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Nonsense-Containing mRNAs That Accumulate in the Absence of a Functional Nonsense-Mediated mRNA Decay Pathway Are Destabilized Rapidly upon Its Restitution

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Nonsense-mediated mRNA decay (NMD) is a conserved proofreading mechanism that protects eukaryotic cells from the potentially deleterious effects of truncated proteins. Studies of *Saccharomyces cerevisiae* imply that NMD is a predominantly cytoplasmic decay pathway, while studies of mammalian systems suggest that decay of most substrate mRNAs may occur while they are still associated with the nucleus, possibly during a round of translation that occurs during their export to the cytoplasm. Complete entry of the latter mRNAs into the cytoplasm appears to render them immune to further NMD; i.e., they escape further susceptibility to this decay pathway. To determine if yeast cytoplasmic nonsense-containing mRNAs that evade decay are subsequently immune to NMD, we examined the consequences of placing each of the three *UPF*/*NMD* genes under the control of a galactose-inducible promoter. The decay kinetics of *ADE2* and *PGK1* nonsense-containing mRNAs were then analyzed when expression of *UPF1*, *NMD2*, or *UPF3* was either repressed or subsequently induced. Results from these experiments demonstrated that activation of NMD caused rapid and immediate degradation of both substrate transcripts, with half-lives of both stable mRNA populations shortened to approximately 7 min. These findings make it unlikely that yeast nonsense-containing mRNAs can escape degradation by NMD and indicate that such mRNAs are available to this decay pathway at each round of translation.

Intricate mechanisms that safeguard against errors in gene expression have been identified in eukaryotes (10, 17, 19, 25, 37, 85). 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 (25, 35, 36, 55, 66, 67). The process of NMD has been studied extensively in *Saccharomyces cerevisiae*, where rapid degradation of nonsense-containing mRNAs involves recognition of a premature translation termination codon, deadenylation-independent decapping, and subsequent 5′→3′ exonucleolytic digestion of the remainder of the mRNA (36, 60). In addition to the decapping enzyme Dcp1p (3, 43) and the exonuclease Xrn1p (32), three additional *trans*-acting factors are essential for NMD in yeast: Upf1p, Nmd2p/Upf2p, and Upf3p (12, 24, 27, 44–46). Consistent with their roles in the response to aberrant translation, all three of the latter *UPF*/*NMD* proteins have been shown to localize predominantly to the cytoplasm and to associate with polyribosomes (1, 2, 53, 64, 73). These observations indicated that yeast NMD occurred in the cytoplasm and was linked to translation, conclusions consistent with other results showing that (i) drugs or mutations that inhibit translation also eliminate NMD (49, 81, 90); (ii) nonsense-containing polysomal mRNAs stabilized in cycloheximide-treated cells reinitiate NMD as soon as the drug is withdrawn (90); and (iii) a dominant-negative form of Nmd2p/Upf2p inhibits decay only when localized to the cytoplasm (24).

In mammalian cells, NMD may not be limited to the cytoplasm. Nonsense-containing derivatives of mammalian β-globin, *APRT*, and *HEXA* mRNAs, as well as *GPx1* mRNA, have been shown to decay in the cytoplasm (39, 54, 58, 59, 68). However, other nonsense-containing mRNAs, e.g., those encoded by the *TPI*, *TCR*-β, and *L*κ genes (7–9, 15, 50), as well as globin mRNAs expressed in nonerythroid cells and a portion of *APRT* mRNA (39, 41, 87, 88), appear to be degraded while still associated with the nucleus. Of particular interest is the observation that the fraction of nonsense-containing *TPI* mRNAs that avoid nucleus-associated decay and are exported to the cytoplasm appear to be as stable as wild-type *TPI* mRNA (9). These findings suggest that recognition of premature nonsense codons in at least some mammalian mRNAs occurs solely in the nucleus, or during nuclear export, and that those mRNAs which escape to the cytoplasm can acquire immunity to further degradation by the NMD pathway (76).

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

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TABLE 1. Yeast strains used in this study

Strain	Genotype	Reference
HFY1200	*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 UPF1 NMD2 UPF3*	24
HFY870	*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 upf1::HIS3 NMD2 UPF3*	27
HFY1300	*MAT*α *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 UPF1 nmd2::HIS3 UPF3*	24
HFY861	*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 UPF1 NMD2 upf3::HIS3*	27
HFY1067	*MAT***a** *ade2-1 HIS3 his4-38 leu2-3,112 trp1-1 ura3-1 can1-100 UPF1 NMD2 UPF3 dcp1::URA3*	F. He and A. Jacobson, unpublished data
HFY1081	*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 UPF1 NMD2 UPF3 xrn::ADE2*	F. He and A. Jacobson, unpublished data
HFY456	*MAT*α *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 rpb1-1 UPF1 nmd2::HIS3 UPF3* [pRS315]	24

residing in the cytoplasm of yeast cells are potentially susceptible to NMD at each round of translation.

MATERIALS AND METHODS

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. (69). Transformation of yeast was done by the rapid method described by Soni et al. (75). DNA manipulations were performed according to standard techniques (71). All PCR amplifications were performed with *Taq* DNA polymerase (84) and confirmed, where appropriate, by DNA sequencing using the method described by Sanger et al. (72). Plasmid DNAs were prepared from *Escherichia coli* DH5α.

RNA extraction, Northern blotting, and mRNA decay rate measurements. For the galactose induction experiments, RNA was isolated from yeast using the hot phenol method (29). Aliquots (20 μg) of each RNA sample were analyzed by Northern blotting. For isolation of RNA from polyribosome fractions, the method described by Benard et al. (5) was used. DNA probes were prepared by either random priming with [α-^{32}P]dCTP (16) or by 5′ end-labeling of single-stranded oligodeoxyribonucleotides with [γ-^{32}P]ATP (71). Relative mRNA 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 *Bgl*II fragment from an *xrn1*::*ADE2* disruption plasmid), *PGK1* (oligonucleotide 1 from reference 66), *CYH2* (a 600-bp *Eco*RI-*Hin*dIII fragment from pGEM4Z-*CYH2* which hybridizes to both the pre-mRNA and the mRNA [29]), and *SCR1* (a 400-bp fragment amplified from yeast genomic DNA using oligonucleotides SCR1-1 and SCR1-2 [52]). mRNA decay rates were measured in wild-type and *nmd2* cells grown in either rich medium (YEPD) or SC–galactose minimal medium, using thermal inactivation of RNA polymerase II to inhibit ongoing transcription (24, 29, 69).

