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Upf1p Control of Nonsense mRNA Translation Is Regulated by Nmd2p and Upf3p

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Upf1p, Nmd2p, and Upf3p regulate the degradation of yeast mRNAs that contain premature translation termination codons. These proteins also appear to regulate the fidelity of termination, allowing translational suppression in their absence. Here, we have devised a novel quantitative assay for translational suppression, based on a nonsense allele of the *CAN1* gene (*can1-100*), and used it to determine the regulatory roles of the *UPF*/*NMD* gene products. Deletion of *UPF1*, *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-number *can1-100* expression in wild-type cells caused an increase in mRNA abundance 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 *UPF1*, and overexpression of *UPF1* in cells with individual or multiple *upf*/*nmd* mutations lowered the level of nonsense suppression without affecting the abundance of the *can1-100* mRNA. Our 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. Consistent with these relationships, Upf1p, Nmd2p, and Upf3p were shown to be present at 1,600, 160, and 80 molecules per cell, levels that underscored the importance of Upf1p but minimized the likelihood that these proteins were associated with all ribosomes or that they functioned as a stoichiometric complex.

The pathways of gene expression include intricate mechanisms that safeguard against the accumulation of aberrant transcripts and proteins (6, 13, 14, 19, 30, 62). In addition to their protective functions, these pathways also contribute additional regulatory facility and complexity (57). 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 (19, 29, 39, 45, 46, 49, 51). NMD also provides the cell with a pathway for the selective degradation of a subset of mRNAs whose coding regions could be considered "normal" (37, 57).

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 (4, 5, 17, 27, 33, 40). Three additional factors are also essential for NMD in yeast: Upf1p, Nmd2p (Upf2p), and Upf3p (7, 20, 22, 34, 35). Mutations in the *UPF1*, *NMD2*, or *UPF3* genes lead to the stabilization of mRNAs containing premature nonsense codons without affecting the rates of decay 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 (22). Substantial support for this conclusion has been derived from protein-protein interaction analyses (11, 22).

A more detailed understanding of the functions of Upf1p, 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 to associate with polyribosomes (3, 38, 46). Upf1p is a 109-kDa protein that contains two putative zinc finger domains near its amino terminus and harbors seven motifs characteristic of RNA-DNA helicase superfamily I (1, 31). In vitro studies demonstrated that purified Upf1p has the ability to bind nucleic acids and that its ATPase and helicase activities are dependent upon nucleic acid binding (10, 60). Upf1p interacts with the polypeptide release factors Sup35p and Sup45p (11) and utilizes the same N-terminal zinc finger region for 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 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 Upf1p 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 (36, 58), allosuppression (9), and enhancement of programmed ribosomal frameshifting (8, 52). To investigate further the possible regulatory roles of Upf1p, Nmd2p, and Upf3p, we devised an assay that quantitatively monitors the effects of *upf*/*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 *upf*/*nmd* strains 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 Upf1p plays a critical role in regulating the efficiency of translation termination and that Nmd2p and Upf3p, in turn, regulate Upf1p activity.

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TABLE 1. Yeast strains

Strain	Genotype	Reference
HFY1200	*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 UPF1 NMD2 UPF3*	20
HFY870	*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 upf1::HIS3 NMD2 UPF3*	22
HFY1300	*MAT*α *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 UPF1 nmd2::HIS3 UPF3*	20
HFY861	*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 UPF1 NMD2 upf3::HIS3*	22
HFY3000	*MAT*α *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 upf1::URA3 nmd2::HIS3 UPF3*	20
HFY872	*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 upf1::URA3 NMD2 upf3::HIS3*	22
HFY874	*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 UPF1 nmd2::URA3 upf3::HIS3*	22
HFY883	*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 upf1::LEU2 nmd2::URA3 upf3::HIS3*	22

