techniques has revealed that the degradation of some transcripts takes place in both cellular compartments (Cheng and Maquat, 1993; Lozano et al., 1994; Maquat, 1995; Kessler and Chastin, 1996). In fact, certain nonsense-containing mRNAs, that are normally degraded by NMD while associated with the nucleus, are capable of eluding the NMD surveillance by simply translocating out of the nucleus. Examples of these mRNAs are the nonsense-containing triose phosphate isomerase (TPI), immunoglobulin (Ig), and T cell receptor (TCR) transcripts (Cheng and Maquat, 1993; Lozano et al., 1994; Carter et al., 1996). Because of the wealth of evidence in yeast linking NMD to translation, it is conceivable that those aberrant mRNAs that evade destruction by NMD are simply unable to be translated efficiently, and therefore they are protected from the bulk of the NMD machinery that was associated with the translation apparatus. Contrary to expectation, however, these cytoplasmic mRNAs were found to associate with polysomes, indicating that stabilization of these cytoplasmic nonsense mRNAs is not a consequence of poor translation efficiency and that mammalian NMD,
like that of yeast, also requires translation (Stephenson and Maquat, 1996). Consistent with this hypothesis are the studies demonstrating that agents commonly used to abrogate NMD in yeast (such as translational inhibitors, hairpin structures in the 5’ region that interfere with translation initiation, and suppressor tRNAs) also effectively stabilize mammalian nonsense mRNAs. Surprisingly, these agents not only stabilize those nonsense mRNAs in the cytoplasmic fraction, but also those found in the nuclear fraction (Cheng et al., 1994). Therefore, perturbations in cytoplasmic translation have consequences on nuclear-associated mRNA degradation. The growing evidence of a nuclear-associated NMD pathway in mammals may implicate the presence of a more complex decay mechanism in higher organisms that is dedicated to ridding cells of unwanted, nonsense mRNAs.

In distinguishing the different mechanisms of NMD, it is necessary to identify the features that differentiate between the lower and higher eukaryotes. One striking feature that distinguishes mammalian and yeast transcripts is the prevalence of introns. In mammalian cells, these noncoding sequences are universally distributed throughout the RNA population while in yeast, very few mRNAs contain introns. The involvement of introns in regulating NMD is implied from the observation that nonsense transcripts generated from genes without introns are generally stable (Cheng et al., 1994). More detailed analyses have demonstrated that mammalian NMD requires not only a premature termination codon but also at least one downstream intron to initiate degradation of substrate transcripts, suggesting a positional dependence for the these regulatory cis-acting elements. The general rule for premature nonsense codons to initiate NMD in
mammals requires that these codons reside more than 50-55 nucleotides upstream of the 3'-most exon-exon junction (Cheng et al., 1994; Nagy and Maquat, 1998; Zhang et al., 1998). As further evidence, mutagenesis of splice sites in the last intron of nonsense TCR-β stabilized the resulting transcripts (Carter et al., 1996). The implication of these findings is that a downstream spliceable intron is required to target nonsense mRNAs to degradation by NMD.

The requirement of a spliceable intron downstream of the premature nonsense codon in mammals indicates that specific cis-acting elements, analogous in function to the DSE in yeast, appear to define the context of “correct” versus “aberrant” termination. In fact, similar to the characterization studies of the yeast DSE, transplanting a spliceable intron downstream of the true termination codon in TCR-β transcript transformed the otherwise wild-type mRNA into an aberrant mRNA that was degraded by NMD (Carter et al., 1996).

Although the pathways of NMD in yeast and mammals appear to be distinct, the identification of elements that are functionally similar in both the systems contributes to the idea that the NMD pathway has been conserved in evolution. Indeed, the identification and characterization of human Upf1p homolog, hUpf1p, provides strong evidence for this notion by demonstrating conservation of both sequence and function for specific trans-acting factors essential for this decay pathway. In an attempt to incorporate the data of both eukaryotic systems, one model proposes that ribosomes and other components of the translation machinery can assemble on mRNA during transport out of the nucleus (Maquat, 1995). Once the translation apparatus is assembled, NMD is
functional. Therefore, according to this model, NMD can target and degrade mRNAs at a very early stage, even before they are able to be released fully into the cytoplasm. The differences observed experimentally might reflect the efficiencies with which the mRNA is transported and released from the nuclear pore. For example, the observations in yeast where degradation appears to limited to the cytoplasm might be due to the rapidity of mRNA transport and translation initiation relative to the onset of NMD (Jacobson and Peltz, 1996).

A physiological role for NMD in controlling the abundance of specific, endogenous transcripts

Although identified as a pathway dedicated to the degradation of errant nonsense-containing mRNAs, it seem unlikely that NMD evolved as a system solely dedicated to the prevention of such rare events. Instead, an emerging view contends that the primary role of the NMD pathway may actually be to regulate the stability of a select population of physiological transcripts. In fact, various studies have recognized several classes of endogenous substrates by identifying the mRNAs that become stabilized in mutant strains that are specifically defective for NMD decay.

One type of substrate identified by such studies are those inefficiently spliced pre-mRNAs that introduce an in-frame premature termination codon due to retention of the intron sequence. This situation, as expected, renders the unspliced pre-mRNA a substrate for the NMD pathway. Examples of this class of substrates in yeast include the CYH2, RP51B, and MER2 pre-mRNAs which are all significantly stabilized when the NMD...
pathway is inactivated (He et al., 1993). Therefore, by the nature of its ability to detect mRNAs containing premature nonsense codons, NMD acts as a surveillance system that prevents the expression of these faulty transcripts. Other substrates subject to the degradation imposed by this protective mechanism are those aberrant transcripts that potentially arise due to errors in transcription or splicing.

A second class of endogenous transcripts targeted by NMD are those harboring a short upstream open reading frame (uORF) within the 5'-untranslated region (UTR) (Oliveira and McCarthy, 1995). It is conceivable that these transcripts are substrates for NMD since the uORF could be misinterpreted as an mRNA containing a premature termination signal. This is best illustrated in a study where a short uORF was cloned into the 5'-UTR of the chloramphenicol acetyltransferase (CAT) gene. The CAT transcript produced from this construct was significantly destabilized by the presence of the uORF (Oliveira and McCarthy, 1995). A comparable experiment generated a short uORF by introducing a mutation that created an ATG in the 5'-UTR of the CYCl gene (Pinto et al., 1992). The resulting CYCl mRNA was also found to be notably destabilized (Pinto et al., 1992). For this class of substrates, NMD may offer a level of gene regulation to selectively limit the abundance of such transcripts.

A third class of substrates include mRNAs in which the ribosome has bypassed the initiator AUG and commenced translation further downstream (Welch and Jacobson, 1999). These transcripts contain a downstream translation initiator start site (that is in a preferred context as compared to the intended/original start site) and an in-frame terminator. For example, experiments analyzing the structure of the SPT10 mRNA
revealed the existence of a downstream AUG that was in a more favorable context for initiation (Welch and Jacobson, 1999). The authors suggest that the ribosome bypasses the actual initiator AUG and initiates at the downstream AUG. Upon encounter of a nonsense codon that is in-frame with the downstream AUG, NMD is activated because it is interpreted as a premature termination event. Changing the context of this downstream AUG to be less favorable than the true initiator was found to stabilize the resulting mRNA (Welch and Jacobson, 1999). Therefore, because the structure of these substrates appears to be similar to that of the uORF-containing substrates, the mechanism involved in triggering rapid decay of these transcripts might also be analogous. A possible hypothesis is that competition for initiation (normal vs. upstream/downstream AUG) affects translation efficiency which ultimately determines decay rates. The targeting of this group of mRNAs supports the presumed surveillance role of NMD in ridding the cell of aberrant transcripts.

An additional, noteworthy observation is that *SPT10* encodes a transcriptional regulator of another factor (encoded by *HHF2*). In mutants that inactivate NMD, *HHF2* mRNA abundance is increased. However, this increased abundance is not coupled with a reduction in its decay rate, suggesting that NMD indirectly affects *HHF2* mRNA abundance through modulation *SPT10* mRNA levels (Welch and Jacobson, 1999). These findings reveal an added regulatory function for NMD to control gene expression by targeting mRNAs that encode factors which subsequently regulate the expression of other factors. Further evidence demonstrating this type of control on gene expression comes from experiments with *C. elegans*. Mutations in the *SMG* genes (that result in
inactivation of the NMD pathway) affect the abundance of two alternatively spliced transcripts, *SRp20* and *SRp30b* (Morrison et al., 1997). These mRNAs are presumably substrates for NMD due to the presence of an alternatively spliced exon that introduces a premature nonsense codon relative to the extended ORF (Morrison et al., 1997). Since *SRp20* and *SRp30b* are themselves *trans*-acting regulators of alternative splicing, the consequence of modulating their relative abundance becomes reflected in their downstream targets. As a result, NMD is able to impose indirectly an extensive level of control over the splicing patterns of pre-mRNAs that are not themselves substrates for this pathway. These and similar observations implicate a powerful mechanism of regulation by NMD wherein gene expression of a broad repertoire of factors can be fine-tuned by targeting a select subset of transcripts (Leeds et al., 1991; Dahsheid et al., 1998; Lelivelt and Culbertson, 1999; Lew et al., 1999).

Another class of aberrant mRNAs identified as endogenous substrates for NMD are those transcripts harboring an abnormally extended 3'-UTR which arise due to a mutation in the polyadenylation site (Pulak and Anderson, 1993; Muhlrad and Parker, 1999). These substrates are unique in that a secondary/aberrant nonsense codon does not act as the trigger for decay. The supposition that these transcripts are *bona fide* substrates of the NMD pathway became apparent when these mRNAs were found to be stabilized under conditions that inactivated NMD (Pulak and Anderson, 1993; Muhlrad and Parker, 1999). Furthermore, *cis*-acting stabilizer elements that have been shown to prevent premature nonsense-containing mRNAs from degradation by NMD also stabilized mRNAs harboring these extended 3'-UTRs (Muhlrad and Parker, 1999). The protective
role of NMD as a surveillance mechanism ensuring quality control of gene expression is consistent with these results as this pathway prevents the expression of an aberrant product. However, the identification of these types of substrates provides additional evidence that discrimination of "premature" versus "normal" termination codons may be dependent upon a spatial relationship between the nonsense codon/termination site and elements residing at the 3'-terminal end of the transcript (see above). These types of substrates have yet to be identified in mammalian cells.

In higher organisms, the physiological importance of the NMD is underscored by its participation in the maturation of the immune system. During this process, immunoglobulin (Ig) and T-cell receptor (TCR) genes are subjected to programmed gene rearrangements where a myriad of combinations composed of different gene segments (termed V, D, and J segments) are linked to a constant element. While programmed gene rearrangement allows for the production of an expansive antigenic repertoire, it is conceivable that some, if not most, rearrangements result in the introduction of a premature nonsense codon. These non-productive gene rearrangements give rise to transcripts that are rapidly degraded by NMD (Carter et al., 1995). In this way, NMD selectively promotes the proliferation of functional receptor gene rearrangements and prevents the expression of proteins that could interfere with functional Ig and TCR proteins.

The identification of natural substrates for this decay pathway prompted the use of high density oligonucleotide arrays to further address the impact of NMD on global gene expression. Initial analyses of the yeast transcriptome using this powerful method
originally identified approximately 225 transcripts that increased in abundance when any of the \textit{UPF/NMD} genes were deleted (Lelivelt and Culbertson, 1999) but a similar experiment using a more sophisticated chip showed approximately 400 affected mRNAs (F. He, unpublished expts.). With 3-6\% of the yeast genome affected by perturbations in NMD, it is clear that this pathway plays a significant role in cellular gene expression.

\textbf{A mechanistic role for NMD factors in translation termination.}

The very nature of the substrates for the NMD pathway implies a mechanism that is triggered by signals occurring at the termination site. Accordingly, the components of the NMD machinery, \textit{UPF1, NMD2, and UPF3}, are all hypothesized to be regulators of events occurring at the translation termination site. Consistent with this notion are experiments which demonstrated that deletion of these genes not only stabilize nonsense mRNAs but also allow for a translational effect termed nonsense suppression (Leeds et al., 1992; Weng et al., 1996a; Weng et al., 1996b). Nonsense suppression occurs when elongating ribosomes translate past the premature nonsense codon and generate a full length product instead of correctly terminating peptide elongation. Under such circumstances, a near cognate tRNA is thought to effectively compete for the translation termination release factors at the premature termination site resulting in amino acid incorporation. Therefore, as a consequence of inactivating NMD, the stringency in recognizing termination codons becomes impaired.

With yeast, nonsense suppression is commonly monitored by utilizing strains that harbor a premature nonsense allele within an essential gene. Therefore, under conditions
where NMD can degrade the substrate transcript, the nonsense mutation is lethal. Suppression of these nonsense alleles is achieved when the level of readthrough past the site of the premature nonsense mutation is sufficient to restore viability.

There are two hypotheses on how inactivation of NMD leads to nonsense suppression. The first contends that nonsense suppression is solely the consequence of the increased mRNA abundance caused by NMD inactivation. As such, the assumption is that the translation termination event is subject to a certain amount of inaccuracy that results in an inherent rate of “leaky” termination. The combination of enhanced mRNA abundance and the inherent rate of nonsense codon readthrough allows the cell to generate a minimal, but sufficient, amount of full length product to sustain viability. Therefore, the nonsense suppression phenotype mediated by deletion of the UPF/NMD factors is an indirect effect simply due to an effective increase in the template mRNA.

The second alternative establishes a more elaborate role for the UPF/NMD proteins by suggesting that they are directly involved in modulating translation termination efficiency. Under this notion, the absence of the UPF/NMD factors impairs mechanisms necessary for effective translation termination. This suggests that the UPF/NMD factors must play an active role in translation termination. Evidence for this hypothesis first became apparent with the identification of upf1 alleles that appeared to separate the mRNA decay phenotype from the nonsense suppression phenotype. One set of upf1 alleles resulted in inactivation of mRNA decay but failed to show a nonsense suppression phenotype, while another set of upf1 alleles promoted normal mRNA decay in spite of displaying a nonsense suppression phenotype (Weng et al., 1996; Weng et al.,
1996). This finding attributed a direct role in translation for \textit{UPF1} that is separate from its role in stabilizing nonsense mRNAs.

More compelling evidence for involvement of the \textit{UPF/NMD} gene products in translation termination was presented when both yeast Upf1p, and hUpf1p were found to be capable of interacting with the polypeptide release factors, Sup35p (eRF3) and Sup45p (eRF1) (Czapinski et al., 1998). This provided physical evidence for the involvement of the \textit{UPF/NMD} factors in the regulation of translation termination. Analyses of the interacting domains on Upf1p showed that interaction with Sup35p interfered with its association with RNA (Czapinski et al., 1998). Therefore, a competition between RNA and Sup35p for Upf1p binding is established and factors that promote preferential binding of Upf1p to one or the other may have a direct influence on termination efficiency. For example, factors that encourage preferential binding of Upf1p to Sup35p might promote translation termination while other factors that encourage Upf1p-RNA interaction might dictate control of termination by regulating its interaction with the release factors. The latter might explain the increase in nonsense suppression under conditions where nonsense mRNAs are stabilized. Due to the increase in competing RNA, binding of Upf1p to RNA is favored which effectively reduces Upf1p-release factor interaction resulting in decreased termination efficiency.

Because Nmd2p and Upf3p interact with Upf1p and strains that harbor deletions of these genes also exhibit nonsense suppression phenotypes, it is believed that Nmd2p and Upf3p may also be regulators of termination efficiency. Their contribution, however, is unclear since neither factor has been shown to interact with the release factors.
C. THESIS OBJECTIVES.

1. Investigation of the decay kinetics of NMD substrates to determine the cellular site of NMD. Although most of the evidence in yeast implicates NMD as a cytoplasmic process, the cellular site of this decay pathway has still been the subject of controversy due to the numerous examples of nuclear-associated NMD in the mammalian system. Therefore, these studies were conducted to address the cellular site of NMD in yeast.

2. Characterization of the nuclear localization signal (NLS) of Nmd2p. The presence of NLS and NES sequences within proteins that are essential for NMD (i.e. Nmd2p and Upf3p) introduced the notion that NMD involves a nuclear component. Because the NLS region of Nmd2p appears to be essential for the protein to function in NMD, I investigated whether mutations affecting this region inactivate decay as a consequence of improper nuclear localization.

3. Investigation of the roles of Upf1p, Nmd2p, and Upf3p in regulating the efficiency of translation termination. With the development of an assay to monitor nonsense suppression, I assayed the effect of deleting the UPF/NMD genes on the efficiency of translation termination and determined the epistatic relationships among Upf1p, Nmd2p, and Upf3p.
Figure I. Pathways of mRNA decay

- General decay
- NMD
- Decapping
- UTR
- Exonucleolytic digestion
- Deadenylation
- Dcp1p
- Xrn1p
- AUG
- UAA
Figure 1. Pathways of mRNA decay in *Saccharomyces cerevisiae*. Shown is a cartoon representation of two types of mRNA decay pathways in yeast. The left side of the cartoon depicts deadenylation-dependent decapping pathway of mRNA decay (general decay of most wild-type mRNAs). The initial step in this pathway is the shortening of the 3'-poly(A) tail to an oligo(A) length of ~10-15 residues (represented by 3 A’s). Following deadenylation, the cap (smiley face) is removed by the action of the *DCPI* gene product (lightning bolt), then subsequently degraded in a 5' to 3' direction by Xrn1p (pac-man). The right side of the figure depicts nonsense-mediated mRNA decay pathway (NMD) which does not require deadenylation to initiate decapping by Dcp1p. These transcripts are decapped, then degraded in a 5' to 3' direction by Xrn1p while retaining long poly(A) tails. This shows that substrates for the NMD pathway are shunted into a late step of a common degradation pathway.
CHAPTER 2

MATERIALS AND METHODS

A. Strains, plasmids and general methods

The yeast strains used in this study are listed in Table 1. Preparation of standard yeast media and methods of cell culture were conducted as described by Rose et al. (1990). Transformation of yeast was done by the rapid method described by Soni et al. (1993). 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, by DNA sequencing using the method described by Sanger et al. (1977). Plasmid DNAs were prepared from Escherichia coli DH5α.