Protein gels, Western blotting, and antibodies. Whole-cell lysates were prepared by collecting culture aliquots equivalent to 0.2 ml at an optical density at 600 nm (OD_{600}) of 1. The appropriate volume was centrifuged for 10 min, resuspended in 10 μl of 1× sample buffer (71), and boiled for 5 min just before loading onto the respective polyacrylamide gel. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described by Laemmli (42). 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 (23). Detection was enhanced by chemiluminescence with either the ECL or ECL(+) kit from Amersham Corp. Antibodies used included polyclonal affinity-purified anti-Upf1p antibody (53), polyclonal affinity-purified anti-Nmd2p antibody (26), and the monoclonal anti-hemagglutinin (anti-HA) antibody, 12CA5 (from Boehringer Mannheim Biochemicals), for detection of Upf1p, Nmd2p, and HA epitope-tagged Upf3p, respectively.

Plasmid constructions. The galactose-inducible *UPF1* construct was made by ligating a 3.6-kb *Eco*RI-*Sal*I fragment from pMA424-*UPF1* (24) to pRS426 (11), 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 3.7-kb *Xba*I-*Sal*I fragment cut from the pRS315-*NMD2* plasmid (27) to the pMW29 vector (91) cut with the same enzymes. The galactose-inducible *UPF3* plasmid was constructed by ligating a 1.7-kb *Nco*I-*Sal*I fragment cut from the pRS314-HA-*UPF3* plasmid (27) to pRS316 (74), containing the *GAL1* promoter, cut with the same enzymes. The latter plasmid was obtained by restriction digestion of pRS314-GALp-HA-*NMD3* [4]).

Preparation of polyribosome fractions. Yeast cell extracts were prepared and fractionated on sucrose gradients as described previously (53).

Galactose induction. Yeast strains containing galactose-inducible *UPF*/*NMD* gene constructs were grown in SC medium (69) without uracil and with raffinose to mid-log phase (OD_{600} = 0.5). Strains containing the inducible *UPF*/*NMD* gene constructs and either of the *pgk1* nonsense alleles were grown in SC medium without uracil and leucine and with raffinose (to maintain selection for the *GAL-UPF*/*NMD* plasmid and the plasmid harboring the *pgk1* allele, respectively) to mid-log phase(OD_{600} = 0.5). Galactose was then added to a final concentration of 2%. Culture aliquots for RNA and protein isolations were taken at 10-min intervals for 40 min.

RESULTS

The *ade2-1* transcript is a substrate for NMD. To address the stability of cytoplasmic nonsense-containing mRNAs, we took advantage of an allele of the *ADE2* gene, *ade2-1*. Earlier studies showed that the *ade2-1* mutation could be suppressed in yeast strains containing an ochre tRNA suppressor (86), suggesting that the *ade2-1* allele was attributable to a nonsense (UAA) mutation and that the *ade2-1* mRNA was likely to be a substrate for NMD. To test the latter possibility, single deletions of *UPF1*, *NMD2*, or *UPF3* were constructed in yeast strains that harbored the *ade2-1* allele, and the effects of these mutations on the abundance of the *ade2-1* transcript were examined. Northern analyses of mRNA steady-state levels demonstrated that mutations in genes regulating the 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. 1) (25). The *ade2-1* mRNA was approximately sevenfold more abundant in *upf*/*nmd* mutant cells than in the isogenic *UPF*/*NMD* (wild-type) strain (Fig. 1). Likewise, deletion of a gene encoding a general factor involved in mRNA decay (i.e.,

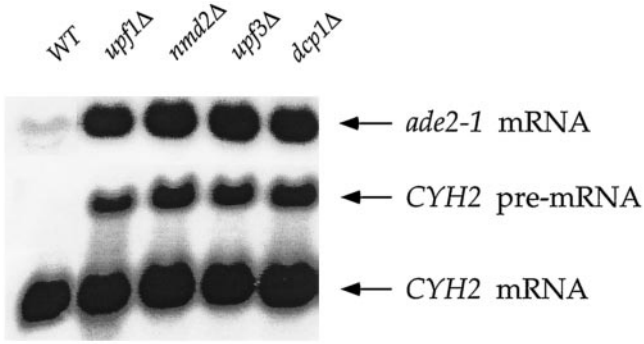

FIG. 1. The *ade2-1* transcript is a substrate for NMD. Total RNA isolated from yeast strains with the indicated *UPF*/*NMD* genotypes was analyzed by Northern blotting with DNA probes that detected the *ade2-1* or *CYH2* transcripts. *WT*, wild type. The yeast strains used for this experiment were grown in YEPD at 30°C and included HFY1200 (*WT*), HFY870 (*upf1*Δ), HFY1300 (*nmd2*Δ), HFY861 (*upf3*Δ), and HFY1067 (*dcp1*Δ).