MATERIALS AND METHODS

Strains, plasmids, and general methods. The isogenic yeast strains used in this study are listed in Table 1. Preparation of standard yeast media and cell culturing were done as described by Rose et al. (50). Transformation of yeast strains was done by the rapid method described by Soni et al. (55). DNA manipulations were performed according to standard techniques (53). All PCR amplifications were performed with *Taq* DNA polymerase (61) and confirmed, where appropriate, by DNA sequencing using the method described by Sanger et al. (54). The *can1-100* allele (28), characterized in this study by DNA sequencing (see Results), was recreated in a YEp24-*CAN1* high-copy-number plasmid and a pRIP-*CAN1* single-copy plasmid by PCR mutagenesis. *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 *Sal*I-*Eag*I HA-containing fragment into the YEp24-*can1-100* plasmid digested with the same enzymes. Plasmid DNAs were prepared from *Escherichia coli* DH5α

RNA extraction and Northern blot analysis. RNA was isolated using the hot phenol method as described by Herrick et al. (24). Aliquots (20 μg) of each RNA sample were analyzed by Northern blotting using radiolabeled probes prepared by random priming (12). 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 *CYH2* (a 600-bp *Eco*RI-*Hin*dIII fragment from pGEM4Z-CYH2 which hybridizes to both the pre-mRNA and the mRNA) (24), *CAN1* (a 1-kb *Eco*RI-*Sal*I fragment from YEp24-*CAN1*), and *SCR1* (a 400-bp fragment amplified from yeast genomic DNA using oligonucleotides SCR1-1 [5′-AGGCTGTAATGGCTTTCTGGTGGGATGGGA-3′] and SCR1-2 [5′-GATATGTGCTATCCCGGCCGCCTCCATCAC-3′]). Immunoprecipitation of capped mRNAs was performed as described by Muhlrad et al. (40) using polyclonal anti-m^7G antibodies generously provided by Elsebet Lund.

Protein gels, Western blots, and antibodies. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described by Laemmli (32). 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 (18). Detection was enhanced by chemiluminescence with an ECL kit from Amersham Corp. Western blots were quantitated by densitometry or by Fluor-S (Bio-Rad) scanning of films exposed for different lengths of time. The anti-HA antibody (12CA5) used for Western blotting was obtained from Boehringer Mannheim Biochemicals.

Purification of recombinant GST-Upf1p and GST-Nmd2p. Extraction steps were carried out at between 0 and 4°C. All buffers included 0.1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, and the protease inhibitors bestatin (0.35 μg/ml), pepstatin (0.4 μg/ml), leupeptin (0.5 μg/ml), and benzamidine (20 μg/ml). Cell pellets were resuspended in 4 volumes of T(50) buffer (30 mM Tris-HCl [pH 7.9] 2 mM EDTA, 5% glycerol, 10 mM $MgCl_2$, 50 mM KCl) per g of cell wet weight and lysed with a French press (cell pressure, 20,000 lb/in^2). Lysates were cleared by centrifugation at 30,000 × *g*. The pellet was resuspended in denaturing buffer (6 M urea, 50 mM Tris-HCl [pH 7.9], 1 mM EDTA, 8 mM DTT), vortexed vigorously, homogenized with a B pestle, and centrifuged at 30,000 × *g*. Chromatography steps were carried out at room temperature. The supernatant was dialyzed against a buffer containing 50 mM Tris-HCl [pH 7.9], 1 mM EDTA, 1 mM DTT, and 20% glycerol. Extracts were bound in batches to glutathione-agarose (Sigma) previously equilibrated in T(50) buffer. After binding for 10 min on a platform shaker, the resin was washed three times with the same buffer. The resin was then transferred to a small column, and the protein was eluted with 10 column volumes of T(50) buffer containing 10 mM glutathione (Sigma). The purity of the protein was assessed by sodium dodecyl sulfate-polyacrylamide electrophoresis and staining with Coomassie blue R-250. Glutathione *S*-transferase (GST)–Upf1p (residues 876 to 971) was greater than 99% pure and was the only protein detected, while GST-Nmd2p was greater than 90% pure, with the majority of the contamination coming from proteolysis.

Quantitation of mRNA decay factors. The relative abundance of Upf1p, Nmd2p, and Upf3p was determined by comparing the Western blot band intensities of the factors present in crude cell extracts to those of specific standards. For Upf1p, purified recombinant GST-Upf1p (residues 876 to 971) was used as a standard; for Nmd2p, purified recombinant Nmd2p was used as a standard; and for Upf3p, cells bearing an HA-*NMD2* allele were used as a standard. For Western blotting, aliquots of crude cell extracts equivalent to 1 ml of cells at an optical density at 600 nm (OD_{600}) of 0.2 were loaded onto polyacrylamide gels. The number of cells in each aliquot was determined by serially diluting and plating the cultures.