B. Construction of NMD2-NLS mutant alleles

All of the mutations in the NMD2 NLS were created by PCR mutagenesis using the QuikChange Site Directed Mutagenesis Kit (Statagene). The mutagenic primers used for single and double point mutation in the Nmd2p NLS are summarized in Table 2.

C. Construction of galactose-inducible NMD plasmids

The galactose-inducible UPF1 construct was made by ligating a 3.6kb EcoRI-SalI fragment from pMA424-UPF1 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 XbaI-SalI 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 NcoI-SalI 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).

D. Construction of YCp and YEp can1-100 alleles

The can1-100 allele was recreated in a YEp24-CANI high-copy-number plasmid and a pRIP-CANI single-copy plasmid by PCR mutagenesis using the QuikChange Site Directed Mutagenesis Kit (Stratagene). The oligo can1-100 (Table X) was the mutagenic primer. CAN1 containing sequences that comprised a 3' triple-hemagglutinin (HA) epitope tag was obtained from Duane Jenness. The 3'-HA-tagged can1-100 allele was constructed by inserting a SalI-EagI HA-containing fragment into the YEp24-can1-100 plasmid digested with the same enzymes.

D. RNA extraction and northern blot analysis

RNA was isolated using the hot phenol method as described by Herrick et al. (1990). Aliquots (20μg) of each RNA sample were analyzed by northern blotting. For isolation of
RNA from polysome fractions, the method as described by Benard et al. (1998) was used. DNA probes were prepared by either random priming with [α-32P]dCTP (Feinberg and Vogelstein, 1983) or by 5' end-labeling of single stranded oligodeoxyribonucleotides with [γ-32P]ATP (Sambrook et al., 1989). mRNA steady-state levels were determined by quantitating northern blots with a Bio-Rad Molecular Imager. The DNA probes used to detect specific transcripts included: ADE2 (a 2 kb BgII fragment from an xrn1::ADE2 disruption plasmid generously provided by Feng He), PGK1 (oligonucleotide 1 from Peltz et al., 1993), CYH2 (a 600 bp EcoRI-HindIII fragment from pGEM4Z-CYH2 which hybridizes to both the pre-mRNA and the mRNA; Herrick et al., 1990), CAN1 (a 1 kb EcoRI-SalI fragment from YEp24-CANI), ADH1 (a 2.0 kb XbaI-BamHI fragment generously provided by Michael Green) and SCR1 (a 400-bp fragment amplified from yeast genomic DNA using oligonucleotides SCR1-1 [5'-AGGCTGTAATGGCTTTCTGGTG GGATGGGA-3'] and SCR1-2 [5'-GATATGTGCTATCCCGGCCGCTCCATCA C-3']). Immunoprecipitation of capped mRNAs were performed as described in Muhlrad et al. (1994), using polyclonal anti-m7G antibodies generously provided by Elsebet Lund.

E. Protein gels, western blots, and antibodies

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described by Laemmli (1970). 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 anti-Pbp1p antibody (a generous gift from David Mangus; Mangus et al., 1998), anti-Swi3p antibody (a generous gift from Craig Peterson), anti-Pgk1p antibody (a generous gift from Duane Jenness), anti-Rpo21p antibody (a generous gift from Judith Jaehning), polyclonal affinity purified anti-Upf1p antibody (Mangus and Jacobson, 1999), polyclonal affinity purified anti-Nmd2p antibody (He et al., 1996), and the monoclonal antihemagglutinin (HA) antibody, 12CA5, (from Boehringer Mannheim Biochemicals) for detection of Upf1p, Nmd2p, and HA-epitope tagged Upf3p, respectively. The antihemagglutinin (HA) antibody (12CA5) was also used for quantitation of the NMD factors.

F. Purification of recombinant GST-Nmd2p for isolation of polyclonal anti-Nmd2p antibody

This procedure was accomplished largely through the advice and expertise of David Mangus. The extraction steps were carried out between 0 and 4°C. All buffers included 0.1mM dithiothreitol (DTT), 1mM phenylmethylsulfonylfluoride (PMSF) and the protease inhibitors bestatin (0.35µg/ml), pepstatin (0.4µg/ml), leupeptin (0.5µg/ml), and benzamidine (20µg/ml). Cell pellets were resuspended in 4 volumes/g cell wet weight T(50) buffer (30mM Tris-HCl, pH 7.9, 2mM EDTA, 5% glycerol, 10mM MgCl2, and 50mM KCl) and lysed with a French press (cell pressure 20,000 psi). Lysates were cleared by centrifugation at 30,000 x g. The pellet was resuspended in denaturing buffer (6M urea, 50mM Tris-HCl, pH 7.9, 1mM EDTA, and 8mM DTT), vortexed vigorously, homogenized with a B pestle and centrifuged at 30,000 x g. The chromatography steps
were carried out at room temperature. The supernatant was dialyzed against buffer (50mM Tris-HCl, pH 7.9, 1mM EDTA, 1mM DTT and 20% glycerol). Extracts were bound in batch to glutathione-agarose (Sigma) previously equilibrated in T(50) buffer. After binding for 10 min. on a platform shaker, the resin was washed 3 times with the same buffer. The resin was then transferred to a small column and the protein eluted with 10 column volumes of T(50) buffer containing 10mM glutathione (Sigma). The purity of the protein was assessed by SDS-polyacrylamide electrophoresis and staining with Coomassie Blue R-250. GST-Nmd2p was greater than 90% pure with the majority of the contamination coming from proteolysis.

G. Preparation of purified yeast nuclei

Yeast nuclei were isolated by osmotic lysis of spheroplasts, followed by banding two times on Ficoll gradients (Guthrie and Fink, 1991). The purity of the nuclei was monitored by Western blotting with, as a criteria, enrichment for nucleus-associated proteins (Rpo21p, Pbp1p, or Swi3p) and the loss of a cytoplasmic protein (Pgk1p).

H. Preparation of polysome fractions

Yeast cell extracts were prepared and fractionated on sucrose gradients as described by Mangus and Jacobson (Mangus and Jacobson, 1999).
I. Galactose induction

Yeast strains containing galactose inducible \textit{UPF/NMD} gene constructs were grown in SC –uracil +raffinose media to mid-log phase (OD$_{600}$=0.5). Strains containing the inducible \textit{UPF/NMD} gene constructs and either of the \textit{pgkl} nonsense alleles were grown in SC –uracil -leucine +raffinose media (to maintain selection for the \textit{GAL-UPF/NMD} plasmid and the plasmid harboring the \textit{pgkl} 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.

J. \textit{canl-100} nonsense suppression assay

Multiple independent isolates of yeast strains to be assayed were grown in selective liquid media to mid-log phase (OD$_{600}$=0.5-0.7). Samples from these cultures were serially diluted (1:10) four times, and aliquots (10μl) of the four dilutions were spotted on SC-arginine plates containing 0 to 500 μg of canavanine per ml. The final aliquots, used as the principal indicators of canavanine sensitivity, each contained approximately 100 cells. Growth on plates, monitored after incubation at 30°C for 2 days, yielded reproducible results for each strain.

K. Arginine uptake assay

The arginine uptake assay was adopted from that previously described by Opekaro and Kubin (1997). Yeast cultures were grown to mid-log phase (OD$_{600}$=0.5-0.7) at 30°C in SC-arg medium and then supplemented with 50mM L-arginine containing 5μCi of L-
[\textsuperscript{3}H]arginine (Amersham). Aliquots of the cultures were then removed at specific intervals, diluted in 2 ml of 100mM LiCl, filtered on GF/C filters (Whatman), and washed with 2ml of water. Radioactive arginine associated with each filter was determined by scintillation counting.
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<th>Strain</th>
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<tr>
<td>HFY1200</td>
<td>( MATa ade2-1 ) his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 &lt;br&gt; UPF1 NMD2 UPF3</td>
<td>He and Jacobson, 1995</td>
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CHAPTER 3

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

Introduction

Intricate mechanisms that safeguard against errors in gene expression exist in all eukaryotes (Yarus, 1992; He et al., 1993; Chin and Pyle, 1995; Friest et al., 1996; Jeon and Agarwal, 1996; Gottesman et al., 1997). 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; Peltz et al., 1993; Pulak and Anderson, 1993; Maquat, 1995; Jacobson and Peltz, 1996). The process of NMD has been studied extensively in *Saccharomyces cerevisiae*, where rapid degradation of nonsense-containing mRNAs involves deadenylation-independent decapping and subsequent 5’—>3’ exonucleolytic digestion of the remainder of the mRNA (Hsu and Stevens, 1993; Muhlrad et al., 1994; Hagan et al., 1995; Beelman et al., 1996; LaGrandeur and Parker, 1998). 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 (Leeds et al., 1991; Cui et al., 1995; He and Jacobson, 1995; Lee and Culbertson, 1995; He et al., 1997). Consistent with their roles in the response to aberrant translation, all three of the latter UPF/NMD proteins have been shown to localize to the cytoplasm and associate with polyribosomes (Peltz et al., 1993; Atkin et al., 1995; Atkin et al., 1997; Shirley et al., 1998; Mangus and Jacobson, 1999). These observations indicate that yeast NMD occur in the cytoplasm and are linked to translation, conclusions consistent with other results showing that: a) drugs or mutations in specific genes that cause inhibition of translation also abrogate NMD (Losson and Lacroute, 1979; Zhang et al., 1997; Welch and Jacobson, 1999); 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).

Studies using mammalian cells have found that mRNAs encoding rabbit and human β-globin show decay kinetics consistent with NMD consistent with NMD targeting all early nonsense-containing mRNAs in the cytoplasm (Maquat et al., 1981; Lim et al., 1989; Shyu et al., 1991; Lim et al., 1992). However, it is still controversial as to whether mammalian NMD is limited solely to the cytoplasm and completely dependent upon conventional translation (Daar and Maquat, 1988; Cheng et al., 1990; Cheng and Maquat, 1993; Aoufouchi et al., 1996). Increasing evidence supports a less stringent, nuclear-associated NMD mechanism that does not appear to affect all
nonsense-containing mRNAs. 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 (Daar and Maquat, 1988; Cheng et al., 1990; Cheng and Maquat, 1993). Additionally, the nonsense-containing TPI mRNAs that are exported into the cytoplasm apparently escape degradation by NMD since they were found to be as stable as the wild-type TPI mRNA (Cheng and Maquat, 1993). These findings suggest that recognition of premature nonsense codons in at least some mammalian mRNAs occurs solely in (or in association with) the nucleus and that those nonsense mRNAs which 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. Two nonsense-containing mRNAs (encoded by the ade2-1 and the pgk1-UAG-2 alleles) 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 mRNA Decay.

To address the stability of cytoplasmic nonsense-containing mRNAs, we took advantage of an allele of the ADE2 gene, ade2-1. Earlier studies have shown that the ade2-1 mutation can be suppressed in yeast strains containing an ochre tRNA suppressor (Zecherle et al., 1996), suggesting that the ade2-1 allele is attributable to a nonsense (UAA) mutation. Therefore, the ade2-1 mRNA is 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 (Fig. 2) (He et al., 1993). The ade2-1 mRNA was approximately seven-fold more abundant in upf/nmd mutant cells as compared to the isogenic UPF/NMD (WT) strain (Fig. 2). Likewise, deletion of genes encoding general factors involved in mRNA decay (i.e., DCP1 and XRNI) also promoted a seven-fold increase in ade2-1 transcript abundance (Fig. 2). These differences in mRNA abundance were consistent with the respective differences in the decay rates of the ade2-1 mRNA in UPF/NMD and upf/nmd mutant cells. The half-life of the ade2-1 mRNA was found to be less than 5 minutes in the UPF/NMD strain and approximately 35 minutes 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, thus, 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 Fig. 3, Upf1p, Nmd2p, and Upf3p was not detected 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 Fig. 3, and others, indicated that: a) Upf1p, Nmd2p, and Upf3p all begin to accumulate approximately 12-14 minutes after galactose addition and b) by 20 minutes 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.
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 (t₁/₂=35 minutes; 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, however, 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 (t₁/₂=19 minutes; Fig. 7B). Only after substantial dilution with newly synthesized mRNA would the population begin to reflect a more rapid decay rate.

These possibilities were evaluated by using northern blot analyses of yeast strains expressing regulatable *UPF1, NMD2*, or *UPF3*. These experiments demonstrate that, as expression of *UPF1, NMD2*, or *UPF3* increases (Fig. 3), the abundance of the *ade2-1* mRNA decreases (Fig. 4A). Subsequent to the time at which the *UPF/NMD* proteins...
begin to accumulate (12-14 minutes post-induction; see above), the ade2-1 mRNA disappears with a half-life of approximately 7 minutes in all three strains. By 30 minutes after galactose induction of any of the three UPF/NMD genes, approximately 20% of the ade2-1 mRNA population remains and, by 40 minutes, 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 (Fig. 4B).

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 minutes post 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. 5A, 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) (Sasnauskas et al., 1987; Stotz and Linder, 1990). Upon restoration of NMD, the ade2-1 mRNA is rapidly degraded (Fig. 4) and is no longer detected in the polyribosome fractions (Fig. 5B). As a control for these experiments, the northern blots of Figs. 5A 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 (Figs. 5A and B).

Results virtually identical to those of Figs. 5A 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 PGKI 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 transcript. The PGKI mRNA is normally very stable, having a half-life of approximately 60 minutes
(Peltz et al., 1993), but a derivative with a nonsense mutation at codon 22 (pgkl-UAG-2) is extremely unstable ($t_{1/2}=6$ minutes) (Peltz et al., 1993). Inactivation of the NMD pathway (by mutations in UPFI, 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., 1993). 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. 6A demonstrates that induction of UPFI, NMD2, or UPF3 (in the respective deletion strains) resulted in rapid disappearance of the pgkl-UAG-2 mRNA (see “early pgk1”). 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 minutes, after a lag for induction of the pathway, and b) by 30 minutes post-induction, most (85%) of the mRNA was degraded (Figs. 6A and 7). 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 (Peltz et al., 1993; Peltz et al., 1993; Hagan et al., 1995; Jacobson and Peltz, 1996). As an additional means to testing whether the post-induction disappearance of the pgkl-UAG-2 and ade2-1 mRNAs was a direct consequence of restoration of NMD, we repeated the NMD induction experiments in cells harboring a pgkl allele with a nonsense mutation at codon 385 (pgkl-UAG-7). This
mutation does not affect the stability of the encoded mRNA ($t_{1/2}>60$ minutes) and does not render it a substrate for NMD (Peltz et al., 1993). As such, the pgkl-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. Fig. 6A shows that galactose induction of UPF1, NMD2, or UPF3 does not affect the abundance of the pgkl-UAG-7 mRNA (see “late pgkl”). 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 pgkl-UAG-7 mRNA is not a substrate for NMD.

Control experiments were also conducted to ensure that destabilization of the pgkl-UAG-2 mRNA subsequent to restoration of NMD was not due to an effect of galactose addition. Fig. 6B shows that upf1Δ, nmd2Δ, and upf3Δ strains containing either the early or late pgkl nonsense alleles and an empty GAL1-vector do not alter the stability of either the early or late pgkl 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, that causes destabilization of the pgkl-UAG-2 mRNA.
Discussion

In both prokaryotes and eukaryotes, the abundance of faulty mRNAs is tightly regulated by the NMD pathway (Leeds et al., 1991; Leeds et al., 1992; He et al., 1993; Peltz et al., 1993; Pulak and Anderson, 1993; Maquat, 1995; Jacobson and Peltz, 1996). The widespread existence of this pathway is significant because it implies that the function of NMD is a basic necessity for the prosperity of all cellular organisms. Although the mechanism by which this decay pathway recognizes and acts to degrade substrate transcripts is currently not known, it is clear that the NMD pathway is an evolutionarily conserved mechanism (see above). Perhaps the most compelling evidence that supports conservation of this mRNA decay pathway is the identification of homologous trans-acting factors in yeast, nemotodes, and mammalians (Pulak and Anderson, 1993; Peltz et al., 1994; Maquat, 1995). These homologues are similar in sequence but, more importantly, are also similar in protein function as evident by the ability of hUpf1p to complement the mRNA decay defect in a strain harboring a deletion of the yeast homologue (Perlick et al., 1996; Applequist et al., 1997). Furthermore, duplication of a yeast Upf1p dominant-negative mutation in its human counterpart also results in a protein with dominant-negative activity (Sun et al., 1998). While the similarities are evident, there are also distinct discrepancies between lower and higher eukaryotic systems which possibly identify the subtle differences in mechanism of mRNA decay. Presently, a significant issue of controversy is the cellular site of NMD.
Studies in yeast have largely contributed to the understanding of the NMD pathway and due to the wealth of evidence, it has long been accepted that NMD functions exclusively in the cytoplasm concurrent with translation (see Chapter 1). In contrast to these findings, numerous studies in mammalian systems have shown that the nucleus plays as an equally important role in the degradation of nonsense mRNAs. This conclusion is supported by the findings of fractionation experiments showing that the reduced abundance of nonsense TPI mRNA was similar in both nuclear and cytoplasmic fractions despite normal rates of transcription (Daar and Maquat, 1988; Cheng et al., 1990; Cheng and Maquat, 1993). Moreover, the nonsense-containing TPI mRNAs that were exported to the cytoplasm were not degraded by NMD and maintained the stability of its wild-type counterpart (Cheng and Maquat, 1993; Stephenson and Maquat, 1996). This argues that nonsense-containing mRNAs that evade the surveillance of NMD and enter the cytoplasm become immune to further susceptibility to degradation (Cheng and Maquat, 1993; Stephenson and Maquat, 1996). In addition to the TPI example, full stability of cytoplasmic immunoglobulin (Ig) and T cell receptor (TCR) nonsense-containing mRNAs have also been observed (Lozano et al., 1994; Carter et al., 1996). Therefore, in contrast to the findings in yeast, these studies suggest that recognition of aberrant nonsense-containing mRNAs and substrate degradation occur at, or in association with, the nucleus.