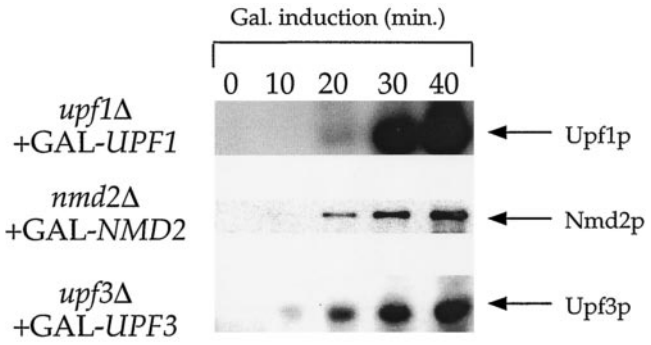

FIG. 2. Galactose-inducible expression of *UPF1*, *NMD2*, and *UPF3*. Yeast strains harboring *upf1*Δ (HFY870), *nmd2*Δ (HFY1300), or *upf3*Δ (HFY861) mutations and the respective, complementary galactose-inducible *NMD* gene construct were grown in SC raffinose liquid medium without uracil to mid-log phase (OD_{600} = 0.5). Galactose was then added to a final concentration of 2%, culture aliquots were taken at 10-min intervals, and all samples were subsequently analyzed by Western blotting. Gal., galactose.

DCP1) (62) also promoted a sevenfold increase in *ade2-1* transcript abundance (Fig. 1). These differences in mRNA abundance were consistent with the respective differences in the decay rates of the *ade2-1* mRNA in wild-type and *upf*/*nmd* mutant cells. In YEPD medium at 37°C, the half-life ($t_{1/2}$) of the *ade2-1* mRNA was found to be less than 5 min in the *UPF*/*NMD* strain and approximately 35 min in *upf*/*nmd* cells (data not shown), suggesting that the wild-type gene, *ADE2*, encodes a relatively stable mRNA. The latter conclusion is supported by previous experiments, reporting $t_{1/2}$s of 30 and 33 min, respectively, for the *ADE2* mRNA (80; htt://web.wi.mit.edu/young/expression/halflife.html). Collectively, these results indicate that the *ade2-1* mRNA requires Upf1p, Nmd2p, Upf3p, and Dcp1p for its degradation and is, therefore, a typical substrate for NMD.

Galactose-inducible expression of *UPF1*, *NMD2*, and *UPF3*. To assess the stability of *ade2-1* transcripts that had avoided degradation by the NMD pathway, we sought a mechanism to regulate the activity of the pathway. To accomplish this, the *UPF1*, *NMD2*, and *UPF3* genes were cloned into either single- or high-copy-number 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. 2, Upf1p, Nmd2p, and Upf3p are not detectable in the respective regulated strains prior to galactose induction, but these proteins accumulate substantially postinduction. Quantitation of each of the Western blots shown in Fig. 2, and others, indicated that (i) Upf1p, Nmd2p, and Upf3p all begin to accumulate approximately 12 to 14 min after galactose addition and (ii) by 20 min after galactose addition, the cellular levels of each of the induced proteins are comparable to those present in the isogenic *UPF*/*NMD* strains (data not shown). From these data, we conclude that use of these constructs allows for inducible expression of *UPF1*, *NMD2*, and *UPF3*.

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. Upon activation of the NMD pathway, the fate of these mRNAs can be monitored by simply measuring their relative abundance over time, leading to an approximation of the decay kinetics of the steady-state *ade2-1* mRNA population. If the accumulated *ade2-1* transcripts are susceptible to NMD, then activation of this decay pathway should result in their rapid degradation. If the *ade2-1* transcripts are immune to NMD, then activation of the decay pathway should have no effect on the stability of these mRNAs. The overall *ade2-1* mRNA population would then consist of newly synthesized mRNAs that are rapidly degraded and the stable cytoplasmic transcripts that had accumulated prior to activation of NMD. Under these circumstances, the expected decay rate of the entire *ade2-1* mRNA population would initially be slow (approximating that of the stabilized *ade2-1* transcripts), and then, after substantial dilution with newly synthesized mRNA, the population would begin to reflect a more rapid decay rate.

These possibilities were evaluated by Northern blot analyses of yeast strains expressing regulatable *UPF1*, *NMD2*, or *UPF3*. As a first step, the $t_{1/2}$ of the *ade2-1* mRNA was measured in *nmd2* cells in SC-galactose medium. In experiments in which temperature-sensitive *rpb1-1*/*nmd2*Δ cells were shifted to 37°C (24), the *ade2-1* mRNA had a $t_{1/2}$ of 22 to 24 min (Fig. 3). Extrapolation from previous comparisons of mRNA decay rates at 30 and 37°C indicates that the $t_{1/2}$ of this mRNA at 30°C must approximate 44 to 48 min (29, 65). The abundance of this stabilized *ade2-1* mRNA was then assessed (at 30°C) as a function of *UPF1*, *NMD2*, or *UPF3* expression. Figure 4A shows that, as expression of these factors increases (Fig. 2), the abundance of the *ade2-1* mRNA decreases (Fig. 4A). Subsequent to the time at which the *UPF*/*NMD* proteins begin to accumulate (12 to 14 min postinduction; see above), the *ade2-1* mRNA disappears, with a $t_{1/2}$ of approximately 7 min in all three strains. By 30 min after galactose induction of any of the three *UPF*/*NMD* genes, approximately 20% of the *ade2-1* mRNA population remains, and by 40 min, the abundance of the *ade2-1* mRNA returns to the low levels characteristic of a *UPF*/*NMD* (wild-type) strain. These experiments show that induction of Upf1p, Nmd2p, or Upf3p restores NMD and results in immediate destabilization of the entire *ade2-1* mRNA population, i.e., the *ade2-1* mRNA molecules present

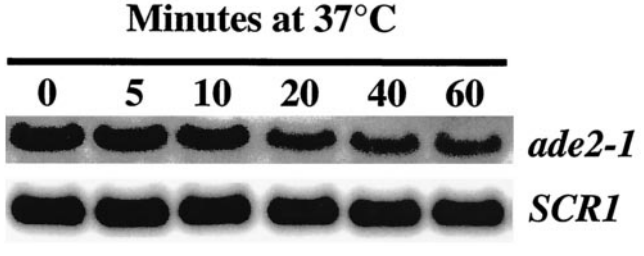

FIG. 3. $t_{1/2}$ of the *ade2-1* mRNA in SC-galactose medium. Yeast cells (HFY456) harboring *nmd2*Δ and *rpb1-1* mutations were grown in SC-galactose medium at 24°C and then shifted to 37°C to inhibit further transcription. RNA was isolated at different times after the temperature shift and subjected to Northern blotting as in Fig. 1. Hybridization to the *SCR1* probe served as a loading control (52).