***can1-100* 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 to 0.7). Samples from these cultures were serially diluted (1:10) four times, and aliquots (10 μl) of the four dilutions were spotted on SC-arg 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.

Arginine uptake assay. The arginine uptake assay was adopted from that previously described by Opekarova and Kubin (43). Yeast cultures were grown to mid-log phase (OD_{600} = 0.5 to 0.7) at 30°C in SC-arg medium and then supplemented with 50 mM L-arginine containing 5 μCi of L-[^{3}H]arginine (Amersham). Aliquots of the cultures were then removed at specific intervals, diluted in 2 ml of 100 mM LiCl, filtered on GF/C filters (Whatman), and washed with 2 ml of water. Radioactive arginine associated with each filter was determined by scintillation counting.

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 *CAN1* gene, which encodes a high-affinity permease (Can1p) responsible for the transport of arginine into cells (26). 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 (28). 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 *CAN1* 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 fourfold more abundant in *upf*/*nmd* cells than in the isogenic *UPF*/*NMD* strain (Fig. 1A). Likewise, deletion of genes encoding general factors involved in mRNA decay (i.e., *DCP1* and *XRN1*) also promoted a fourfold increase in *can1-100* transcript abundance (Fig. 1A). These differences in mRNA abundance were consistent with the respective differences in the rates of decay of the *CAN1* and *can1-100* mRNAs in wild-type cells (half-lives of 8 and 2 min, respectively; data not shown). As a control for the experiments shown in Fig. 1A, the abundance of an endogenous substrate of the NMD pathway (19) was monitored. As expected, this experiment showed that the *CYH2* pre-mRNA was barely detectable in wild-type cells and was abundant in all of the mutants. These results indicate that the *can1-100*