While the involvement of the nucleus in NMD has rarely been addressed in yeast, the notion of a nuclear-associated mRNA decay pathway in yeast cannot be completely dismissed since there have been several intriguing observations that do introduce nuclear-
associated NMD as formal possibility (see Chapter 4). As such, experiments presented here address the possibility that the actions of nonsense recognition, mRNA surveillance, and subsequent decay occur at the nucleus.

Because this hypothesis precludes nonsense mRNA degradation in the cytoplasm, we investigated the decay kinetics of the ade2-l and early-nonsense pgkl transcripts in response to activating NMD. Since these mRNAs are substrates for NMD, they show a significant difference in stability when NMD is inactive \( t_{1/2}=35-60 \text{ minutes} \) versus when NMD is induced \( t_{1/2}=2-6 \text{ minutes} \) (Fig. 2 and Peltz et al., 1993). This large difference in half-life makes it possible to address if the cytoplasmic nonsense mRNAs are either rapidly degraded by, or immune to, NMD.

The findings of these experiments supported previous conclusions contending that NMD does not solely occur in association with the nucleus. Induction of any of the UPF/NMD proteins in their respective deletion strains caused rapid and immediate degradation of the ade2-l and pgkl substrate transcripts such that by the 40 minute time point, the steady state levels of both substrate mRNAs were down to levels observed in a UPF/NMD strain (Figs. 4 & 6). This makes it unlikely that a stable mRNA population exists since the half life of the same RNA in the absence of NMD is greater than 30 minutes. Additionally, since induction of the UPF/NMD proteins actually occurs \( \sim 12-14 \) minutes after galactose addition (as determined from Figs. 3 & 7), the abundance of the ade2-l and pgkl substrate transcripts were actually reduced to UPF/NMD levels in less than 30 minutes.
Upon induction of the NMD pathway, the half lives of both the \textit{ade2-1} and \textit{pgk1} substrates at steady state were calculated to be approximately 7 minutes (as determined by the slopes in Fig. 7). This finding makes it highly improbable that the cytoplasmic mRNA population is immune to degradation. This is because if two populations of the same mRNA degrading at different rates were present (newly synthesized, nuclear associated mRNAs, \(t_{1/2} \leq 5\) min, and cytoplasmic mRNAs, \(t_{1/2} = 35-60\) minutes) then the overall decay rate of the that mRNA would be expected to approximate the average of the two subset populations. Upon comparison with the data obtained in these experiments, it is clear that the overall half lives of both substrate mRNAs (\(t_{1/2} = 7\) minutes; Fig. 7A&B) are significantly shorter than their hypothetical half lives (if two subset populations existed for each mRNA; Fig. 7B). Additionally, for the total mRNA population to show a 7 minute half life, the newly synthesized population would have to comprise at least 85% of the total mRNA. Because the transcription rate of \textit{ADE2} was determined to be 3.5 mRNAs/hour (Holstege et al., 1998), it is not possible to generate the amount of newly synthesized mRNA necessary to bring the total \textit{ade2-1} mRNA population half life down to 7 minutes during the course of these experiments. In contrast to the relatively slow transcription rate of \textit{ADE2}, the transcription rate of \textit{PGK1} was found to be 110.5 mRNAs/hour (Holstege et al., 1998). Interestingly, both the \textit{ade2-1} and \textit{pgk1} mRNA populations show identical half-lives regardless of the large differences in their transcription rates. Therefore, we believe that the transcription rate does not make a large contribution to the overall decay kinetics of the mRNA populations in our experiments. These findings clearly demonstrate that the cytoplasmic nonsense mRNA populations are
susceptible to degradation by NMD and, furthermore, turnover of these substrate transcripts occurs on polysomes (Fig. 5), again, consistent with NMD occurring in the cytoplasm.

Although these results suggest that NMD is not solely limited to the nucleus, they do not completely rule out the existence of a nuclear-associated decay mechanism in yeast. Differences in the efficiency of RNA processing and export may explain the contrasting findings between yeast and mammals. Under one notion, the degradation machinery of is located in the cytoplasm while the necessary processes that recognize and target a substrate RNA to this decay pathway reside in or at the nucleus. Assuming that the higher eukaryotes appear to have more efficient mechanisms in “preparing” the RNA, the decay machinery is able act rapidly on substrate RNAs. As a result, the turnover of most substrate transcripts occurs while still associated with the nucleus during transport. By this model, the less efficient mechanisms for preparing substrates for NMD in yeast increase the probability that substrate transcripts escape to the cytoplasm. Once in the cytoplasm, only those RNAs that have been appropriately processed become rapidly degraded by the NMD machinery. This model would also assume that efficient preparation of the substrate for degradation requires full/near exit from the nucleus. To accommodate this, factors involved in preparing the substrate in this model might be located on the cytoplasmic side of the nuclear membrane.

Alternatively, the mechanisms of RNA processing and transport may occur more rapidly in yeast due to a lower level of complexity involved in the “preparation” of targeted substrates. The outcome is expected to be the same where in yeast, rapid
transport out of the nucleus results in predominantly cytoplasmic degradation. In mammals, if it is assumed that RNA processing and transport out of the nucleus is slower, degradation would be observed mainly in association with the nucleus.

Consistent with the idea that substrate mRNAs must be prepared/processed appropriately for degradation by NMD, recent studies have identified a factor, Hrp1p, that appears to target transcripts for selective degradation by NMD (Kessler et al., 1997; Shen et al., 1998; Gonzalez et al., 2000). This RNA-binding protein is capable of shuttling between the nuclear and cytoplasmic compartments and can interact with both Upf1p and the cis-acting downstream element (DSE) that appears to be required for degradation of some nonsense mRNAs (Kessler et al., 1997; Shen et al., 1998; Gonzalez et al., 2000). According to this model, Hrp1p binds to newly synthesized transcripts. Subsequent cytoplasmic remodeling of the mRNA and translation of the open reading frame result in displacement of Hrp1p. However, events that result in the failure to translate an mRNA completely (i.e. premature termination) prevents displacement of Hrp1p. Bound Hrp1p then interacts with Upf1p to initiate rapid degradation of the errant transcript (Czapinski et al., 1999; Gonzalez et al., 2000). This model incorporates the findings in mammalian systems that contend immunity for nonsense mRNAs that escape nuclear retention. In this case, such mRNAs could somehow avoid interaction with Hrp1p. Devoid of this "mark", these transcripts should be immune from further susceptibility to rapid decay.

Additional findings presented in this thesis, however, do not support this model of bound Hrp1p acting as the "mark" responsible for targeting substrates for NMD (see
Chapter 5). These results demonstrate that inactivation of NMD by mutations in any of the \textit{UPF/NMD} genes reduces the efficiency of translation termination which, in turn, enhances translational readthrough past the premature nonsense codon. Therefore, under conditions where NMD is inactivated by \textit{UPF/NMD} gene mutations, Hrp1p is still capable of being displaced by the translating ribosomes. According to the tenets of the model presented, devoid of Hrp1p, these nonsense mRNAs that are exported to the cytoplasm are expected to remain stable upon restitution of the NMD pathway. The experiments presented in this chapter show that activation of the NMD pathway results in immediate degradation of cytoplasmic nonsense mRNAs (Figs. 4, 6, & 7) making it unlikely that bound Hrp1p targets mRNAs for degradation by NMD.

Due to the interest in understanding the fundamental differences between yeast and mammalian NMD, the cellular site of NMD has recently emerged as a key focus of investigation. Although the results of these and other experiments clearly contend that the action of this decay pathway is localized to the cytoplasm in yeast, it is apparent that the nuclear compartment plays a significant role in mammalian NMD. The involvement of the nucleus in yeast NMD remains unclear and determination of the precise subcellular location of this decay pathway is important as it not only provides insight into the molecular mechanisms of function, but also aids in the identification of other factor/processes that might contribute to the efficiency of this quality-control mechanism.
Figure 2. The *ade2-1* transcript is a substrate for NMD
**Figure 2. The ade2-1 transcript is a substrate for NMD.** Total RNA isolated from yeast strains with the indicated *UPF/NMD* genotypes was analyzed by Northern blotting with DNA probes that detected the *ade2-1* and *CYH2* transcripts. *WT*, wild type. Yeast strains used in this experiment were: HFY1200, HFY870, HFY1300, HFY861, HFY1067, and HFY1081.
Figure 3. Galactose inducible expression of UPF1, NMD2, and UPF3
Figure 3. Galactose inducible expression of UPF1, NMD2, and UPF3. The upf1Δ, nmd2Δ, and upf3Δ yeast strains harboring the appropriate galactose inducible NMD gene constructs were grown in SC -uracil, raffinose liquid media to mid log phase (OD₆₀₀=0.5). Galactose was then added to a final concentration of 2% and aliquots were taken at 10 minute intervals for protein isolations. Isolated protein samples were then analyzed by Western blotting. Yeast strains used in this experiment were: HFY1200, HFY870, HFY1300, and HFY861.
Figure 4. The ade2-1 transcript is rapidly degraded upon activation of NMD.

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Figure 4. The ade2-1 transcript is rapidly degraded upon activation of NMD. (A) Activation of NMD causes rapid degradation of 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, or 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 plasmid (GAL-vector) was analyzed by Northern blotting with DNA probes that detected the ade2-1 transcript. Yeast strains used in this experiment were: HFY1200, HFY870, HFY1300, and HFY861.
Figure 5. Degradation of the \textit{ade2-1} mRNA population occurs on polysomes.

A.
Figure 5. Degradation of the *ade2-1* mRNA population occurs on polysomes. (A) The *ade2-1* mRNA is detected in the polysome fractions before galactose induction. Total RNA isolated from polysome fractions collected before the addition of galactose was analyzed by Northern blotting with DNA probes that detected the *ade2-1* mRNA and the *SCRI* RNA (the latter to serve as a control to ensure that RNA was isolated from the polysome fractions). (B) The *ade2-1* mRNA is no longer detected in the polysome fractions upon activation of NMD. Total RNA isolated from polysome fractions collected 30 minutes after the addition of galactose was analyzed by Northern blotting as described above in (A). The results depicted in this figure were obtained from the *upf1Δ* yeast strain (HFY870) harboring the galactose-inducible *UPF1* construct.
Figure 6. Early nonsense *pgkl* mRNA degrades rapidly upon activation of NMD.

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Figure 6. 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 transcript. Total RNA isolated from yeast strains with the indicated UPF/NMD genotypes harboring the appropriate galactose-inducible NMD gene construct (GAL-UPF1, GAL-NMD2, or GAL-UPF3) 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 UPF/NMD genotypes harboring only the vector plasmid (GAL-vector) was analyzed by Northern blotting as described above in (A). Yeast strains used in this experiment were: HFY1200, HFY870, HFY1300, and HFY861.
Figure 7. Activation of NMD results in the rapid decay of substrate transcripts.

A.
B.

![Graph showing the percentage of relative mRNA level over time for different genotypes. The x-axis represents time in minutes (0 to 50), and the y-axis represents the percentage of relative mRNA level (from 1 to 1000). The graph includes lines for ade2-l (hypothetical), ade2-l, pgk1 early (hypothetical), and pgk1 early.](image)
Figure 7. Activation of NMD results in the rapid decay of substrate transcripts.

(A) Graphical depiction of the relative levels of the $ade2\text{-}1$ (○), early nonsense $pgkl$ (□), and late nonsense $pgkl$ (■) mRNAs with the relative level of induced protein, Nmd2p (●), upon activation of NMD (by the addition of galactose). The data for the construction of this graph was quantitated from the Northern blots of Figure 4A ($ade2\text{-}1$) and Figure 6A (early and late $pgkl$) and from the Western blot of Figure 2 (Nmd2p). (B) Graph comparing a hypothetical model where cytoplasmic nonsense mRNAs ($ade2\text{-}1$ (●) and early nonsense $pgkl$ (■) mRNAs; dashed lines) remain stable upon restitution of the NMD pathway to the actual data obtained in these experiments. For $ade2\text{-}1$, the half life for the overall population would be expected to be between 18 and 20 minutes and for $pgkl\text{-}UAG\text{-}2$ (early nonsense $pgkl$), the half life for the overall population would be expected to be approximately 33 minutes.
CHAPTER 4

CHARACTERIZATION OF THE NUCLEAR LOCALIZATION SIGNAL OF Nmd2p

Introduction

In yeast, there is a tremendous amount of evidence that supports the notion of NMD as a translation-dependent decay mechanism (see above). As such, the cellular site of NMD is postulated to be solely the cytoplasmic compartment. However, structural analyses of the essential trans-acting components of the yeast NMD pathway revealed some surprising motifs. Nmd2p was found to harbor a motif characteristic of a putative bipartite nuclear localization sequence (NLS) at its amino terminus (Fig. 8) and Upf3p was found to contain several domains resembling NLS and nuclear export signals (NESs) (Cui et al., 1995; He and Jacobson, 1995; Lee and Culbertson, 1995; He et al., 1997). The existence of these sequence elements within these proteins introduces the possibility that NMD is not solely limited to the cytoplasm, but may also involve a nuclear component. Consistent with this notion are findings in mammalian systems that identify examples of transcripts susceptible to nuclear-associated NMD (Daar and Maquat, 1988; Cheng et al., 1990; Cheng and Maquat, 1993; see Chapter 3). Additionally, Upf3p has been shown to be capable of shuttling between the nucleus and the cytoplasm, and its translocation between these cellular compartments appears to play an important role for its function in mRNA decay (Lee and Culbertson, 1995; Shirley et al., 1998). In fact,
mutations in Upf3p that cause mislocalization of the protein were found to coincide with a loss of NMD function (Lee and Culbertson, 1995; Shirley et al., 1998).

The importance of the Nmd2p NLS region is underscored by analyses of mutations in NMD2 demonstrating that deletion of the 21 amino acid region comprising the putative NLS region is sufficient to inactivate NMD (He and Jacobson, 1995). This finding suggests that the putative NLS region of Nmd2p is essential for the protein’s function in decay and further supports the possibility of a nuclear component involved in mRNA decay. Therefore, experiments were conducted to address the legitimacy of the Nmd2p NLS by determining if the decay function of Nmd2p was dependent upon nuclear localization of the protein.

Initial deletion analyses of the putative Nmd2p NLS found that its basic regions, previously characterized as residues critical for proper nuclear targeting, were also critical for the decay function of Nmd2p. Subcellular fractionation was then used to show that, while the bulk of Nmd2p is present in the cytoplasm, a small proportion of the protein could be detected in purified nuclei. When the entire putative NLS region was deleted from NMD2, the amount of protein detected in the nuclear fraction was reduced, implying an interdependence between Nmd2p’s decay function and its nuclear localization. However, other nmd2 NLS-mutants that showed identical decay defects as the nmd2 NLS deletion mutant did not show comparable reductions in the amount of protein in purified nuclear fractions. Therefore, these findings do not correlate the decay function of Nmd2p with its nuclear localization.
Results

Independent deletion of either basic region of the putative NLS inactivates Nmd2p function in decay.

The protein sequence of Nmd2p spanning residues 26-46 exhibits a motif characteristic of a bipartite NLS. This putative NLS region is comprised of two clusters of basic amino acids separated by a spacer region of 12 amino acids (Fig. 8). Previous characterization of similar nuclear targeting sequences has demonstrated that the two clusters of basic residues are critical for proper nuclear localization (Laskey and Dingwall, 1993). Further studies showed that the amino acids located in the intervening spacer region (usually containing between 10-15 residues) were random and could be mutated without affecting nuclear targeting, regardless of whether or not they were replaced with basic residues (Laskey and Dingwall, 1993).