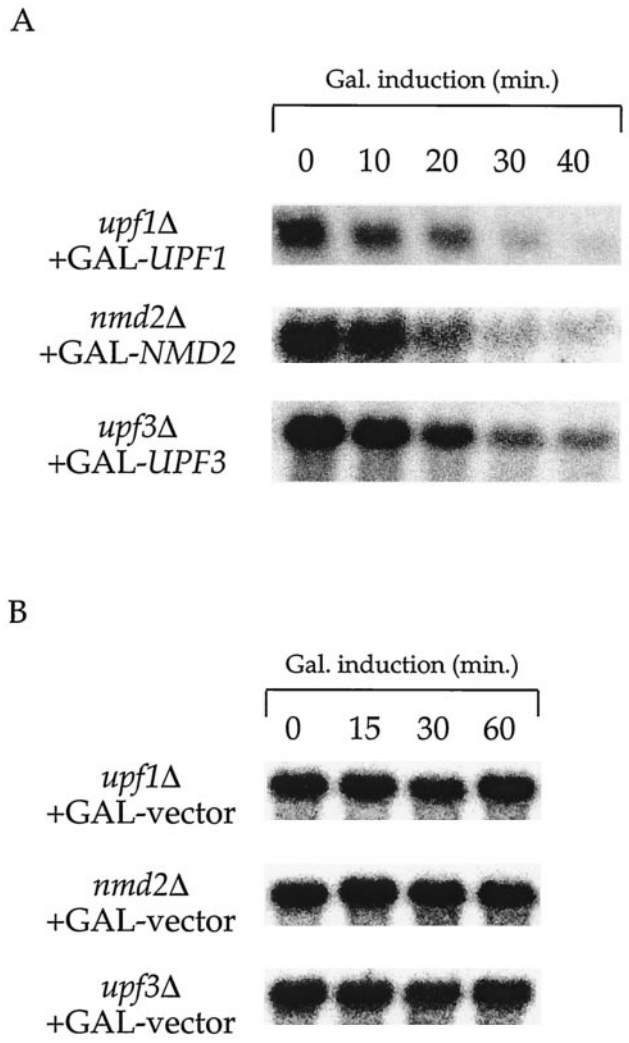

FIG. 4. The *ade2-1* transcript is rapidly degraded upon activation of NMD. (A) Activation of NMD causes rapid degradation of *ade2-1* mRNA. Total RNA was isolated from yeast strains (HFY870, HFY1300, and HFY861) harboring *upf1*Δ, *nmd2*Δ, or *upf3*Δ mutations and the respective, complementary galactose-inducible *NMD* gene constructs (GAL-*UPF1*, GAL-*NMD2*, or GAL-*UPF3*). Galactose induction was performed as described in Fig. 2, and RNA was analyzed by Northern blotting with a DNA probe 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, and harboring only the vector plasmid (GAL-vector), was analyzed by Northern blotting as in Fig. 4A. Gal., galactose.

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 *upf1*Δ, *nmd2*Δ, and *upf3*Δ 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). Side-by-side Northern analyses of *ade2-1* mRNA in these control cells with those obtained in uninduced cells harboring *GAL1-UPF1*, *GAL1-NMD2*, or *GAL1-UPF3* plasmids yielded virtually identical levels (data not shown), a result indicating that the observed induction is a bona fide switch from inactive to active states of NMD.

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 (90), the association of the *ade2-1* mRNA population with ribosomes was investigated under conditions where NMD was either inactive or active. Cytoplasmic extracts were prepared from a strain containing galactose-inducible *UPF1*, both prior to galactose induction and 30 min postinduction, 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 to 7), suggesting that these transcripts are associated with actively translating ribosomes. The association of these transcripts with an average of four to five ribosomes is consistent with premature translational termination within a large mRNA (2.2 kb [77]). 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 Fig. 5A and B were also probed for the *SCR1* RNA. The latter blots demonstrate that the qualities and quantities of RNA isolated from the two sets of gradients (0 and 30 min post-galactose induction) were similar (Fig. 5A and B).

Results virtually identical to those of Fig. 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 *PGK1* transcripts with early but not late nonsense codons. To substantiate our findings with the *ade2-1* mRNA, we investigated the effect that restoration of NMD had on the decay kinetics of another nonsense-containing transcript. The *PGK1* mRNA is normally very stable, having a $t_{1/2}$ in *rpb1-1* temperature-shift experiments of 60 to 70 min (66, 80), but a derivative with a nonsense mutation at codon 22 (*pgk1*-UAG-2) is extremely unstable ($t_{1/2}$ = 6 min [66, 81]). Inactivation of the NMD pathway (by mutations in *UPF1*, *NMD2*, or *UPF3*) restores the stability of this nonsense-containing mRNA ($t_{1/2}$ >35 min [66]), confirming that it is a substrate for NMD. The large differences in the $t_{1/2}$s 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.

Figure 6A demonstrates that induction of *UPF1*, *NMD2*, or *UPF3* (in the respective deletion strains) resulted in rapid disappearance of the *pgk1*-UAG-2 mRNA (see "early *pgk1*" panel). The decay kinetics for the steady-state population of this mRNA were comparable to those of the *ade2-1* mRNA, such that (i) it disappeared with a $t_{1/2}$ of approximately 7 min, after a lag for induction of the pathway, and (ii) by 30 min postinduction, most (85%) of the mRNA was degraded (Fig. 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.