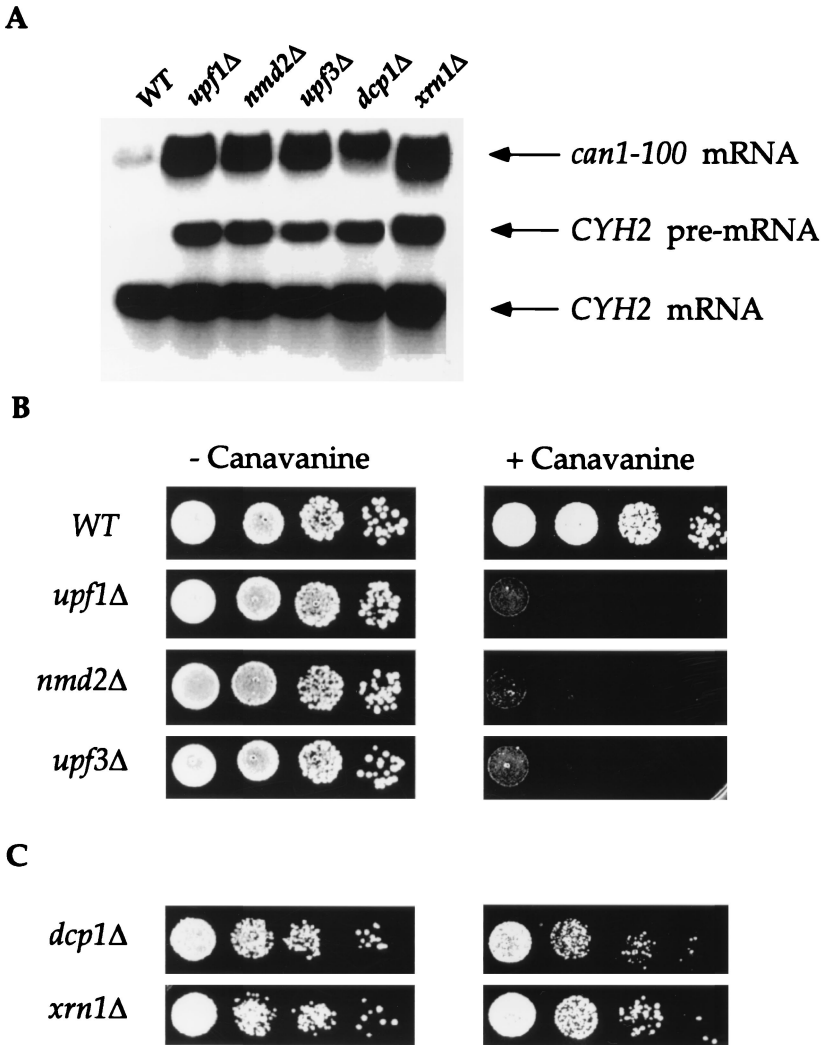


FIG. 1. Deletion of *UPF1*, *NMD2*, or *UPF3* stabilizes the *can1-100* transcript and promotes nonsense suppression. (A) Deletion mutants that inactivate NMD stabilize the *can1-100* transcript. Total RNA isolated from yeast strains with the indicated *UPF*/*NMD* genotypes was analyzed by Northern blotting 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 (10 μl) 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 without or with 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 *xm1*Δ strain at 30°C for 3 days and the *dcp1*Δ strain at 30°C for 4 days.

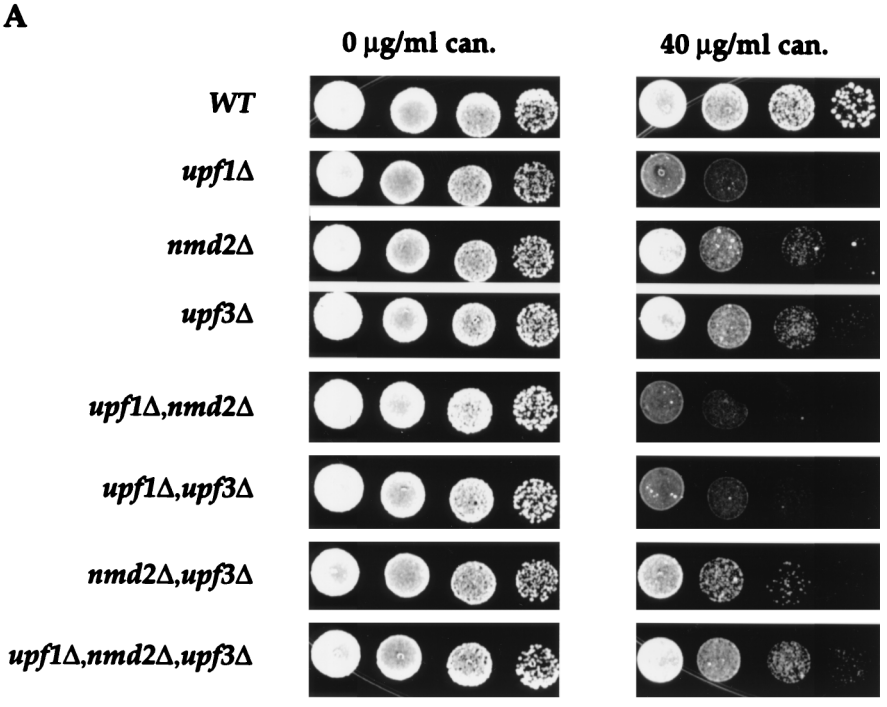
mRNA requires Upf1p, Nmd2p, Upf3p, Dcp1p, and Xrn1p for its degradation and that it is thus a typical substrate for NMD.

Quantitative assay for nonsense suppression. Mutations in the *UPF1*, *NMD2*, or *UPF3* genes have been found to lead not only to increased abundance of substrate mRNAs but also to suppression of certain nonsense alleles, including *leu2-2* and *tyr7-1* (36, 58). To investigate nonsense suppression of the *can1-100* allele, we took advantage of the observation that canavanine, a toxic arginine analog, is also transported into cells via Can1p (15). *can1-100* cells are thus phenotypically canavanine resistant, and sensitivity to canavanine is indicative of *can1-100* suppression.