Because the clusters of basic residues were identified as the most critical in determining proper nuclear targeting, these regions of the Nmd2p NLS were initially chosen as targets for mutagenesis. This was accomplished by site-directed mutagenesis of a single-copy NMD2-containing plasmid. The mutated plasmids were then introduced into a yeast strain harboring a deletion of the NMD2 gene and the function of these alleles determined by their ability to promote the degradation of a endogenous substrate transcript of the NMD pathway, the CYH2 pre-mRNA (He et al., 1993; He and Jacobson, 1995). (Since the fully spliced, mature CYH2 mRNA does not contain a premature
nonsense codon, it is unaffected by perturbations in the NMD pathway and serves as an internal control for the northern blot assay).

As demonstrated in Fig. 9, independent deletion of either basic region of the putative NLS was found to increase the accumulation of the CYH2 pre-mRNA, suggesting a NMD defect. Therefore, the amino acids that comprise the basic clusters of the Nmd2p NLS (residues 26-29 and residues 42-46) are critical for the protein’s function in mRNA decay. The severity of the mutations’ effects were quantitated by comparing the extent of stabilization of the CYH2 pre-mRNA with that of the strain that harbors a complete NMD2 deletion and the isogenic NMD2 (wild type) strain. Deletion of the first group of basic residues (nmd2-Δ1) displayed an intermediate phenotype and therefore did not completely abolish activity of Nmd2p (Fig. 9). However, deletion of the distal basic cluster (nmd2-Δ2) exhibited a phenotype identical to that of an nmd2Δ strain (Fig. 9) suggesting complete abrogation of Nmd2p activity. These results implicate residues 42-46 as an essential group of amino acids for the function of Nmd2p in decay. To verify that the mutations did not produce unstable proteins, western blot assays were conducted on lysates prepared from strains containing the respective nmd2 alleles. These experiments demonstrated that all of the nmd2 alleles that exhibited an mRNA decay defect produced stable proteins (Fig. 12).

The nmd2 alleles were then introduced into the isogenic NMD2 strain in order to determine if the mutations had dominant negative activities. As shown in Fig. 9, deletion of either basic cluster has no effect on the accumulation of the CYH2 pre-mRNA suggesting that these mutations are not dominant negative.
Mutations in the distal basic region of the Nmd2p NLS affect mRNA decay function. Since removal of either basic cluster of the Nmd2p NLS adversely affected activity of the protein, a second round of mutagenesis was conducted in an attempt to identify point mutations that also abrogated Nmd2p decay function. In these experiments, each of the amino acids spanning the two basic regions was replaced with alanine. The effect of these alanine substitutions on the resulting protein’s function was again monitored by assaying the accumulation of the CYH2 pre-mRNA. The results from this set of experiments demonstrated that alanine scanning of either basic region did not have any significant effects on the accumulation of the CYH2 pre-mRNA (Fig. 10). All of these nmd2 alleles showed phenotypes identical to that of the isogenic N1v2 strain. Therefore, substitution of alanine into any of the positions within the basic regions of the putative NLS was not sufficient to inactivate Nmd2p function for mRNA decay.

Because single point mutations did not adversely affect Nmd2p function, a subsequent round of mutagenesis was conducted to generate double point mutations. From previous studies of bipartite NLSs, it was determined that the first amino acid in the second cluster of residues was one of the more crucial residues in assuring proper nuclear localization (Laskey and Dingwall, 1993). As such, the lysine to alanine substitution at this position (denoted as position 5 in Figs. 10 and 11) was retained as the primary point mutation and the secondary mutation was generated by systematically replacing each of the remaining residues of the two basic clusters with alanine. The effect of these mutations on Nmd2p activity was again monitored by measuring CYH2 pre-mRNA accumulation. Double point mutations harboring the secondary mutation in the first basic
region had no effect on Nmd2p activity since the strains containing these *nmd2* alleles showed a phenotype comparable to that of the isogenic *NMD2* strain (*nmd2-mu1,5-mu4,5; Fig. 11). When the secondary mutation was placed in the distal basic cluster, two mutants were identified that did show a significant effect on Nmd2p activity: the *nmd2-mu5,6* mutant (alanine substitution of the first two adjacent residues of the second cluster) and the *nmd2-mu5,9* mutant (alanine substitution of the terminal residues of this cluster with alanine) (Fig. 11). The mRNA decay defect of these double point mutants was slightly less than that of a *nmd2Δ* strain and more comparable to the *nmd2-Δ1* mutant. Therefore, these experiments demonstrated that only double point mutations in the distal cluster of basic residues could adversely affect Nmd2p activity, suggesting that this group of amino acids is of greater importance for decay function than those located in the first cluster. This notion is supported by the previous finding that deletion of the second cluster of basic residues shows a more pronounced decay defect than deletion of only the first cluster of basic residues (Fig. 9). Western blot assays again verified that the point mutations that resulted in a decay defect (i.e. *nmd2-mu5,6* and *nmd2-mu5,9*) still produced stable, full-length proteins (Fig. 12).

Taken together, analysis of *nmd2*-NLS mutants demonstrated that mutation of residues previously identified to be important for nuclear targeting in the context of the NLS also had adverse affects on Nmd2p function. Therefore, it seemed plausible that the reduction in Nmd2p activity may be a consequence of the failure to properly localize to the nucleus. The experiments described below address the effects of these NLS mutations on nuclear localization of Nmd2p.
A fraction of Nmd2p is detected in yeast nuclei.

Because it is has been widely accepted that NMD is a cytoplasmic process, the amount of nuclear localized Nmd2p had yet to be investigated. Therefore, in order to address the effects of mutations in the Nmd2p NLS on nuclear localization, an assay was initially needed to establish that Nmd2p was localized to the nucleus under wild type conditions. As such, subcellular fractionation experiments were used to determine the extent of Nmd2p nuclear localization. Differential centrifugation through 30-50% Ficoll gradients allowed for the separation of nuclei from the remainder of cellular organelles/compartments. All of the collected fractions were then analyzed by Western blotting. Fractionation of a NMD2 extract demonstrated that although Nmd2p was predominantly found in the fractions representative of cytoplasmic compartments, a small amount of protein (approximately 5-10%) could be detected in the purified nuclei fraction (Fig. 13). Since these results show that Nmd2p is indeed present in purified nuclei, it is possible that Nmd2p performs a nuclear-associated function that is essential for its function in NMD.

To ensure that the fractionation procedure was successful in separating the nuclei from cytoplasmic components, several controls were utilized. Rpo21p, the α-subunit of RNA polymerase (Himmelfarb et al., 1987), and Pbp1p, a poly-A binding protein interactor (Mangus et al., 1998), were used as the nuclear markers and were both found to be enriched in the purified nuclear fraction (Fig. 13). Pgk1p, a soluble, cytoplasmic protein involved in glycolysis (Lam and Marmur, 1977), was used as the cytoplasmic marker and was detected in all fractions except for the purified nuclei (Fig. 13).
Therefore, the fractionation procedure proved to be a reliable assay for nuclear association.

Mutations in the putative NLS that abrogate NMD do not affect nuclear localization of Nmd2p

Because isolation of purified yeast nuclei using the subcellular fractionation protocol allowed for the detection of nuclear-associated Nmd2p in wild type cells (Fig. 13), this method was used to directly assay the effects of the nmd2-NLS mutations on the nuclear targeting of the resulting protein. The objective of these experiments was to determine whether it was possible to correlate Nmd2p function with its nuclear localization. To minimize experimental error, fractionation of the NMD2 strain was always done in parallel with the nmd2-NLS mutant strain to be tested. The nuclear protein, Swi3p (Peterson and Herskowitz, 1992), was used as an internal loading control to quantitatively compare the amount of Nmd2p in purified nuclear fractions isolated from NMD2 cells and nmd2-NLS mutant cells.

The first of these studies investigated the consequence of deleting the entire Nmd2p NLS region (amino acids 26-46) on nuclear localization by comparing the amount of protein in nuclear fractions isolated from the nmd2-ANLS mutant with that of the isogenic NMD2 strain. These experiments demonstrated that the amount of Nmd2p in the nuclear fraction was approximately 4 fold greater in the NMD2 strain than in the nmd2-ANLS strain (Fig. 14). These results are consistent with the Nmd2p putative NLS acting as a bona fide nuclear targeting sequence since deletion of the putative nuclear
targeting sequence coincided with a reduction in the amount of nuclear localized protein. When another nuclear protein (Pbp1p) was used as the internal control, the results were the same as those as exhibited in Fig. 14 (data not shown). Therefore, since this mutant also displayed a null phenotype for mRNA decay, these findings suggest that the function of Nmd2p in NMD is dependent upon proper nuclear localization.

The \textit{nmd2-}\textit{A2} mutant was found to have a decay defect identical to that of the \textit{nmd2A} and the \textit{nmd2-}\textit{ANLS} strains (Fig. 9 and He and Jacobson, 1995). As such, the effect of this mutation on nuclear localization was expected to be similar to those obtained in the \textit{nmd2-}\textit{ANLS} strain. However, when these experiments were repeated with the \textit{nmd2-}\textit{A2} mutant, the amount of nuclear associated Nmd2p was found to be comparable to that present in the isogenic \textit{NMD2} strain (Fig. 14). These results suggest that decay function and nuclear localization are not interdependent in this mutant. Similar findings were obtained when comparing any of the \textit{nmd2-NLS} mutants that exhibited a NMD defect (i.e. \textit{nmd2-mu5,6} and \textit{nmd2-mu5,9}) to that of the isogenic \textit{NMD2} strain (data not shown). Collectively, these findings demonstrate that the effects of these mutations on the activity of Nmd2p are not related to the nuclear localization of the protein.

**Deletion of \textit{UPF3} affects nuclear localization of Nmd2p**

Structural analyses of the \textit{UPF3} gene product revealed three putative NLS motifs within its protein coding region. More recently, studies have demonstrated that all of the NLS sequences can target reporter proteins to the nucleus (Shirley et al., 1998). Because
Upf3p and Nmd2p are interacting components of the NMD machinery (He et al., 1997), we became interested in determining if deletion of UPF3 would have any effect on nuclear targeting of Nmd2p. As shown in Fig. 15, isolation of purified nuclei from a upf3Δ strain resulted in a 2-3 fold reduction in the amount of nuclear associated Nmd2p when compared to the isogenic NMD2 strain. These results are consistent with Upf3p playing a role in localizing Nmd2p to the nucleus.

Since deletion of UPF3 and deletion of the NLS region of Nmd2p showed similar effects of Nmd2p nuclear localization, we then investigated whether the two mutations together would have an additive effect and further reduce localization of Nmd2p to the nucleus. To accomplish this, the nmd2-NLSΔ allele was introduced into an isogenic strain harboring a deletion of both NMD2 and UPF3. A comparison of the nuclear Nmd2p levels between the NMD2 strain and the strain harboring both the nmd2-NLSΔ and the upf3Δ mutations showed a 2-fold reduction in the mutant (Fig. 15). These results suggest that the two mutations in combination do not cause an additive reduction in the amount of nuclear localized Nmd2p since this decrease is no more significant than either mutation independently.

**Deletion of NMD2 and UPF3 do not affect Upf1p nuclear localization**

Additional experiments were conducted to address the possibility of a nuclear component involved in NMD. The third essential component of the NMD machinery is Upf1p. It is postulated that both Nmd2p and Upf3p interact with Upf1p to regulate its activity (see Chapter 5). Unlike Nmd2p and Upf3p, structural analyses of Upf1p did not reveal the
presence of any nuclear targeting sequences within its protein coding region. Therefore, if nuclear localization of these interacting proteins played a critical role in NMD, we believed that transport to the nuclear compartment was mediated by either Nmd2p or Upf3p or both factors. Because of this, we sought to investigate the effect of deleting NMD2 and UPF3 on Upflp nuclear localization. Previous fractionation experiments (David Mangus, unpublished expts) demonstrated that the cellular distribution of Upflp was very similar to that of Nmd2p, that is, the majority of the protein resided in the cytoplasm with a small fraction associated with the nucleus (data not shown). Subcellular fractionation of a nmd2Δ,upf3Δ strain and the isogenic NMD2, UPF3 strain demonstrated no significant difference in the amount of Upflp in the respective purified nuclear fractions, suggesting that Nmd2p and Upf3p do not contribute to Upflp nuclear localization (data not shown). Therefore, the defect in NMD of a nmd2Δ,upf3Δ strain is not due to the inability of Upflp to localize to the nucleus. Taken together, these results make it unlikely that an essential nuclear component for NMD exists.

Discussion

The sequence of the Nmd2p NLS is similar to other nuclear targeting sequences

Nuclear proteins are targeted to the cell nucleus because they contain nuclear localization signals (NLSs) that allow for selective recognition and translocation through the nuclear pore complex (Dingwall and Laskey, 1986; Goldfarb, 1989; Melchoir and Gerace, 1995). The first nuclear targeting signal to be analyzed at the amino acid level was that of the
SV40 large T antigen where a short basic sequence (PKKKRKV) was found to be sufficient to direct reporter proteins to the cell nucleus (Kalderon et al., 1984; Kalderon et al., 1984). Following the discovery of this NLS, another nuclear targeting signal was identified consisting of two essential domains of basic amino acids that function in an interdependent manner and a spacer region of at least 10 amino acids that separates the two basic domains (Dingwall et al., 1988; Dingwall et al., 1989). The significance of this type of nuclear targeting sequence was evident from the observation that it is found in almost half of the nuclear proteins in the SwissProt database and in less than 5% of nonnuclear proteins (Robbins et al., 1991). Therefore, the presence of this type of NLS within a protein's coding region appears to be, at the very least, suggestive of a nuclear protein.

Amino acid sequence analyses of Nmd2p identified a motif that was very similar to this consensus bipartite NLS and comparison of this sequence to the NLSs of proteins that have been characterized as bona fide nuclear proteins demonstrated that the sequence of the Nmd2p NLS was quite similar to that of several nuclear proteins (Fig. 16). Although the presence of a nuclear targeting sequence in Nmd2p was somewhat unanticipated since yeast NMD is considered to be strictly a cytoplasmic, translation-dependent event, the notion of NMD taking place in the nuclear compartment is not novel. Evidence presented in the previous chapter (Chapter 3) highlighted some of the studies conducted in mammalian systems that implicated NMD of specific substrates occurring in the nucleus. In spite of the fact that mammalian NMD is presumed to be a more intricate mechanism, a number of studies have shown that homologues of the same
trans-acting factors that are essential for yeast NMD exist in the mammalian system implying evolutionary conservation of this decay pathway (Perlick et al., 1996; Applequist et al., 1997). Consistent with this are studies demonstrating that expression of a dominant-negative mutant of a human UPF1 homologue in mammalian cells abrogated NMD (Sun et al., 1998). Therefore, if the mechanism of NMD is truly conserved, the existence of a nuclear targeting signal in a necessary component of the yeast decay pathway and the involvement of the nucleus in yeast NMD should not be so unexpected. Accordingly, studies presented in this chapter were conducted to determine if the 21 amino acid sequence of Nmd2p that resembled a NLS was indeed a nuclear targeting signal.

Is Nmd2p a nuclear protein?

Initial analyses of NMD2 mutants found that deletion of the putative NLS region from this protein resulted in a null phenotype, suggesting that the residues that comprise the putative NLS of Nmd2p were indeed important for the function of this protein in decay (He and Jacobson, 1995). To characterize critical residues of the Nmd2p putative NLS, further mutational analyses were conducted on those amino acids previously identified as being necessary for nuclear targeting. The findings of these studies demonstrated that mutation of residues previously characterized as being necessary for proper nuclear localization coincided with a loss of Nmd2p function. Furthermore, these nmd2-NLS mutations still produced proteins that cosedimented with the polysome fractions on sucrose gradients suggesting that these particular mutations did not disrupt the interaction
of Nmd2p with cytoplasmic, translating ribosomes (data not shown). Therefore, it was conceivable that the consequence of these mutations was the mislocalization of Nmd2p, leading to a loss of function phenotype. It seemed plausible that the function of Nmd2p in decay would be dependent upon proper localization to the nucleus.

*NMD2* mutations that cause loss of NMD function do not correlate with perturbations in nuclear localization

In an attempt to test the hypothesis that Nmd2p requires nuclear localization for proper function in NMD, fractionation experiments were conducted on the *nmd2*-NLS mutants that exhibited an NMD defect to determine if these mutations also prevented Nmd2p localization to the nucleus. While deletion of the entire putative NLS region appeared to significantly reduce Nmd2p localization to nucleus, the remaining *nmd2*-NLS mutants which showed similar decay defects (i.e. *nmd2*-Δ1, -Δ2, -mu5,6 and mu -5,9) had no effect on nuclear accumulation of Nmd2p (Figs. 9-11 and 14). Consequently, a correlation between Nmd2p function and its nuclear localization could not be established.