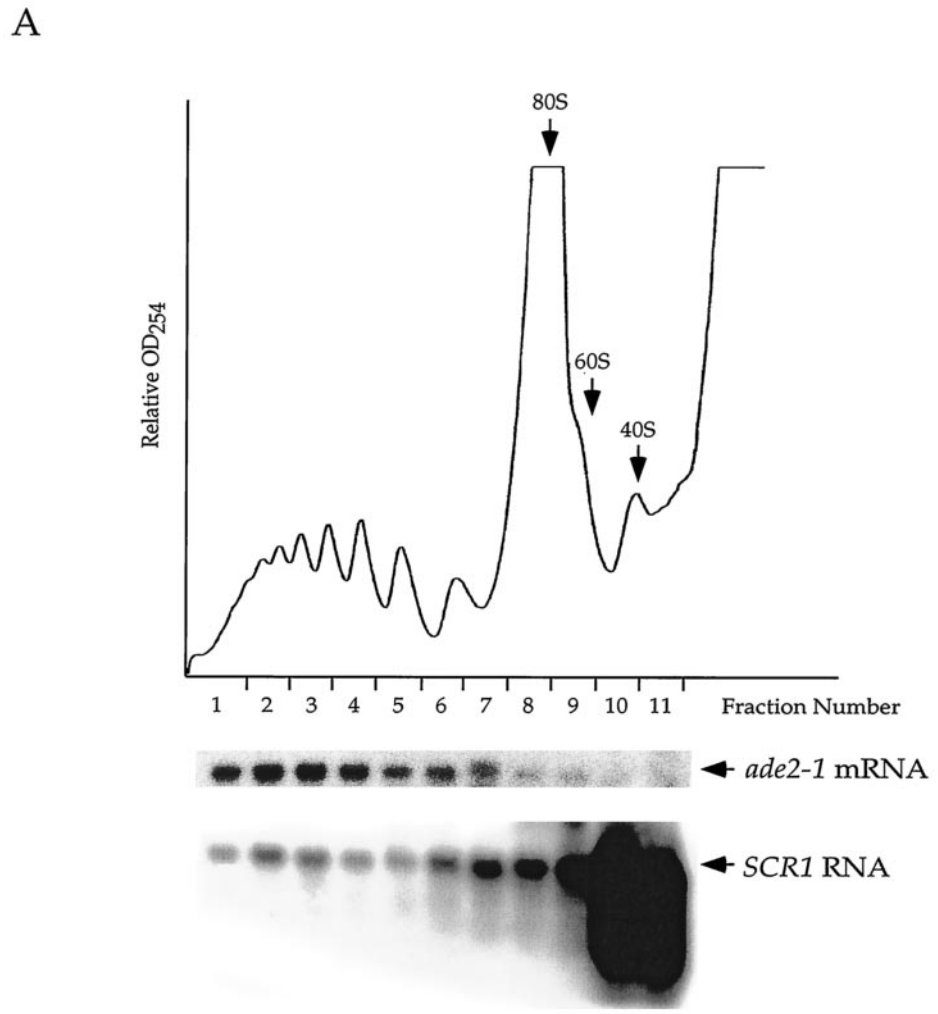

FIG. 5. Degradation of the *ade2-1* mRNA occurs on polysomes. (A) The *ade2-1* mRNA is detected in the polysome fractions before galactose induction. Total RNA isolated from polysome fractions collected before the addition of galactose was analyzed by Northern blotting with DNA probes that detected the *ade2-1* mRNA and the *SCR1* 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 min after the addition of galactose was analyzed by Northern blotting as described for panel A. The results depicted in this figure were obtained from the *upf1*Δ yeast strain (HFY870) harboring the galactose-inducible *UPF1* construct.

Destabilization of mRNAs by premature nonsense codons is a position-dependent phenomenon wherein mRNAs with nonsense codons occurring in the last 20 to 30% of the coding region retain their wild-type decay rates (21, 35, 36, 66). As an additional means to determining whether the postinduction disappearance of the *pgk1*-UAG-2 and *ade2-1* mRNAs was a direct consequence of restoration of NMD, we repeated the NMD induction experiments in cells harboring a *pgk1* allele with a nonsense mutation at codon 385 (*pgk1*-UAG-7). This mutation does not affect the stability of the encoded mRNA ($t_{1/2} > 35$ min) and does not render it a substrate for NMD (66). As such, the *pgk1*-UAG-7 transcript serves as an ideal control to test whether galactose induction of the *UPF*/*NMD* genes results in selective degradation of bona fide NMD substrates. Figure 6A shows that galactose induction of *UPF1*, *NMD2*, or *UPF3* does not affect the abundance of the *pgk1*-UAG-7 mRNA (see "late *pgk1*"). This result demonstrates that the decay pathway activated by induction of the *UPF*/*NMD* genes remains specific for proper substrate mRNAs and reiterates the finding that the *pgk1*-UAG-7 mRNA is not a substrate for NMD.

Control experiments were also conducted to ensure that destabilization of the *pgk1*-UAG-2 mRNA subsequent to restoration of NMD was not due to an effect of galactose addition. Figure 6B shows that *upf1*Δ, *nmd2*Δ, and *upf3*Δ strains containing either the early or late *pgk1* nonsense alleles and an empty *GAL1* vector do not alter the stability of either the early or late *pgk1* nonsense mRNAs in response to galactose addition to the growth medium. Therefore, it is activation of the NMD pathway and not simply the addition of galactose that causes destabilization of the *pgk1*-UAG-2 mRNA.