Figure 1B illustrates the canavanine resistance of *can1-100* cells and demonstrates that deletion of *UPF1*, *NMD2*, or *UPF3* results in a canavanine-sensitive phenotype when these cells are grown on media containing 100 μg of canavanine per ml. Although deletion of *DCP1* and *XRN1* led to *can1-100* mRNA stabilization comparable to that seen in *upf1*Δ, *nmd2*Δ, or *upf3*Δ mutants (Fig. 1A), strains with the former deletions did not exhibit canavanine sensitivity (Fig. 1C). These results indicate that deletion of any of the *UPF*/*NMD* genes allows for suppression of the *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 *UPF1*, *NMD2*, or *UPF3* promoted different extents of *can1-100* suppression. For example, Fig. 2A shows that 40 μg of canavanine per ml was sufficient to kill *upf1*Δ cells but was only partially toxic to comparable numbers of *nmd2*Δ or *upf3*Δ cells.

A



B

Mutation	Can. Sensitivity (μg/ml)
WT	300
upf1Δ	25
nmd2Δ	35
upf3Δ	35-40
upf1Δ,nmd2Δ	25
upf1Δ,upf3Δ	25
nmd2Δ,upf3Δ	35
upf1Δ,nmd2Δ,upf3Δ	50

FIG. 2. 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 or 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.

Similar assays consistently demonstrated that the highest levels of nonsense suppression occurred in *upf1*Δ cells, which exhibited 12-fold greater sensitivity to canavanine than the isogenic wild-type strain (Fig. 2B). Suppression was found to be lower in *nmd2*Δ and *upf3*Δ cells, which exhibited 1.5-fold less sensitivity than *upf1*Δ cells (Fig. 2). Although the canavanine sensitivities of the *nmd2*Δ and *upf3*Δ strains were almost identical, subtle differences were detected which indicated that the *nmd2*Δ mutation was a slightly more effective suppressor than the *upf3*Δ mutation (Fig. 2).

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 [^{3}H]arginine in wild-type and mutant cells. Consistent with the suppression assays of Fig. 1 and 2, these experiments demonstrated that deletion of *UPF1*, *NMD2*, or *UPF3* allowed for enhanced transport of arginine (Fig. 3A).

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 suppression phenotypes identical to those of strains containing the same plasmid lacking the triple-HA tag (data not shown). Figure 3B shows that Can1p-HA was barely detectable in wild-type cells (lower panel, lane 3) but increased approximately 10-fold in abundance in *upf1*Δ, *nmd2*Δ, and *upf3*Δ 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. 3A) 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. 3B 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. 3B 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