Coincident with the experiments presented above were several other experiments that directly addressed Nmd2p cellular localization. The first of these studies incorporated the use of green fluorescent protein (GFP) to observe the localization of Nmd2p in live yeast cells. For these experiments, a plasmid containing the GFP sequence fused to the amino terminal end of the *NMD2* gene was constructed and transformed into a *nmd2Δ* strain. The GFP-*NMD2* fusion was able to restore normal NMD to the *nmd2Δ* strain, demonstrating that inclusion of the GFP reporter did not
inhibit Nmd2p activity (F. He, unpublished expts). Microscopy of strains harboring this plasmid (at single- or high-copy number) showed that GFP-tagged Nmd2p was predominantly dispersed throughout the cytoplasm and seemingly excluded from the nuclei (F. He, unpublished expts). To substantiate these conclusions, purified nuclei were subsequently isolated from this strain to determine if any fluorescence emitted by the tagged-Nmd2p could be visualized in the nuclei. Microscopy of purified nuclei isolated from the nmd2Δ strain containing GFP-NMD2 plasmid (at single- or high-copy number) demonstrated no detectable difference in fluorescence when compared to the isogenic strain containing the same NMD2 plasmid without the GFP tag (data not shown). These results imply that either Nmd2p does not normally localize to the nucleus or that very little Nmd2p localizes to the nucleus such that detection by microscopy is not possible. Although these results do not completely rule out the possibility of Nmd2p shuttling between the nucleus and the cytoplasm, they do demonstrate that the localization and function of Nmd2p is predominantly in the cytoplasm. This argument is strengthened by the previous finding that a dominant-negative mutant of Nmd2p inactivates NMD when localized to the cytoplasm but fails to stabilize nonsense-containing mRNAs when fused to a nuclear localization signal (He and Jacobson, 1995)

The possibility of a nuclear function in yeast NMD continues to be an intriguing subject of study. Although most of the evidence supports a cytoplasmic pathway, there are implications that the nucleus plays an important role in NMD. Studies on Upf3p, for example, have identified sequences resembling nuclear import and export signal sequences and some mutations in the stretches of amino acids making up the export
signals coincided with mislocalization of the mutant protein (nucleolar localization) and a loss of NMD function (Shirley et al., 1998). While this finding implies that the NMD pathway is reliant upon proper cellular localization of the necessary \textit{trans}-acting factors involved, and that the nucleus may play an important role, it is evident that the function of NMD appears to be dependent on transport to the cytoplasm. Clearly, new methods to address nuclear versus cytoplasmic mRNA degradation are necessary to fully understand the mechanisms involved in this important process as well as the role and importance of the nucleus in NMD.
Figure 8: Structural Features of Nmd2p.
Figure 9. Deletion of either basic region in the putative NLS of Nmd2p results in loss of Nmd2p function in decay.

<table>
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<tr>
<th>Allele</th>
<th>Amino Acid Sequence</th>
<th>CYH2 pre-mRNA/mRNA</th>
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</thead>
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<tr>
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<td>Δ2</td>
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A.

B.

![Diagram showing CYH2 pre-mRNA and mRNA levels for different alleles](image-url)
Figure 9. Deletion of either basic region in the putative NLS of Nmd2p results in loss of Nmd2p function in decay. (A) Deletion of the basic regions has an effect on Nmd2p function. The basic amino acids are shown in bold and the basic regions are boxed. The *CHY2* pre-mRNA/mRNA ratio is used to assess decay defect; a higher ratio is indicative of a decay defect. (B) Total RNA isolated from yeast strains with the indicated *UPF/NMD* genotypes was analyzed by Northern blotting with DNA probes that detected the *CYH2* transcripts. Yeast strains used in this experiment were: HFY1200 and HFY1300.
Figure 10. Point mutations in the basic regions of the NLS do not alter the activity of Nmd2p

A.

<table>
<thead>
<tr>
<th>Allele</th>
<th>Amino Acid Sequence</th>
<th>CYH2 pre-mRNA/mRNA</th>
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</thead>
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<td>NMD2</td>
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</tr>
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<td>9</td>
<td>KSKK...KKLKA</td>
<td>0.09</td>
</tr>
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B.

NMD2

nmd2

1 2 3 4 5 6 7 8 9

← CYH2 pre-mRNA

← CYH2 mRNA
Figure 10. Point mutations in the basic regions of the NLS do not alter the activity of Nmd2p. (A) Alanine scanning of the basic regions does not affect Nmd2p decay function. The alanine substitutions and basic amino acids are shown ("...." represents the twelve residues located between the two basic regions). The CHY2 premRNA/mRNA ratio is used to assess decay defect; a higher ratio is indicative of a decay defect. (B) Total RNA isolated from nmd2Δ strains transformed with the indicated mutant plasmid was analyzed by Northern blotting with DNA probes that detected the CYH2 transcripts. Yeast strains used in this experiment were: HFY1200 and HFY1300.
Figure 11. Specific double point mutations in the NLS affect Nmd2p decay function

### A.

<table>
<thead>
<tr>
<th>Allele</th>
<th>Amino Acid Sequence</th>
<th>CYH2 pre-mRNA/mRNA</th>
</tr>
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### B.

![Diagram showing CYH2 pre-mRNA and CYH2 mRNA]
Figure 11. Specific double point mutations in the NLS affect Nmd2p decay function.

(A) Multiple alanine substitutions in the second basic region affect Nmd2p decay function. The alanine substitutions and basic amino acids are shown ("....." represents the twelve residues located between the two basic regions). The CHY2 pre-mRNA/mRNA ratio is used to assess decay defect; a higher ratio is indicative of a decay defect. (B) Total RNA isolated from nmd2Δ strains transformed with the indicated mutant plasmid was analyzed by Northern blotting with DNA probes that detected the CYH2 transcripts. Yeast strains used in this experiment were: HFY1200 and HFY1300.
Figure 12. Mutations in the Nmd2p NLS produce stable proteins.
Figure 12. Mutations in the Nmd2p NLS produce stable proteins. Whole cell lysates were prepared from the nmd2\Delta yeast strain harboring the indicated mutant plasmid. The yeast cells were grown to log phase and harvested when they reached an OD\textsubscript{600} of 0.6-1.0. Equal amount of cells were collected from each strain based on OD\textsubscript{600} (0.2mL collected/OD\textsubscript{600}; Mangus et al., 1998). The protein samples were then analyzed by Western blotting using a polyclonal anti-Nmd2p antibody to detect the protein. Yeast strains used in this experiment were: HFY1200 and HFY1300.
Figure 13. Although predominantly cytoplasmic, some Nmd2p can be detected in purified nuclei.

<table>
<thead>
<tr>
<th>Lysate</th>
<th>Ficoll input</th>
<th>Lipid layer</th>
<th>20% Ficoll</th>
<th>20-30% Interface</th>
<th>Ficoll pellet</th>
<th>40% Ficoll, 2X (nuclei)</th>
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</thead>
<tbody>
<tr>
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<td>( \alpha )-Pgk1p</td>
</tr>
</tbody>
</table>
Figure 13. Although predominantly cytoplasmic, some Nmd2p can be detected in purified nuclei. Yeast nuclei were isolated from a wild-type (NMD2) strain by osmotic lysis of spheroplasts, followed by banding two times on Ficoll gradients. The composition of each fraction presented in this figure is as follows: the Lysate = lysed yeast extract: contains total cellular proteins; Ficoll input = lysate before it is loaded onto the Ficoll gradient: contains total cellular proteins; Lipid layer = topmost layer of the Ficoll gradient post fractionation: contains primarily lipids/membranes; 20% Ficoll fraction: contains cytosolic proteins and polysomes; 20-30% Interface: contains vacuoles, mitochondria, membrane vesicles, and polysomes; Ficoll pellet: contains spheroplasts and cell wall fragments; 40% Ficoll: contains nuclei. Isolated protein samples were analyzed by Western blotting using the following antibodies: polyclonal anti-Nmd2p (to detect Nmd2p), anti-Tcm1p (a ribosomal protein; used as a nuclear/cytoplasmic control), anti-Rpo21p (α-subunit of RNA polymerase II; used as a nuclear control), and anti-Pgk1p (a cytoplasmic protein involved in glycolysis; used as a cytoplasmic control). The initial fractionation experiments (shown in this figure) were conducted by David Mangus, Ph.D. His helpful advice was invaluable and is well appreciated. Yeast strains used in this experiment were: HFY1200 and HFY1300.
Figure 14. Mutants that abrogate decay function do not correlate with a loss in nuclear localization.
Figure 14. Mutants that abrogate decay function do not correlate with a loss in nuclear localization. (A) Yeast nuclei were isolated from a wild-type (NMD2) strain and isogenic strains harboring mutations in the Nmd2p NLS by osmotic lysis of sphereoplasts, followed by banding two times on Ficoll gradients. The composition of each fraction presented in this figure is as follows: the Lysate = lysed yeast extract: contains total cellular proteins; Ficoll input = lysate before it is loaded onto the Ficoll gradient: contains total cellular proteins; Lipid layer = topmost layer of the Ficoll gradient post fractionation: contains primarily lipids/membranes; 20% Ficoll fraction: contains cytosolic proteins and polysomes; 20-30% Interface: contains vacuoles, mitochondria, membrane vesicles, and polysomes; Ficoll pellet: contains spherooplasts and cell wall fragments; 40% Ficoll: contains nuclei. The protein samples were then analyzed by Western blotting using the polyclonal anti-Nmd2p to ensure proper fractionation. (B) The amount of Nmd2p in the nuclei fractions from wild-type (NMD2) and NLS mutant yeast strains was compared by Western blotting using polyclonal anti-Nmd2p (to detect Nmd2p) and anti-Swi3p (subunit of the SWT/SNF complex; used to standardize loading of the nuclear fractions). Yeast strains used in this experiment were: HFY1200 and HFY1300.
Figure 15. Deletion of *UPF3* has an effect on Nmd2p nuclear localization.
Figure 15. Deletion of \textit{UPF3} has an effect on Nmd2p nuclear localization. Yeast nuclei were isolated from a \textit{upf3Δ} strain by osmotic lysis of spheroplasts, followed by banding two times on Ficoll gradients. The composition of each fraction presented in this figure is as follows: the Lysate = lysed yeast extract: contains total cellular proteins; Ficoll input = lysate before it is loaded onto the Ficoll gradient: contains total cellular proteins; Lipid layer = topmost layer of the Ficoll gradient post fractionation: contains primarily lipids/membranes; 20\% Ficoll fraction: contains cytosolic proteins and polysomes; 20-30\% Interface: contains vacuoles, mitochondria, membrane vesicles, and polysomes; Ficoll pellet: contains spheroplasts and cell wall fragments; 40\% Ficoll: contains nuclei. The protein samples were then analyzed by Western blotting using the polyclonal anti-Nmd2p to ensure proper fractionation. (B) The amount of Nmd2p in nuclei fractions from wild-type (\textit{NMD2}) and \textit{upf3Δ} yeast strains was compared by Western blotting using polyclonal anti-Nmd2p (to detect Nmd2p) and anti-Swi3p (subunit of the \textit{SWI/SNF} complex; used to standardize loading of the nuclear fractions). Yeast strains used in this experiment were: HFY1200 and HFY861.
Figure 16. Comparison of Nmd2p Nuclear Localization Signal with Nuclear Targeting Sequences of Characterized Nuclear Proteins.

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<tr>
<th>Protein</th>
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Basic residues are shown in bold print.
CHAPTER 5

Upf1p CONTROL OF NONSENSE mRNA TRANSLATION IS REGULATED BY Nmd2p AND Upf3p

Introduction

The pathways of gene expression include intricate mechanisms that safeguard against the accumulation of aberrant transcripts and proteins (Yarus, 1992; He et al., 1993; Chin and Pyle, 1995; Friest et al., 1996; Jeon and Agarwal, 1996; Gottesman et al., 1997). In addition to their protective functions, these pathways also contribute additional regulatory facility and complexity (Welch and Jacobson, 1999). The phenomenon of nonsense-mediated mRNA decay (NMD) exemplifies such mechanisms. NMD minimizes the synthesis of truncated polypeptides by eliminating mRNAs containing premature nonsense codons within their protein coding regions (He et al., 1993; Peltz et al., 1993; Peltz et al., 1993; Pulak and Anderson, 1993; Maquat, 1995; Jacobson and Peltz, 1996; Ruiz-Echevarria et al., 1996). NMD also provides the cell with a pathway for the selective degradation of a subset of mRNAs whose coding regions could be considered “normal” (Lelivelt and Culbertson, 1999; Welch and Jacobson, 1999).

In the yeast, Saccharomyces cerevisiae, the rapid degradation of nonsense-containing mRNAs proceeds from deadenylation-independent removal of the 5’ cap by the decapping enzyme, Dcp1p, to 5’→3’ digestion of the remainder of the mRNA by the exoribonuclease, Xrn1p (Hsu and Stevens, 1993; Muhlrad et al., 1994; Hagan et al.,
Three additional factors are also essential for NMD in yeast: Upflp, Nmd2p (Upf2p), and Upf3p (Leeds et al., 1991; Cui et al., 1995; He and Jacobson, 1995; Lee and Culbertson, 1995; He et al., 1997). Mutations in the UPF1, NMD2, or UPF3 genes lead to the stabilization of mRNAs containing premature nonsense codons without affecting the decay rates of most wild-type mRNAs. Since single or multiple mutations within UPF1, NMD2, or UPF3 yield similar decay phenotypes, all three gene products have been considered to be functionally related and to act in a common pathway (He et al., 1997). Substantial support for this conclusion has been derived from protein:protein interaction analyses (He et al., 1997; Czapinski et al., 1998).

A more detailed understanding of the functions of Upflp, Nmd2p, and Upf3p has been sought in several ways. Consistent with their roles in responding to aberrant translation, all three proteins have been shown to localize to the cytoplasm and associate with polyribosomes (Peltz et al., 1993; Atkin et al., 1997; Mangus and Jacobson, 1999). Upflp is a 109-kDa protein that contains two putative zinc finger domains near its amino terminus and harbors seven motifs characteristic of the RNA/DNA helicase superfamily I (Altamura et al., 1992; Koonin, 1992). In vitro studies demonstrated that purified Upflp has the ability to bind nucleic acids and that its ATPase and helicase activities are dependent upon nucleic acid binding (Czapinski et al., 1995; Weng et al., 1998). Upflp interacts with the polypeptide release factors Sup35p and Sup45p (Czapinski et al., 1998), and utilizes for this interaction the same N-terminal zinc finger region also involved in Nmd2p interaction, intramolecular interaction, and homodimerization (F. He.
and A. Jacobson, unpublished data). Little is known about the biochemical activities of the 127-kDa Nmd2p and and 45-kDa Upf3p polypeptides.

The involvement of the UPF/NMD genes in regulating the stability of mRNAs containing premature nonsense codons, and the interactions of Upflp with Nmd2p, Upf3p, Sup35p, and Sup45p, suggest that UPF1, NMD2, and UPF3 may all be regulators of translation termination and/or fidelity. Consistent with this notion are experiments which indicate that deletion of these genes leads to nonsense suppression (Leeds et al., 1992; Weng et al., 1996b), allosuppression (Culbertson et al., 1980), and enhancement of programmed ribosomal frameshifting (Cui et al., 1996; Ruiz-Echevarria et al., 1998). To investigate further the possible regulatory roles of Upflp, Nmd2p, and Upf3p, we devised an assay that quantitatively monitors the effects of upfl/nmd mutations on suppression of the can1-100 nonsense allele. Deletion of the genes encoding each of these factors was found to stabilize the can1-100 transcript and promote nonsense suppression. Strains harboring a deletion of UPF1 showed the highest levels of suppression, and overexpression of UPF1 in upfl/nmd strains significantly lowered the levels of nonsense suppression significantly without altering the steady-state levels of the can1-100 mRNA. These data and determinations of the abundance of all three factors indicate that Upflp plays a critical role in regulating the efficiency of translation termination and that Nmd2p and Upf3p, in turn, regulate Upflp activity.
Results

The can1-100 transcript is a substrate for NMD

To address the roles of UPF1, NMD2, and UPF3 in translation termination, we devised a quantitative assay for nonsense suppression, i.e., readthrough of a premature termination codon. This assay exploited the yeast CANI gene, which encodes a high affinity permease (Can1p) responsible for transport of arginine into cells (Hoffmann, 1985). Previous studies indicated that a can1 allele, can1-100, was attributable to a nonsense mutation because it could be suppressed in strains containing an ochre suppressor tRNA (Hurt et al., 1987). We confirmed this conclusion by sequence analysis of the can1-100 allele, identifying a single A-to-T mutation that results in the substitution of a lysine codon at position 47 of the CANI coding region with a UAA codon (data not shown).

The occurrence of a premature termination codon in the can1-100 mRNA led us to predict that it would be a substrate for NMD. To test this possibility, single deletions of UPF1, NMD2, or UPF3 were constructed in yeast strains that harbored the can1-100 allele, and the effects of these mutations on the abundance of the can1-100 transcript were examined. Northern analyses of mRNA steady-state levels demonstrated that the can1-100 transcript was approximately four-fold more abundant in upf/nmd cells than in the isogenic UPF/NMD strain (Fig. 17A). Likewise, deletion of genes encoding general factors involved in mRNA decay (i.e., DCP1 and XRNI) also promoted a four-fold increase in can1-100 transcript abundance (Fig. 17A). These differences in mRNA abundance were consistent with the respective differences in the decay rates of the CANI
and \textit{canl-100} mRNAs in wild-type cells (half-lives of 8 and 2 min., respectively; data not shown). As a control for the experiments of Fig. 17A, the abundance of an endogeneous substrate of the NMD pathway (He et al., 1993) was monitored. As expected, these experiments showed that the \textit{CYH2} pre-mRNA was barely detectable in wild-type cells and was abundant in all of the mutants. These results indicate that the \textit{canl-100} mRNA requires Upflp, Nmd2p, Upf3p, Dcp1p, and Xrn1p for its degradation and that it is thus a typical substrate for NMD.