DISCUSSION

Inducible NMD in yeast. In the absence of a functional NMD pathway, yeast nonsense-containing transcripts exhibit $t_{1/2}$s characteristic of their stable, wild-type counterparts (24,

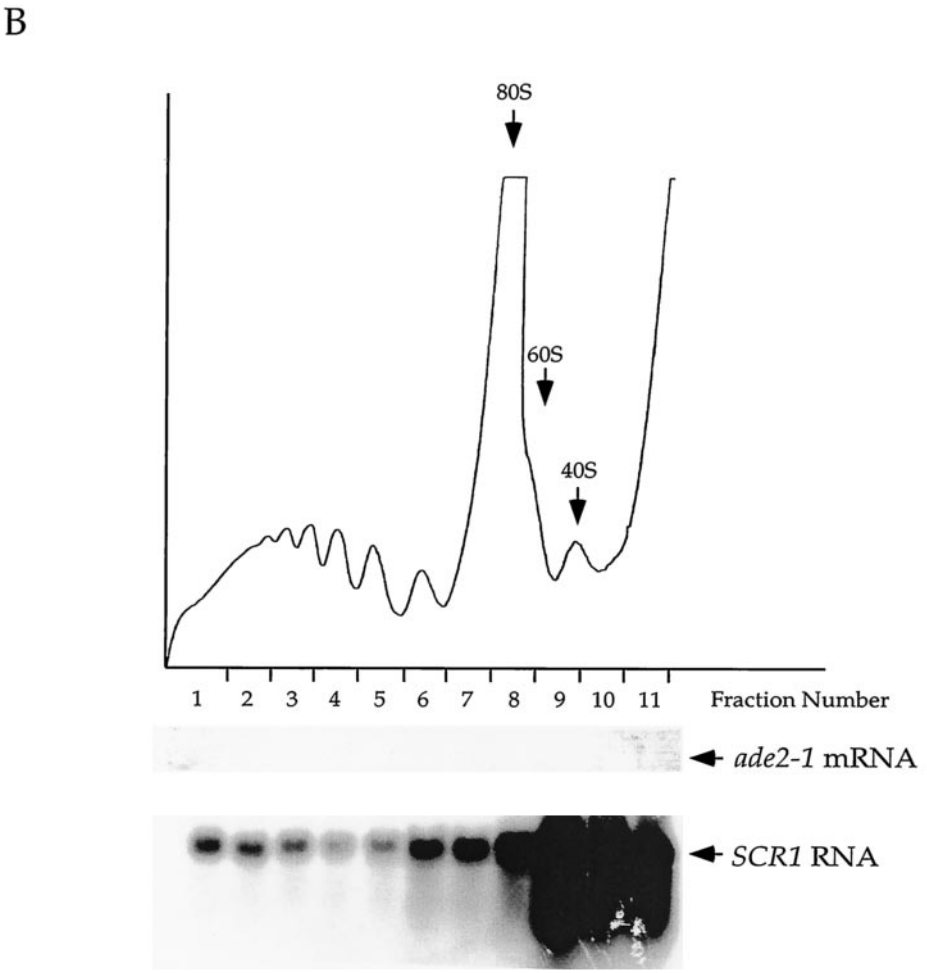

FIG. 5—*Continued.*

46, 81). To determine whether nonsense-containing mRNAs that accumulate in NMD-deficient cells retain their ability to be recognized and degraded by the NMD pathway, we created a series of yeast strains in which NMD was galactose inducible. We then analyzed the cellular levels of specific nonsense-containing mRNAs when expression of *UPF1*, *NMD2*, or *UPF3* was repressed, or subsequently induced. Induction of any of the *UPF*/*NMD* genes in their respective deletion strains resulted in a 12- to 14-min lag before any of the factors could be detected, followed by a rapid and immediate reduction of the levels of *ade2-1* and *pgk1*-UAG-2 substrate transcripts. By 40 min postinduction, the levels of both substrate mRNAs in all induced strains were decreased to levels observed in wild-type cells (Fig. 4, 6, and 7). These results indicate that de novo synthesis of the respective missing factors reconstituted the NMD pathway in each of the strains analyzed, leading to a reduction in $t_{1/2}$s of preexisting nonsense-containing mRNAs. Several considerations suggest that the observed reductions in mRNA levels reflect a uniform switch in mRNA $t_{1/2}$s, from >35 min to approximately 7 min (Fig. 7), as opposed to the average of the behaviors of multiple mRNA populations. If, for example, the steady-state populations of *ade2-1* and *pgk1*-UAG-2 mRNAs remained stable and only the newly synthesized mRNA decayed rapidly, then the newly synthesized population would have to comprise at least 85% of the total mRNA of each species to accommodate the observed changes in absolute levels. Such massive accumulation of the newly synthesized mRNA species would be highly unlikely in light of (i) the constraints imposed by their short $t_{1/2}$ (22) and (ii) the apparent differences in the rates of transcription of the *ADE2* and *PGK1* genes (31).

Further support for the notion that the *ade2-1* and *pgk1*-UAG-2 mRNA populations are uniformly destabilized after *UPF1*, *NMD2*, or *UPF3* induction is obtained from the sucrose gradient analysis of Fig. 5. In strains in which NMD was inactive, *ade2-1* mRNA was found to cosediment with polyribosomes. Induction of the NMD pathway for 30 min, however, led to a quantitative and selective disappearance of the transcripts harboring early nonsense codons from the polysome fractions. If two separate subpopulations of mRNA existed (i.e., stable preinduction mRNA and unstable postinduction mRNA), we should have detected the residual stable species on Northern blots performed after 30 min of galactose induction.