\textbf{Quantitative assay for nonsense suppression}

Mutations in the \textit{UPF1}, \textit{NMD2}, or \textit{UPF3} genes have been found to lead not only to increased abundance of substrate mRNAs, but also to suppression of certain nonsense alleles, including \textit{leu2-2} and \textit{tyr7-1} (Leeds et al., 1992; Weng et al., 1996b). To investigate nonsense suppression of the \textit{canl-100} allele, we took advantage of the observation that canavanine, a toxic arginine analog, is also transported into cells via Can1p (Gutherie et al., 1991). \textit{canl-100} cells are thus phenotypically canavanine-resistant, and sensitivity to canavanine is indicative of \textit{canl-100} suppression.

Figure 17B illustrates the canavanine resistance of \textit{canl-100} cells and demonstrates that deletion of \textit{UPF1}, \textit{NMD2}, or \textit{UPF3} results in a canavanine-sensitive phenotype when these cells are grown on media containing 100\(\mu\)g of canavanine per ml. Although deletion of \textit{DCP1} and \textit{XRNI} led to \textit{canl-100} mRNA stabilization comparable to that seen in \textit{upf1A}, \textit{nmrd2A}, or \textit{upf3A} mutants (Fig. 17A), strains with the former deletions did not exhibit canavanine sensitivity (Fig. 17C). These results indicate that
deletion of any of the \textit{UPF/NMD} genes allows for suppression of the \textit{can1-100} nonsense mutation and that increased mRNA abundance alone is not sufficient to promote suppression (see below).

To quantitate the extent of nonsense suppression in the different mutant strains, they were grown on plates containing increasing amounts of canavanine and the concentration at which each strain exhibited a canavanine-sensitive phenotype was determined. In this assay, canavanine-sensitivity is defined as the minimum concentration of canavanine required to kill all cells at the end point of a serial dilution, i.e., approximately 100 cells. These experiments demonstrated that deletion of \textit{UPF1}, \textit{NMD2}, or \textit{UPF3} promoted different extents of \textit{can1-100} suppression. For example, Fig. 18A shows that 40\,\mu g of canavanine per ml was sufficient to kill \textit{upf1\Delta} cells but was only partially toxic to comparable numbers of \textit{nmd2\Delta} or \textit{upf3\Delta} cells. Similar assays consistently demonstrated that the highest levels of nonsense suppression occurred in \textit{upf1\Delta} cells, which exhibited 12-fold greater sensitivity to canavanine than the isogenic wild-type strain (Fig. 18B). Suppression was found to be lower in the \textit{nmd2\Delta} and \textit{upf3\Delta} cells, which exhibited 1.5-fold less sensitivity than \textit{upf1\Delta} cells (Fig. 18). Although the canavanine sensitivities of the \textit{nmd2\Delta} and \textit{upf3\Delta} strains were almost identical, subtle differences were detected which indicated that the \textit{nmd2\Delta} mutation was a slightly more effective suppressor than the \textit{upf3\Delta} mutation (Fig. 18).
Accumulation of functional Can1p correlates with nonsense suppression of can1-100

To ensure that the respective differences in canavanine-sensitivity reflected comparable changes in the extent of synthesis of functional Can1p, arginine permease activities were determined by monitoring the rate of uptake of $[^3H]$arginine in wild-type and mutant cells. Consistent with the suppression assays of Fig. 17 and 18, these experiments demonstrated that deletion of UPFI, NMD2, or UPF3 allowed for enhanced transport of arginine (Fig. 19A).

To test whether increased suppression and transport activity reflected enhanced synthesis of full-length Can1p, the expression of an HA epitope-tagged allele of can1-100 was monitored by Western blotting. As a control, we showed that all strains containing the can1-100-HA plasmid exhibited phenotypes identical to those strains containing the same plasmid lacking the triple-HA tag (data not shown). Figure 19B shows that Can1p-HA was barely detectable in wild-type cells (lower panel, lane 3) but increased approximately 10-fold in abundance in upf1Δ, nmd2Δ, and upf3Δ cells (compare lane 3 to lanes 4 to 6). Suppression of can1-100 yielded Can1p levels that were approximately 20-fold lower than those obtained from expression of the wild-type CAN1 gene, a result consistent with the high rate of arginine transport in CAN1 cells (Fig. 19A) and the sensitivity of the same cells to 0.7 µg of canavanine per ml (data not shown). Quantitation of the blot shown in Fig. 19B also provided an estimate of the reduction in CAN1 expression caused by the premature termination codon. Since the levels of Can1p in lanes 1 and 3 of Fig. 19B differ by approximately 10-fold and the sample in lane 1 is a 20-fold dilution, premature termination of CAN1 translation caused a 200-fold reduction
in Can1p synthesis. The data in Fig. 19B also demonstrate that Can1p accumulation and the results of the plate assay for canavanine sensitivity approximate a linear relationship. This conclusion is drawn from the observation that wild-type cells harboring the CAN1 gene, wild-type cells harboring can1-100, and upf1Δ cells harboring can1-100 are sensitive to 0.7, 300, and 25 μg of canavanine per ml, respectively, and accumulate 200-, 1-, and 10-fold relative units of Can1p (Fig. 18B and 19B and data not shown).

**can1-100 nonsense suppression by mutations in UPF1, NMD2, or UPF3 is only partially attributable to increases in mRNA abundance**

Since the can1-100 mRNA was stabilized in upf1Δ, nmd2Δ, or upf3Δ mutants (Fig. 17), suppression might be attributable to a constant but low rate of "leaky" termination that becomes functionally significant as mRNA levels increase. To directly address the contribution of mRNA abundance to the suppression phenotypes, the can1-100 allele was subcloned into single-copy and high-copy-number plasmids that were then introduced into cells that were wild-type for NMD and already harbored a genomic copy of the can1-100 allele. Levels of the can1-100 mRNA were then measured by northern analysis (Fig. 20A and B), and the respective suppression phenotypes (i.e., canavanine sensitivities) of the different strains were determined (Fig. 20C). Wild-type cells expressing an additional copy of can1-100 (YCp can1-100) showed a slight (1.4-fold) increase in can1-100 mRNA levels (Fig. 20A and B), but this increase did not alter the suppression phenotype of wild-type cells containing either the single-copy or high-copy-number vectors without inserts (Fig. 20C, compare WT-YCp can1-100 with WT-YEp;
also, data not shown). Wild-type cells transformed with the high-copy-number plasmid containing the canl-100 allele showed a 12-fold increase in canl-100 mRNA abundance compared to the same cells containing only the vector (Fig. 20A and B, compare WT-YEp canl-100 with WT-YEp). Accompanying this increase in mRNA levels was a six-fold increase in sensitivity to canavanine (Fig. 20C).

The same phenomena were exhibited when this experiment was repeated with upflΔ, nmd2Δ, and upf3Δ mutants. All strains expressing an additional copy of the canl-100 allele exhibited modest increases in canl-100 mRNA levels (15-50%; Fig. 20A and B) but showed approximately three-fold increases in their respective levels of suppression (Fig. 20C, compare YCp canl-100 with YEp for all three mutants). When the canl-100 allele was expressed in these mutants from the high-copy-number plasmid, there was a 10-fold increase in the abundance of its mRNA (Fig. 20A and B) and a comparable increase in the level of nonsense suppression (Fig. 20C). These results indicate that increased mRNA abundance contributes to nonsense suppression but is not its sole determinant. This conclusion is illustrated further by direct comparisons of mRNA levels and extents of suppression in mutant and wild-type cells. For example, UPF/NMD wild-type cells overexpressing canl-100 (WT-YEp canl-100) had two- to three-fold higher levels of canl-100 mRNA than any of the upf/nmd mutant strains (Fig. 20A and B), yet the level of suppression in the WT-YEp canl-100 strain was still lower than that in any of the mutants (Fig. 20C).

Additional support for the notion that increased mRNA abundance is not sufficient for canl-100 nonsense suppression is the finding that single deletions of UPFL,
NMD2, UPF3, DCP1, or XRNI were found to stabilize the can1-100 mRNA to comparable levels (approximately four-fold; Fig. 17A), yet there were substantial differences in the canavanine sensitivities of the respective strains (Fig. 17B, 17C, and 18B). Collectively, the data in Fig. 17 to 20 provide strong support for the notion that the UPF/NMD genes regulate not only the decay rates of nonsense-containing mRNAs but also their efficiencies of translation.

Different efficiencies of suppression are not attributable to changes in the fraction of capped can1-100 mRNA

Recent experiments have indicated that deletions of UPF1, NMD2, or UPF3 inhibit the decay of nonsense-containing mRNAs prior to the decapping step, i.e., such deletions increase the steady-state ratio of capped to uncapped mRNAs (He and Jacobson, unpublished). Since the upf/nmd mutations affected the efficiency of translational suppression (see above), we considered the possibility that this effect, in turn, reflected substantial alterations in the relative percentages of capped can1-100 mRNA in wild-type and mutant cells. Immunoprecipitation experiments with anti-cap antibodies were used to examine the 5' cap status of the can1-100 mRNA and a control (ADH1) mRNA in wild-type, upf1Δ, nmd2Δ, and upf3Δ strains. In both wild-type and mutant, the ADH1 mRNA was predominantly capped (Fig. 21). However, the can1-100 mRNA was predominantly uncapped in wild-type cells, and deletion of UPF1, NMD2, or UPF3 led to a slight increase in their percentage of capped molecules (Fig. 21). These changes in the ratios of capped to uncapped can1-100 mRNA do not correlate with the suppression
data of Fig. 17 to 20 and indicate that variations in suppression efficiencies must reflect events unrelated to mRNA cap status. This conclusion is underscored by experiments indicating that *dcp1Δ* and *xrn1Δ*, two mutations which have negligible effects on *can1-100* suppression (Fig. 17), lead to the accumulation of mRNAs that are predominantly capped or uncapped, respectively (Hsu and Stevens, 1993; Muhlrad et al., 1994; Beelman et al., 1996; also data not shown).

The relative distributions of capped and uncapped *can1-100* mRNA species differed not only from that observed for the *ADH1* mRNA but also from that seen with nonsense-containing *PGK1, MER2*, and *CYH2* transcripts (Muhlrad and Parker, 1994; He and Jacobson, unpublished). This finding was unexpected and may reflect the possibility that, for some mRNAs, decapping is not immediately followed by exonucleolytic digestion. This conclusion is supported by experiments showing that at least one other NMD substrate, the *his4-38* mRNA, behaves similarly (He and Jacobson, unpublished) and that uncapped mRNAs accumulate in a temperature-sensitive eukaryotic initiation factor 5A mutant (Zuk and Jacobson, 1998).

**Epistatic relationships of Upf1p, Nmd2p, and Upf3p in nonsense suppression**

Since the different *upf/nmd* mutations showed small but highly reproducible differences in the extents of *can1-100* suppression that they promoted (see Fig. 18B), we were able to exploit those differences to determine epistatic relationships of Upf1p, Nmd2p, and Upf3p. To resolve epistatic relationships, mutants containing double deletions of the *UPF1, NMD2*, or *UPF3* genes were constructed and assayed for their sensitivity to
canavanine. Analyses of these mutants demonstrated that any strain harboring a deletion of UPF1 exhibited the highest levels of suppression (i.e., sensitivity to 25-µg/ml of canavanine) and, conversely, that strains harboring a wild-type UPF1 gene showed lower levels of suppression (i.e., sensitivity to 35 µg of canavanine per ml). As shown in Fig. 18, double deletion of UPF1 and either NMD2 or UPF3 resulted in a suppression phenotype identical to that caused by upf1Δ alone. This result indicates that combining an nmd2Δ or upf3Δ mutation with upf1Δ does not have an additive effect on nonsense suppression and that the upf1Δ phenotype supersedes the nmd2Δ and upf3Δ phenotypes. Of the double mutants, the nmd2Δ,upf3Δ mutant showed the lowest level of suppression, displaying a phenotype like that of an nmd2Δ strain (compare nmd2Δ,upf3Δ to nmd2Δ in Fig. 18A). These results indicate that Upf1p is epistatic to Nmd2p and Upf3p and suggest a role for Upf1p in affecting the efficiency of premature translation termination.

While the suppression phenotypes of the double mutants suggested relatively straightforward epistatic relationships, the phenotype of the triple mutant, lacking UPF1, NMD2, and UPF3, was somewhat surprising. This mutant showed a lower level of suppression than any of the upf1/nmd mutants tested (sensitivity to 50 µg of canavanine per ml; Figures 18), demonstrating that the efficiency of translation termination is greater in the absence of all three UPF/NMD gene products than in the presence of any one of them. This result suggests either the existence of an alternate mechanism of termination fidelity that functions in the absence of the UPF/NMD gene products or that the presence of one of the UPF/NMD factors without the other two acts dominantly to prevent proper termination.
Overexpression of *UPF1* decreases the efficiency of nonsense suppression without altering *can1-100* mRNA levels

As an additional approach to characterizing the functional relationships of Upf1p, Nmd2p, and Upf3p, these gene products were overexpressed in all of the *upf/nmd* mutant backgrounds, and the resulting effects on nonsense suppression were examined. Overexpression was accomplished by cloning *UPF1*, *NMD2*, or *UPF3* under the control of the strong *ADH1* promoter on a high-copy-number plasmid (He et al., 1997). Expression of the *UPF/NMD* genes from these constructs was found to increase the accumulation of the respective proteins at least 10-fold (data not shown). As controls for these experiments, we utilized mutant strains transformed with only the high-copy-number vector. The presence of this plasmid did not alter the suppression phenotypes of any of the mutant strains (compare Table 3 [YEps column] with Fig. 18B).

Overexpression of *UPF1* in all of the single, double, and triple mutant strains (not including the *upf1Δ* control) was found to lower suppression levels two- to three-fold (Table 3, compare YEps and YEps-*UPF1* columns). These results are consistent with the notion that Upf1p can, by itself, enhance termination fidelity and also implicate a regulatory role for Nmd2p and Upf3p, since Upf1p can lower suppression in the absence of either of the other proteins. Overexpressing *UPF3* complemented its own deletion, had no effect on any other single or double mutation, and did not change the phenotype of the triple mutant. The latter phenomenon, however, could be considered to reflect a modest increase in canavanine resistance over that observed in a *upf1Δ nmd2Δ* strain (Table 3). Overexpression of *NMD2* had comparable effects, except that, in *upf3Δ* cells,
it also enhanced suppression to a level comparable to that obtained in upf1Δ cells (Table 3). This result suggests that Nmd2p may be a negative regulator of the activity of Upf1p or is capable of simply titrating available Upf1p.

Since the overexpression of UPF1 altered the suppression phenotypes of all of the mutants, we investigated whether these effects might be caused by restoration of the rapid rate of decay of the can1-100 transcript. To this end, steady-state levels of the can1-100 mRNA were examined in upf/nmd mutants overexpressing UPF1. As expected, overexpression of UPF1 in the upf1Δ strain restored NMD to wild-type levels, resulting in a four-fold decrease in can1-100 mRNA levels (Fig. 22, compare upf1Δ-YEp to upf1Δ-YEp+UPF1). Accompanying this restoration of decay function was the restoration of the wild-type suppression phenotype (Table 3). In all of the other upf/nmd mutant strains, UPF1 overexpression did not significantly alter can1-100 mRNA levels compared to those seen in the starting mutant strains that contained the vector only (Fig. 22). Additionally, overexpression of NMD2 or UPF3 in any of the mutant backgrounds had no effect on steady-state can1-100 mRNA levels, other than those involving direct complementation of the respective single deletions (data not shown). These results demonstrate that changes in the suppression phenotype caused by overexpression of UPF1 are not attributable to changes in can1-100 mRNA levels. These observations are consistent with the proposed role of Upf1p in controlling the efficiency of translation termination, provide further support for a regulatory function for Nmd2p and Upf3p, and comprise additional evidence for the separation of the activities of Upf1p in mRNA decay and translation (Weng et al., 1996a; Weng et al., 1996b; Czaplinski et al., 1998).
Upf1p is considerably more abundant than Nmd2p or Upf3p, but is not stoichiometric with ribosomes.

The suppression analyses described above indicated that Upf1p was a critical regulator of termination fidelity and that Nmd2p and Upf3p regulated the activity of Upf1p. These putative regulatory relationships are consistent with previous protein-protein interaction analyses (He et al., 1996; Weng et al., 1996a; He et al., 1997; Czapinski et al., 1998), but raise the question as to whether these interactions occur as part of a stoichiometric complex or are more transient events. To address this issue further, we determined the cellular abundance of each of these factors. Western blotting was used to compare the amount of epitope-tagged Upf1p, Nmd2p, or Upf3p in a fixed number of cells with those present in purified samples of each of the two proteins. Relative levels of Nmd2p and Upf3p in crude extracts were determined by comparing the relative Western blot intensities of the two proteins when each harbored the same epitope tag. Using this approach, Upf1p was found to be the most abundant of the three factors, with approximately 1600 molecules of Upf1p/cell (Table 4). Nmd2p was found to be 10-fold less abundant than Upf1p (160 molecules of Nmd2p/cell) and Upf3p was found to be the least abundant of the NMD factors (80 molecules of Upf3p/cell) (Table 4). These experiments indicate that the cellular concentrations of Upf1p, Nmd2p, and Upf3p differ greatly and do not approach the cellular levels of ribosomes, release factors, or the major cellular exonuclease, Xrn1p (Hereford and Rosbash, 1977) (Table 4). These data are, however, consistent with the putative role of Upf1p as a regulator of termination fidelity, as well as the implied roles of Nmd2p and Upf3p as regulators of Upf1p.
Discussion

Suppression of the can1-100 nonsense allele is enhanced by upf1Δ, nmd2Δ, and upf3Δ mutations

The UPF1, NMD2, and UPF3 genes regulate NMD (Leeds et al., 1991; Leeds et al., 1992; Peltz et al., 1993; Cui et al., 1995; He and Jacobson, 1995; Lee and Culbertson, 1995; He et al., 1997). Mutations in any of these genes generally promote the stabilization of nonsense-containing mRNAs by reducing the rate at which recognition of a premature termination codon by the translation apparatus triggers mRNA decapping (He and Jacobson, unpublished). These effects of upf/nmd mutations on mRNA stability and parallel enhancing effects on nonsense suppression (Culbertson et al., 1980; Leeds et al., 1992; Weng et al., 1996a; Weng et al., 1996b) and programmed ribosomal frameshifting (Cui et al., 1996; Ruiz-Echevarria et al., 1998) suggested a regulatory role in translation termination and/or fidelity for Upf1p, Nmd2p, and Upf3p. Strong support for this conclusion was obtained from experiments demonstrating interactions between Upf1p and the polypeptide release factors Sup35p and Sup45p (Czaplinski et al., 1998).

To characterize further the roles of the UPF/NMD gene products in translation termination, we developed an assay that examined the effects of upf/nmd mutations on suppression of the can1-100 allele. A single A→T mutation in this allele leads to the synthesis of a transcript in which codon 47 has been changed to UAA. As a consequence, the can1-100 mRNA is a substrate for NMD. Mutations in UPF1, NMD2, or UPF3 not only stabilized the can1-100 transcript, but also promoted its suppression.
Quantitative measurement of the extent of *can1-100* suppression by these mutations was achieved by varying the canavanine concentration of the growth media and determining the specific concentration that effectively killed diluted samples of the respective mutants. Since the degree of suppression (i.e., enhanced canavanine-sensitivity) was found to correlate with the level and activity of Can1p in the cells, we conclude that the *can1-100* system provides a reliable assay for nonsense suppression. Further support for the reliability of this assay was provided by experiments showing that the qualitative aspects of the *can1-100* suppression were comparable to those obtained in independent assays with the *leu2-1* (UAA) and *tyr7-1* (UAG) nonsense alleles (data not shown).

We initially investigated the effects of single deletions of *UPF1*, *NMD2*, and *UPF3* on *can1-100* nonsense suppression. Individual deletions of each of these genes was shown to have comparable stabilizing effects on the *can1-100* mRNA, but to produce differential effects on suppression. Strains harboring the *upf1Δ* mutation consistently showed a higher level of nonsense suppression than strains harboring either the *nmd2Δ* or *upf3Δ* mutation. We inferred from this observation that Upf1p might play a more direct role in regulating the translation of nonsense-containing mRNAs than the other two factors; further experimentation appears to have substantiated this conclusion (see below).

*can1-100* nonsense suppression: a loss in termination fidelity?

Mutations in the *UPF/NMD* genes have previously been shown to promote the suppression of *leu2*, *tyr7*, *met8*, and *his4* nonsense alleles (Leeds et al., 1992; Cui et al.,
Since these mutations invariably led to increases in the levels of the corresponding mRNAs (Leeds et al., 1991; Leeds et al., 1992) but failed to generate evidence for an effect on the readthrough of premature stop codons, it was initially concluded that suppression was due solely to the combination of enhanced mRNA abundance and an inherent rate of readthrough that was sufficient to generate the minimal amount of protein required for function of the respective genes (Leeds et al., 1992; Peltz et al., 1994). However, the experiments of Weng et al. (Weng et al., 1996a; Weng et al., 1996b) suggested that an alternative explanation was more likely. They generated a large set of upf1 alleles and identified several in which the effects on mRNA decay and translational suppression could be separated. More specifically, they identified two significant classes of upf1 alleles: (i) those which, when expressed at high copy number, inactivated mRNA decay but failed to allow suppression (e.g., DE572AA) (Weng et al., 1996b) and (ii) those which, when expressed in a single copy, promoted normal mRNA decay but allowed suppression to occur (e.g., C84S) (Weng et al., 1996b). The phenotypes of these mutants indicated that suppression was unlikely to be caused solely by changes in mRNA levels and established the notion that Upf1p could regulate both mRNA decay and translation. Since upf1 mutations had no effect on polysome profiles (Leeds et al., 1991; He et al., 1993) and since Upf1p was known to be of relatively low abundance, it was considered likely that the translational effects were not targeted to general initiation or elongation but rather to the premature termination event.
On the basis of the results of Weng et al. (Weng et al., 1996a; Weng et al., 1996b), we anticipated that the suppression of *can1-100* by *upf/nmd* mutations would also be attributable to more than simple increases in mRNA levels. This assumption was substantiated by several new lines of experimentation which demonstrated that: (i) *xrn1Δ* and *dcp1Δ*-mediated increases in *can1-100* mRNA abundance, to levels comparable to those obtained in *upf/nmd* mutant cells, did not promote canavanine-sensitivity; (ii) high-copy-number expression of the *can1-100* allele in wild-type cells, leading to *can1-100* mRNA levels which exceeded those obtained in *upf/nmd* mutant cells 2- to 3-fold, was less effective in promoting canavanine-sensitivity than single *upflΔ, nmd2Δ*, or *upf3Δ* mutations; (iii) when *UPFI* was overexpressed, large changes in the extent of nonsense suppression could be attained without significant alterations in *can1-100* mRNA abundance; (iv) in *upf/nmd* mutant cells harboring an additional copy of the *can1-100* allele, 2- to 3-fold increases in canavanine-sensitivity were obtained when levels of the corresponding mRNA only increased 50% or less; and (v) high-copy-number expression of the *can1-100* allele led to 3- to 4-fold higher levels of the corresponding mRNA in *upf/nmd* mutant cells than in wild-type cells but to 16- to 25-fold higher levels of suppression. Interestingly, the observation that the canavanine sensitivity of wild-type cells increased at all in response to enhanced abundance of the *can1-100* mRNA indicates that mRNA abundance contributes to suppression and that the premature termination codon in the *can1-100* mRNA must be leaky. The latter conclusion is substantiated by the identification of small amounts of full-length Can1p in wild-type cells harboring the *can1-100* allele (Fig. 19B).
Given that the premature termination codon in the *can1-100* allele has an intrinsic, albeit low, rate of readthrough, two explanations for the mechanism of suppression appear plausible. In the first, translation initiation of the *can1-100* mRNA is somehow increased and, in the second, the efficiency of the premature termination event is decreased. While there is no evidence supporting global effects on translation initiation by *upf/nmd* mutants (Leeds et al., 1991; He et al., 1993), inactivation of the NMD pathway has been shown to promote a modest increase in the translational efficiency of nonsense-containing mRNAs (Muhlrad and Parker, 1999). Moreover, recent studies have demonstrated that the *upf1Δ*, *nmd2Δ*, and *upf3Δ* mutations alter the distribution of capped and uncapped transcripts (He and Jacobson, unpublished). Therefore, suppression by deletion of *UPF1*, *NMD2*, or *UPF3* could, in principle, have been caused by subtle increases in the translational efficiency of the *can1-100* mRNA, possibly because of changes in its extent of capping. However, deletion of *UPF1*, *NMD2*, or *UPF3* produced differential effects on the amounts of capped and uncapped *can1-100* mRNAs (Fig. 21) that did not correlate with their respective suppression phenotypes, therefore it is unlikely that suppression is dependent on changes in the fraction of capped *can1-100* mRNA. We therefore consider it likely that suppression caused by deletion of *UPF1*, *NMD2*, or *UPF3* is due either to a loss in termination fidelity at the premature nonsense codon or to additional rounds of translational initiation on an mRNA with an inherent, low rate of leaky termination. The demonstration of interactions between Upf1p and the polypeptide factors (Czapinski et al., 1998) suggests that the former model is more likely.
Upflp plays a central role in regulating nonsense suppression

The finding that deletion of UPF1 resulted in a greater extent of suppression than deletion of NMD2 or UPF3 either implicates Upflp as the most critical of the three factors for the maintenance of termination fidelity or suggests that Upflp and either Nmd2p (i.e., as in the upf3Δ strain) or Upf3p (i.e., as in the nmd2Δ strain) may enhance termination fidelity cooperatively. To distinguish between these possibilities, strains harboring double mutations were constructed, and nonsense suppression by these strains was monitored. Any double mutant harboring a deletion of UPF1 showed the highest levels of suppression and, alternatively, the nmd2Δ,upf3Δ mutant (the only double mutant expressing UPF1) showed lower suppression levels. Therefore, the suppression phenotype mediated by the deletion of UPF1 supersedes an additional mutation of NMD2 or UPF3, suggesting that, of the three proteins, Upflp is the central factor involved in regulating the translational efficiency of nonsense-containing mRNAs. This conclusion was significantly reinforced by analyses of the consequences of UPF1 overexpression (see below). Interestingly, since deletion of both NMD2 and UPF3 does not have an additive effect on suppression, it appears that Nmd2p and Upf3p may act in concert, as opposed to independently, to regulate Upflp activity.

Deletion of all three UPF/NMD genes resulted in significantly lower levels of suppression than that seen in any of the other mutants tested. This result was surprising, since this mutant was expected to exhibit a phenotype characteristic of upf1Δ strains. Since deletion of the genes encoding all three factors enhances termination efficiency,
either an alternate fidelity pathway may function in the absence of the *UPF/NMD*-mediated mechanism or any one of the *UPF/NMD* factors without the other two may act in a dominant-negative manner.

**Overexpression of *UPF1* restores termination fidelity without affecting mRNA decay**

As noted above, Weng *et al.* (Weng *et al.*, 1996a; Weng *et al.*, 1996b) showed that specific *upfI* alleles could separate the translation and turnover functions of Upf1p, i.e., some alleles resulted in normal mRNA decay but impaired translational fidelity whereas others resulted in the opposite phenotype. Curiously, these phenotypes are not reproduced in the *canl-100* system. Strains with *upfl* alleles shown to result in normal decay but impaired fidelity (e.g., C84S) behaved like the wild-type strain in the *canl-100* system, and strains with alleles resulting in inactive mRNA decay but functional fidelity (e.g., DE572AA) behaved just like *upflA* strains (data not shown). However, we have been able to obtain independent evidence for the separation of the two putative functions of Upf1p. In analyses of the effects of overexpressing each of the *UPF/NMD* genes in the different mutant backgrounds, we observed that high-copy-number expression of *UPF1* led to substantial decreases in *canl-100* nonsense suppression without having any significant effect on *canl-100* mRNA levels. This finding underscores the existence of a separate translational role for Upf1p, reinforces the notion of Upf1p as the preeminent of the three factors in regulating termination fidelity, and implies a regulatory role for
Nmd2p and Upf3p (since overexpression of Upf1p has the ability to enhance fidelity even in the absence of Nmd2p or Upf3p).

The overexpression of NMD2 in a upf3Δ strain enhanced nonsense suppression to an extent comparable to that observed in strains harboring only a UPF1 deletion. This result suggests that Nmd2p may negatively regulate the activity of Upf1p, such that an excess of this negative regulator renders Upf1p inactive. Alternatively, since Nmd2p and Upf1p interact (He et al., 1996; He et al., 1997), the overexpression of NMD2 may simply sequester Upf1p molecules and prevent their proper functioning by hindering additional interactions. The latter hypothesis leaves open the possibility that Nmd2p and Upf3p are actually activators of Upf1p activity, a model consistent with the decreases in suppression observed when UPF1 was overexpressed. If Nmd2p and Upf3p are indeed such activators, then the results of their respective overexpression would indicate that high levels of either factor alone are not sufficient to promote such activation.

**Cellular concentrations of Upf1p, Nmd2p, and Upf3p are consistent with their apparent regulatory interactions**

Earlier studies recognized that the UPF/NMD factors were relatively low in abundance (Peltz et al., 1993; Atkin et al., 1997; Lelivelt and Culbertson, 1999), but their actual cellular concentrations were not determined previously. Here, using Western blotting of crude cell extracts and purified proteins as standards, we found approximately 1600, 160, and 80 molecules of Upf1p, Nmd2p, and Upf3p per cell, respectively. The abundance of these factors is consistent with the proposed central role of Upf1p in regulating
termination fidelity, as well as with the hypothesis that Nmd2p and Upf3p regulate the activity of Upf1p. Although Upf1p, Nmd2p, and Upf3p have all been shown to be interacting proteins that associate with polyribosomes (Peltz et al., 1993; He and Jacobson, 1995; He et al., 1996; Weng et al., 1996a; Weng et al., 1996b; Atkin et al., 1997; He et al., 1997; Mangus and Jacobson, 1999), these data make it unlikely that these proteins exist in a stable complex or that they associate with all ribosomes. Rather, their interactions and ribosome association must be transient, with the latter limited to those ribosomes recognizing newly synthesized mRNAs or termination codons. An association with ribosomes actively recognizing termination codons would be consistent with recent studies demonstrating that Upf1p interacts with the peptidyl release factors Sup35p and Sup45p (Czaplinski et al., 1998).

**Possible functions of Upf1p, Nmd2p, and Upf3p in translation termination**

Taken together, the findings presented are consistent with Upf1p playing an important role in regulating the efficiency of translation termination, with Nmd2p and Upf3p serving as codependent activators of Upf1p’s function (Fig. 23). The importance of UPF1 is highlighted by the observations that deletion of UPF1 results in the highest levels of suppression, overexpression of UPF1 can restore termination fidelity, and Upf1p is most abundant of the three proteins involved in NMD. Further, homologs of Upf1p have been identified in other organisms, including *Caenorhabditis elegans* (Pulak and Anderson, 1993; Page et al., 1999) and human (Perlick et al., 1996; Applequist et al., 1997), indicating evolutionary conservation of this factor. It is possible that Upf1p’s role
in translation termination simply involves stimulation of the activity of the peptide release factors (K. Czaplinski et al., submitted for publication), such that efficient release allows for enhanced fidelity. Alternatively, Upflp may play a more elaborate role at termination, including the regulation of ribosome release and recycling and the stimulation of decapping concurrent with premature nonsense codon recognition. Experiments to be described elsewhere suggest that these activities are also within the realm of Upflp (R. Ganesan, F. He, and A. Jacobson, unpublished data; He and Jacobson, unpublished).
Figure 17. Deletion of UPFl, NMD2, or UPF3 stabilizes the canl-100 transcript and promotes nonsense suppression.

A

WT, upf1Δ, nmd2Δ, upf3Δ, dcp1Δ, xrn1Δ

→ canl-100 mRNA

→ CYH2 pre-mRNA

→ CYH2 mRNA

B

- Canavanine

+ Canavanine

WT

upf1Δ

nmd2Δ

upf3Δ

C

dcp1Δ

xrn1Δ
Figure 17. Deletion of UPF1, NMD2, or UPF3 stabilizes the can1-100 transcript and promotes nonsense suppression. (A) Deletion mutants that inactivate NMD stabilize the can1-100 transcript. Total RNA isolated from yeast strains with the indicated UPF/NMD genotypes was analyzed by Northern blotting with DNA probes that detected the can1-100 and CYH2 transcripts. WT, wild type. (B) Deletion of UPF1, NMD2, or UPF3 leads to a canavanine sensitive phenotype. Aliquots (10μl) of each of four 1:10 dilutions of liquid cultures of each yeast strain were spotted on SC-arg plates containing either 0 or 100 μg of canavanine per ml (-Canavanine or +Canavanine, respectively) and grown at 30°C for 2 days. (C) Deletion of DCP1 or XRN1 does not suppress the can1-100 mutation. Aliquots of serial 1:10 dilutions of each yeast strain were spotted on plates with or without canavanine as in panel B. Because these two mutants had slow doubling times, growth comparable to that of wild-type cells was obtained by maintaining the xrn1Δ strain at 30°C for 3 days and the dcp1Δ strain at 30°C for 4 days. Yeast strains used in this experiment were: HFY1200, HFY870, HFY1300, HFY861, HFY1067, and HFY1081.
Figure 18. Deletion of *UPF1* promotes higher levels of *can1-100* nonsense suppression than deletion of *NMD2* or *UPF3*.

### A

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### B

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Figure 18. Deletion of UPF1 promotes higher levels of can1-100 nonsense suppression than deletion of NMD2 or UPF3. (A) Growth of yeast strains with different UPF/NMD genotypes on SC-arg plates containing either 0 ot 40 µg of canavanine (can.) per ml. Cells were grown for 2 days at 30°C. WT, wild-type. (B) Canavanine sensitivities of different yeast strains. Suppression assays analogous to those shown in panel A were used to determine the minimum concentration of canavanine required to kill approximately 100 cells of the respective yeast strains (Can. Sensitivity) after 2 days of growth at 30°C. Yeast strains used in this experiment were: HFY1200, HFY870, HFY1300, HFY861, HFY3000, HFY872, HFY874, and HFY883.
Figure 19. Accumulation of functional Can1p correlates with nonsense suppression of *can1-100*. 