What comprises a substrate for nonsense-mediated decay of yeast mRNAs? Yeast mRNAs containing premature translation termination codons are rapidly degraded via the NMD

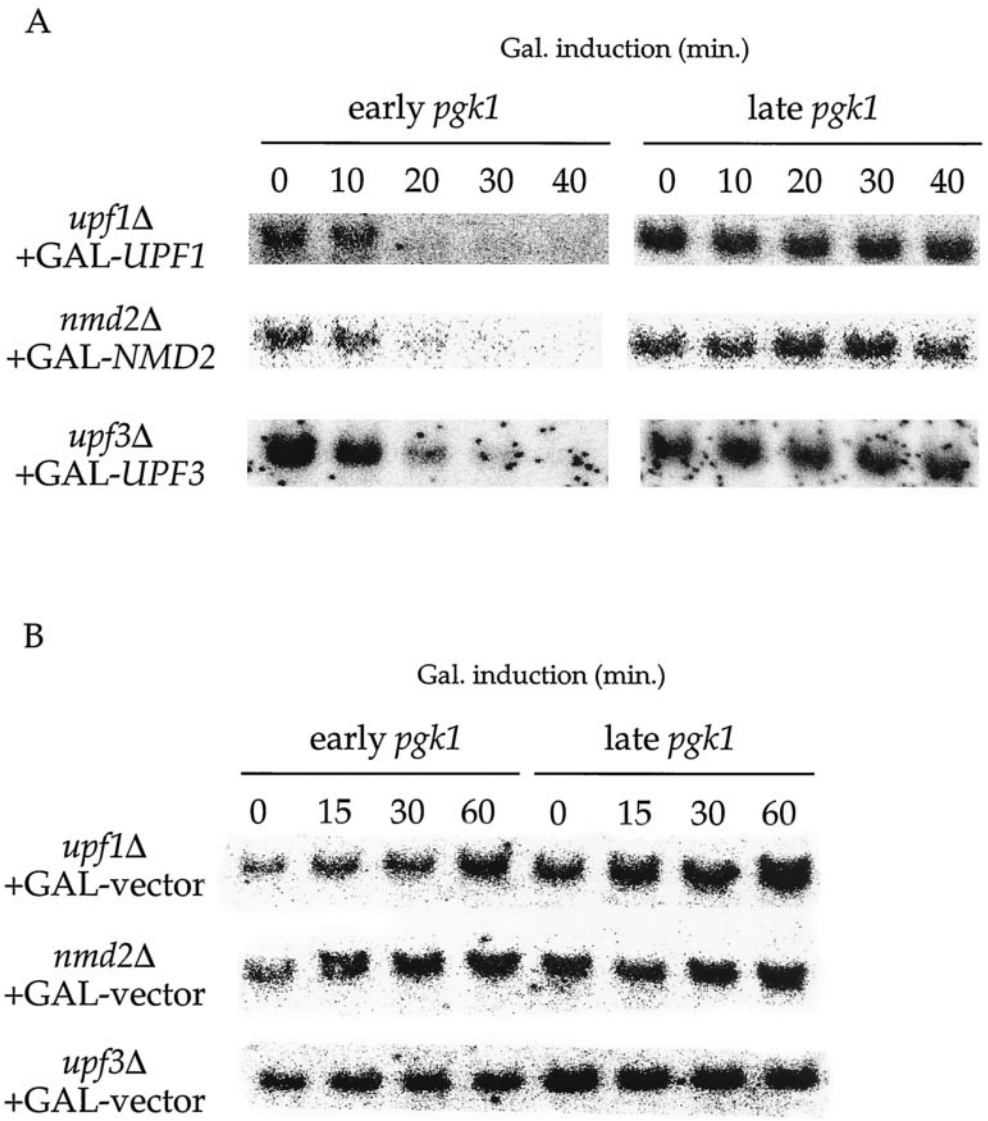

FIG. 6. *pgk1* mRNA harboring an early nonsense codon degrades rapidly upon activation of NMD. (A) Activation of NMD results in degradation of a *pgk1* transcript containing an early nonsense codon but does not destabilize a *pgk1* transcript with a late nonsense codon. Total RNA isolated from yeast strains (HFY870, HFY1300, and HFY861) with the indicated *UPF*/*NMD* genotypes harboring complementing galactose-inducible *NMD* gene constructs (GAL-*UPF1*, GAL-*NMD2*, or GAL-*UPF3*) was analyzed by Northern blotting with a DNA probe that detected nonsense-containing *pgk1* transcripts. (B) The addition of galactose does not destabilize *pgk1* mRNA harboring an early nonsense codon. 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 for panel A. Gal., galactose.

pathway when several criteria are met. The termination codon in question must occur within the first two-thirds to three-quarters of the mRNA coding region and be 5′ proximal to an essential sequence element (the downstream element [64, 66, 89]). Moreover, the nonsense-containing mRNA needs to be translated (90), and several factors essential to the NMD process need to be present and functional (12, 24, 27, 44, 46). The nonsense codon that promotes mRNA destabilization can occur within a conventional coding region (49, 66) or be derived from an upstream open reading frame (12), present within an unprocessed intron (25), recognized only during out-of-frame translation caused by leaky scanning (81), or be the normal termination codon in an mRNA with an extended 3′ untranslated region (UTR) (61). Since NMD has been shown to occur without significant prior shortening of the mRNA poly(A) tail (60), it has been suggested that the decay-initiating event can occur very early in the functional lifetime of the mRNA (35). It has been unclear, however, whether this is obligatory or if an mRNA qualifies as an NMD substrate at any time during its cellular life cycle.

One perspective, originally derived from data in mammalian cells, suggests that spatial relationships reflect temporal relationships, i.e., that the apparent nuclear proximity of NMD (55, 56) and the deposition of factors essential for NMD during pre-mRNA splicing (34, 40, 47, 48, 51) must reflect a decay process that occurs during an early round of translation or not at all (9, 76). Experiments utilizing regulated expression of the *Gpx1* mRNA suggest that this rule may only apply to tran-

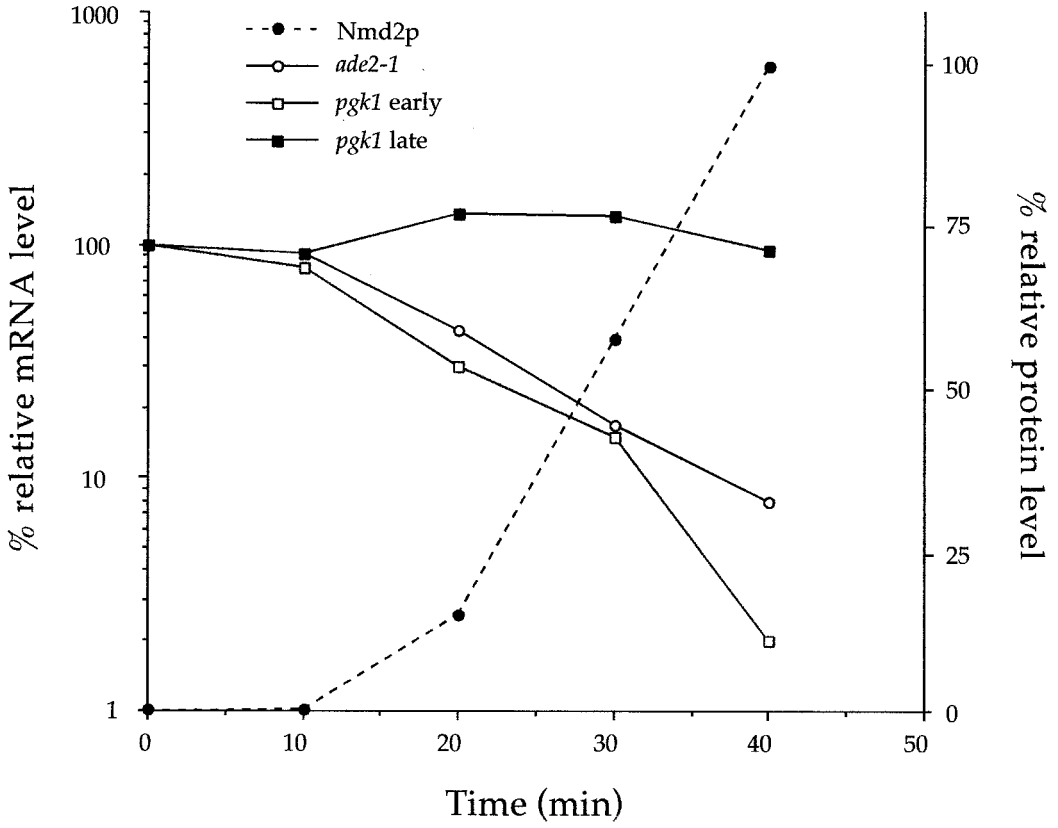

FIG. 7. Activation of NMD results in the rapid decay of substrate transcripts. Quantitation of the relative levels of Nmd2p (●) and nonsense-containing mRNAs before and after the activation of NMD (by the addition of galactose): *ade2-1* mRNA (○), *pgk1* mRNA harboring an early nonsense codon (■), and *pgk1* mRNA harboring a late nonsense codon (□). The data for this graph were derived from the Northern blots of Fig. 4A (*ade2-1*) and 6A (early and late *pgk1*) and from the Western blot of Fig. 2 (Nmd2p).

scripts for which NMD is nucleus associated (78). The notion of a limited opportunity for decay of nonsense-containing transcripts has also been considered in yeast, where it has been shown that the RNA-binding protein Hrp1p associates with Upf1p and with the downstream element of a *PGK1* nonsense-containing transcript, and that these associations appear to promote NMD (18). The known roles of Hrp1p in mRNA processing and export (28, 38, 57) led to a model in which Hrp1p association with newly synthesized mRNA would be disrupted by the initial round of translation but maintained if translation were interrupted by premature termination (18). In the latter event, Hrp1p bound to an mRNA was postulated to stimulate rapid mRNA decay as a consequence of its ability to interact with the Upf1p-containing surveillance complex (13, 14, 18).

A second, related model for yeast NMD postulates that mRNA decay is triggered by a ribosome's failure to terminate adjacent to a properly configured 3′ UTR (30, 36). This "faux-UTR" model suggests that proper termination of translation and normal rates of mRNA decay only occur in the context of interactions between a terminating ribosome and a specific RNP domain or set of factors localized 3′ to the stop codon (6, 30, 36). The mammalian and surveillance complex models both imply that at least some nonsense-containing mRNAs are only capable of being degraded during their initial rounds of translation, after which they acquire immunity to NMD. However, the data presented here demonstrate that, at least in yeast, NMD is not limited to an early round of mRNA translation and can occur at any time during an mRNA's life cycle. This suggests that (i) there are no NMD-essential factors that are shed during readthrough translation in NMD-deficient strains (45, 52, 82, 83), (ii) factors that are shed can reassociate with an mRNA while it remains in the cytoplasm, or (iii) marking is not critical for NMD of *ade2* and *pgk1* mRNAs and that their mode of decay more closely approximates the tenets of the faux-UTR model (36).

The continual availability of substrates for the yeast NMD apparatus implies that their decay occurs in the cytoplasm. The mere presence of a premature nonsense codon within a transcript is not sufficient to promote its degradation. Destabilization of yeast nonsense-containing mRNAs requires their translation, a conclusion that follows from observations that NMD, its principal factors, and decay intermediates are all localized to polysomes (1, 2, 53, 64, 73, 81, 90; see also Fig. 5) and that decay can be antagonized by drugs or mutations that interfere with protein synthesis (70, 90) or by tRNAs that suppress termination (20, 49). While a role for translation has generally been regarded to imply a cytoplasmic event, recent results with mammalian cells (33, 63) demand firmer evidence for such a spatial assignment. Experiments providing additional substantiation include those showing that (i) mutations of the *UPF*/*NMD* genes not only lead to the stabilization of

nonsense-containing mRNAs but also promote nonsense suppression (45, 52, 79, 82, 83), (ii) dominant-negative Nmd2p is only active when localized to the cytoplasm (24), and (iii) Upf1p interacts with the polypeptide release factors Sup35p (eRF3) and Sup45p (eRF1) to modulate termination (13, 14, 79). The data of this paper provide yet another indication of the cytoplasmic nature of yeast NMD: nonsense-containing transcripts are capable of being degraded long after they have been synthesized and exported to the cytoplasm. In the absence of evidence for reassociation of these transcripts with nuclei, their continual availability to the NMD apparatus is not consistent with a nuclear, or nucleus-associated, decay pathway.

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