*nmol arginine*

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Figure 19. Accumulation of functional Can1p correlates with nonsense suppression of can1-100. (A) \(^3\)H-labeled arginine uptake in yeast strains with the indicated UPF/NMD and CAN1 genotypes. The control yeast strain harboring the CAN1 allele is PLY148 (Leeds et al., 1992). WT, wild-type. Each time point was done in triplicate and error bars indicate standard deviations. The rates of arginine uptake were standardized by conducting the experiment with the different yeast strains at comparable OD\(_{600}\). (B) Western analysis of Can1p levels. Lysates of yeast strains with the indicated UPF/NMD genotypes and bearing either CAN1 or can1-100 plasmids were analyzed by Western blotting with HA-specific antibodies. The lower panel is a longer exposure of the same blot shown in the upper panel. Yeast strains used in this experiment were: HFY1200, HFY870, HFY1300, and HFY861.
Figure 20. *can1-100* nonsense suppression is only partially attributable to increased mRNA abundance.
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Relative mRNA levels
Figure 20. *can1-100* nonsense suppression is only partially attributable to increased mRNA abundance. (A) Northern analysis of *can1-100* mRNA levels. RNA isolated from yeast strains of the indicated genotypes was analyzed by Northern blotting with probes specific for transcripts for *can1-100* mRNA and *SCR1* RNA (the latter to serve as an internal loading control). Each of the indicated strains contained either: a high-copy-number *can1-100* plasmid (YEp *can1-100*), a single copy *can1-100* plasmid (YCp *can1-100*), or an empty vector as a control (YEp). *WT*, wild-type. (B) *can1-100* steady-state mRNA levels. Data from the blot in panel A were quantitated by phosphorimaging, standardized to *SCR1* RNA levels, and normalized to data for the *upflA* strain. (C) Canavanine-sensitivities of strains harboring single-copy or high-copy-number plasmids. Suppression assays analogous to those shown in Figure 18 were used to define the canavanine (Can.) sensitivities of cells with different *UPF/NMD* genotypes. Yeast strains used in this experiment were: HFY1200, HFY870, HFY1300, and HFY861.
Figure 21. Suppression phenotypes are not a consequence of changes in the relative fractions of capped *can1-100* mRNA.
**Figure 21.** Suppression phenotypes are not a consequence of changes in the relative fractions of capped *can1-100* mRNA. (A) Northern analysis of mRNAs fractionated by 5'-cap immunoprecipitation. Total RNA from yeast strains with the indicated genotypes was separated into capped and uncapped fractions by use of polyclonal anti-m^7^G antibodies and analyzed by Northern blotting with DNA probes for either the *ADHI* mRNA or the *can1-100* mRNA. I, input RNA; S, RNA in the supernatant fraction (represents the uncapped fraction); P, RNA in the pellet fraction (represents the capped fraction). *WT*, wild-type. (B) Relative amounts of capped and uncapped *can1-100* and *ADHI* transcripts. RNA in the S and P fractions of panel A was quantitated by phosphorimaging, and the relative percentages of capped or uncapped transcripts were determined by calculating the fraction each sample represented of its respective total (S+P). Yeast strains used in this experiment were: HFY1200, HFY870, HFY1300, and HFY861.
Figure 22. Overexpression of UPF1 in upf/nmd mutant strains does not affect canl-100 mRNA abundance.
Figure 22. Overexpression of UPFI in upf/nmd mutant strains does not affect canl-100 mRNA abundance. (A) Northern analysis of canl-100 mRNA levels. Total RNA isolated from yeast strains of the indicated genotypes was analyzed by Northern blotting as described in the legend to Figure 20. Each of the mutant strains contained either a high-copy-number UPFI plasmid (YEp-UPFI) or an empty vector as a control (YEp). (B) Quantitation of canl-100 steady-state mRNA levels. canl-100 mRNA levels were determined, standardized to SCRI RNA, and normalized to the upf1Δ strain, as described in the legend to Figure 20. Yeast strains used in this experiment were: HFY1200, HFY870, HFY1300, HFY861, HFY3000, HFY872, HFY874 and HFY883.
Figure 23. Model for functional relationships of Upf1p, Nmd2p, and Upf3p in translation termination.
Figure 23. Model for functional relationships of Upf1p, Nmd2p, and Upf3p in translation termination. Upf1p is depicted as a positive regulator of the efficiency of translation termination mediated by Sup35p and Sup45p. The activity of Upf1p is postulated to be dependent on the function of both Nmd2p and Upf3p. Regulation of Upf1p by Upf3p and Nmd2p is postulated to occur as a consequence of either the combined or the sequential action of Upf3p and Nmd2p. The left and right complexes depict translation termination with and without nonsense decay factors, respectively, with the breadth of the large arrows indicating the relative efficiencies of the two events. E, P, and A represent the exit, peptidyl, and aminoacyl sites on the ribosome (dark grey ovals).
<table>
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Suppression assays are summarized, with the concentration of canavanine at which each strain began to exhibit the canavanine-sensitive phenotype indicated (canavanine sensitive). Yeast strains of the indicated genotypes were transformed with the high-copy-number vector alone (YEp: control) or high-copy-number plasmids expressing UPF1, NMD2, and UPF3, respectively.

Table 3. Overexpression of UPF1, NMD2, or UPF3 in upf1, nmd2 and upj3 strains.
Table 4. Cellular levels of Upf1p, Nmd2p, and Upf3p.

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^1The relative abundance of Upf1p and Nmd2p was determined by comparing Western blot band intensities of the UPF/NMD factors present in crude cell extracts to those of the individual proteins. For Upf1p and Nmd2p, highly purified recombinant GST-Upf1p and GST-Nmd2p were used as standards, respectively. For Upf3p, cells bearing an HA-NMD2 allele were used as the standard. Calculations used to derive protein abundance are summarized in Materials and Methods. This table was generated by David Mangus, Ph.D. at University of Massachusetts Medical Center.

^2Cellular Sup45p levels determined by Gygi et al. (1999).

^3Cellular Xrn1p levels determined by Heyer et al. (1995).

^4Cellular levels of ribosomes determined by Waldron and Lacroute (1975).
ADDENDUM TO CHAPTER 5

Deletion of \textit{XRNI} enhances nonsense suppression of \textit{can1-100}

\textit{XRNI} encodes the 5' to 3' exoribonuclease responsible for the degradation of most transcripts in yeast (Hsu and Stevens, 1993). Consistent with its role in general mRNA degradation, deletion of \textit{XRNI} was found to globally stabilize most mRNAs. Because deletion of \textit{XRNI} was found to stabilize the \textit{can1-100} transcript to the same extent as any of the \textit{UPF/NMD} deletion mutants (Fig. 17), we became interested in determining if deletion of \textit{XRNI} would also have an effect on suppression. It is clear from the findings presented in Figure 17 that deletion of \textit{XRNI} does not enhance suppression of \textit{can1-100} to the same levels as deletion of any of the \textit{UPF/NMD} factors. However, application of the suppression assay demonstrated that a \textit{xrn1Δ} strain was at least 2 fold more sensitive to canavanine as compared to its isogenic \textit{XRNI} strain suggesting that deletion of \textit{XRNI} does indeed contribute to \textit{can1-100} nonsense suppression (\textit{xrn1Δ} sensitive to 125 μg/ml, Table 5 and \textit{XRNI} sensitive to 300 μg/ml, \textit{WT} strain in Fig. 17).

This enhancement in canavanine sensitivity could be due to several reasons. Deletion of \textit{XRNI} may have created a situation wherein factors that are necessary for efficient termination become limiting. Under these conditions, there is a higher probability of readthrough past the premature termination codon of \textit{can1-100} contributing to an increase in suppression. Alternatively, the accumulation of uncapped \textit{can1-100} mRNA may contribute in some way to suppression. Under this notion, the uncapped \textit{can1-100} transcripts are able to be translated at some, albeit low, level of efficiency. The
findings of Fig. 19B indicated that some leaky termination does occur in the \textit{UPF/NMD} strain. Therefore, the elevated \textit{canl-100} mRNA levels induced by deletion of \textit{XRNI} in combination with leaky termination could conceivably contribute to the enhancement of suppression.

To corroborate the conclusion that the \textit{UPF/NMD} factors contribute to translational fidelity, suppression was monitored in strains harboring a deletion of the \textit{XRNI} gene in combination with a single deletion of any of the \textit{UPF/NMD} genes. If Upf1p, Nmd2p, and Upf3p are involved in the maintenance of translational fidelity then the absence of any of these factors in combination with a \textit{xrn1\Delta} mutation should still enhance canavanine sensitivity as compared to a strain containing only the \textit{xrn1\Delta} mutation. Suppression assays summarized in Table 5 showed that strains containing a deletion of either \textit{UPF1}, \textit{NMD2}, or \textit{UPF3} in combination with a deletion of \textit{XRNI} were more sensitive to canavanine than the isogenic \textit{xrn1\Delta} strain. Moreover, the level of \textit{canl-100} nonsense suppression was found to be highest when \textit{UPF1} was deleted in the \textit{xrn1\Delta} background (12 fold increase in suppression, Table 5). Comparatively, the \textit{xrn1\Delta,nmd2\Delta} and \textit{xrn1\Delta,upf3\Delta} strains showed lower levels of suppression exhibiting 2.5-fold less sensitivity than the \textit{xrn1\Delta,upfl\Delta} strain (Table 5). Therefore, the relative differences between the \textit{upf/nmd} mutants in the \textit{xrn1\Delta} background were similar to those obtained in the \textit{XRNI} background where, in both instances, suppression was highest in the strains harboring a deletion of \textit{UPF1}. These findings further support the notion of Upf1p playing the central role (of the three factors) in affecting efficiency of translation termination. Although the suppression phenotypes between the \textit{xrn1\Delta,upf/nmd} double
mutants differed significantly from the \textit{xrn1}\Delta strain, northern blot analyses demonstrated that all mutants stabilized the \textit{can1-100} transcript to the same extent (data not shown). This finding is in agreement with previous experiments supporting the notion that the decay and translation effects mediated by the \textit{UPF1NMD} gene products can be separated (Fig. 22 and Weng et al., 1996b).

\textbf{Epistatic relationships of Upflp, Nmd2p, and Upf3p are reproduced in strains harboring a deletion of XRNI}

The reproducible differences in the extents of \textit{can1-100} suppression promoted by the different \textit{xrn1}\Delta,upfl/nmd strains (Table 5) made it possible to investigate the epistatic relationships between Upflp, Nmd2p, and Upf3p. Mutants containing double deletions of the \textit{UPF1}, \textit{NMD2}, or \textit{UPF3} genes were constructed in strains already harboring a deletion of \textit{XRNI} and subsequently assayed for their sensitivity to canavanine. Analyses of these mutants demonstrated that any strain harboring a deletion of \textit{UPF1} (i.e. \textit{xrn1}\Delta,upfl\Delta,nmd2\Delta; \textit{xrn1}\Delta,upfl\Delta,upf3\Delta; and \textit{xrn1}\Delta,upfl\Delta,nmd2\Delta,upf3\Delta) exhibited the highest levels of suppression (sensitivity to 10 \( \mu \)g/ml canavanine) and, conversely, the only strain that harbored a wild-type \textit{UPF1} gene, the \textit{xrn1}\Delta,nmd2\Delta,upf3\Delta strain, showed the least amount of suppression (sensitivity to 50 \( \mu \)g/ml canavanine) (Table 5). Additionally, double deletion of \textit{UPF1} with either \textit{NMD2} (i.e. as in the \textit{xrn1}\Delta,upfl\Delta,nmd2\Delta strain) or \textit{UPF3} (i.e. as in the \textit{xrn1}\Delta,upfl\Delta,upf3\Delta strain) or triple deletion of all three genes resulted in a suppression phenotype identical to that of \textit{upf1}\Delta alone suggesting that combination of a \textit{nmd2}\Delta and/or \textit{upf3}\Delta mutation(s) with a \textit{upf1}\Delta
does not have an additive effect on nonsense suppression (Table 5). Therefore, the upf1Δ phenotype supersedes that of the nmd2Δ and upf3Δ phenotypes.

The suppression phenotype of the xrn1Δ nmd2Δ, upf3Δ mutant was interesting in that this mutant showed roughly three-fold less suppression that either single upf1/nmd mutation (i.e. as compared to either the xrn1Δ nmd2Δ or the xrn1Δ upf3Δ mutant). This finding suggests that in the absence of Xrn1p, Upf1p is more proficient in enhancing the efficiency of translation termination.

The observation that was not consistent with that found in the XRNI background was triple deletion of all the UPF/NMD genes. In the XRNI background, the level of suppression in the triple upf1/nmd mutant was reproducibly found to be lower that any of the upf1/nmd mutants (Fig. 18). However, when XRNI was deleted, the triple upf1/nmd mutant showed a phenotype like that of the xrn1Δ upf1Δ mutant which is completely consistent with Upf1p being epistatic to Nmd2p and Upf3p (Table 5). If, as suggested above, an alternate fidelity mechanism becomes activated in the absence of the UPF/NMD mediated fidelity mechanism, these findings imply that this alternate mechanism of fidelity cannot function in the absence of Xrn1p. It may be that Xrn1p is an integral part of this fidelity pathway or that Xrn1p plays a role in stimulating proficient termination. This idea where Xrn1p may be involved in the termination process also addresses the alternative hypothesis presented above contending that a dominant negative condition may be caused by the presence of any one of the UPF/NMD factors without the other two. These results suggest that such a situation does not occur in the absence of Xrn1p attributing some aspect of fidelity to Xrn1p. Taken together,
these results further strengthen and support the previous findings suggesting that the function of Upflp is epistatic to that of Nmd2p and Upf3p.

Overexpression of *UPF1* in *upf/nmd,xrn1Δ* mutants decreases the efficiency of nonsense suppression without altering *can1-100* mRNA levels

In order to further characterize the functional relationships of Upflp, Nmd2p, and Upf3p, these gene products were overexpressed in all of the *xrn1Δ,upf/nmd* mutant backgrounds as described above and the resulting effects on nonsense suppression were examined. As anticipated, overexpression of any of the *UPF/NMD* genes in their respective deletion strains restored the levels of suppression to that of the *xrn1Δ* strain (Table 5). Significant differences as a result of *UPF/NMD* gene overexpression were found to be similar to those reported above. Most importantly, overexpression of *UPF1* in all of the strains was found to lower suppression levels at least two- to three-fold (Table 5, compare [Yep] and [YEp-UPF1] columns) consistent with the notion that Upflp can, by itself, enhance translational fidelity. Overexpressing *UPF3* in any strain had no significant effect on suppression. Like the findings in the *XRN1* background, overexpression of *NMD2* showed an effect in *xrn1Δ,upf3Δ* cells, where it enhanced suppression to a level comparable to that obtained in *xrn1Δ,upf1Δ* cells (Table 5). In these sets of experiments, there was an effect of overexpressing *NMD2* in the *xrn1Δ,upf3Δ* strain that was not seen in the *XRN1* background where suppression was enhanced 2-fold as compared to the starting strain without the overexpression plasmid. These results would indicate that Nmd2p by itself also has the ability to enhance termination fidelity. This interpretation,
however, is complicated by finding that same phenomenon is not observed when \( NMD2 \) is overexpressed in the \( xrn1\Delta nmd2\Delta \) or the \( xrn1\Delta upf3\Delta \) mutant. Clearly, in order to fully understand these observations, other methods are necessary to further dissect the regulatory relationships between the \( UPF/NMD \) factors. Nonetheless, these results are similar the findings of experiments conducted in a \( XRNI \) background.

Finally, the steady-state levels of the \( can1-100 \) mRNA were examined in \( xrn1\Delta upf/nmd \) mutants overexpressing \( UPF1 \) since overexpression of \( UPF1 \) altered the suppression phenotypes of all of the mutants. \( UPF1 \) overexpression did not significantly alter \( can1-100 \) mRNA levels when compared to the starting mutant strains that contained the vector only (data not shown) demonstrating that changes in the suppression phenotype caused by overexpression of \( UPF1 \) were not attributable to changes in \( can1-100 \) mRNA levels. In total, these results maintain the previously noted prominent role of Upf1p in controlling the efficiency of translation termination, support a regulatory role for Nmd2p and Upf3p, and provide additional evidence that the mRNA decay and translation functions of Upf1p are separable.
Table 5. Overexpression of UPF1, NMD2, or UPF3 in xrn1, upf/nmd strains

<table>
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<tr>
<th>Strain</th>
<th>Canavanine Sensitivity (µg/ml)</th>
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|        | YEp                           | YEp-UPF1 | YEp-NMD2 | YEp-NMD2
| YEp    | 10                            | 125      | 125      | 125
|        | 25                            | 150      | 125      | 125
|        | 50                            | 75       | 75       | 75
|        | 10                            | 75       | 75       | 75
|        | 10                            | 25       | 25       | 25
|        | 25                            | 75       | 75       | 75
|        | 125                           | 125      | 125      | 125
|        | 25                            | 125      | 125      | 125
|        | 125                           | 125      | 125      | 125

Suppression assays are summarized, with the concentration of canavanine at which each strain began to exhibit the canavanine-sensitive phenotype indicated (canavanine sensitive). Yeast strains of the indicated genotypes were transformed with the high-copy-number vector alone (YEp; control) or high-copy-number plasmids expressing UPF1, NMD2, and UPF3 (YEp-UPF1, YEp-NMD2, and YEp-UPF3, respectively).
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