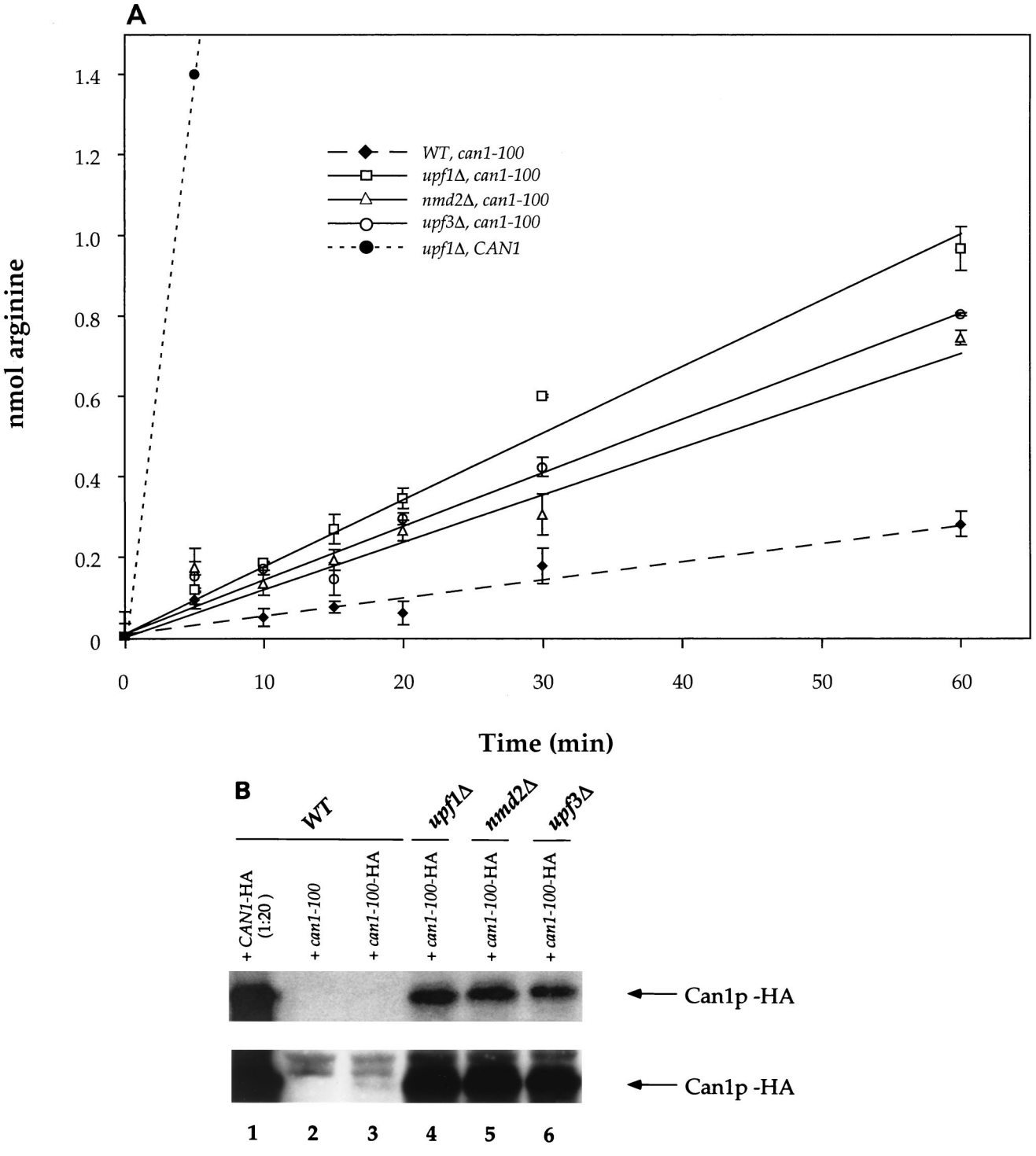
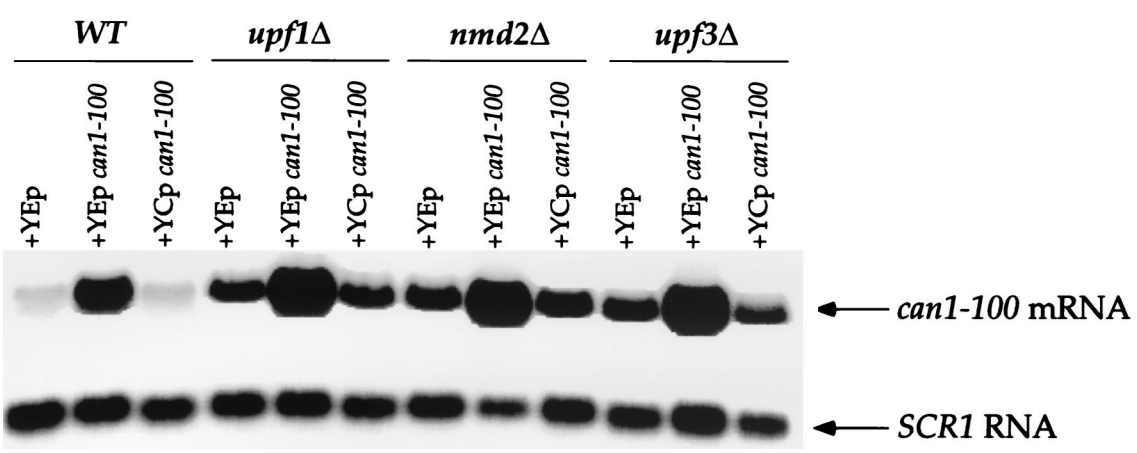


FIG. 3. Accumulation of functional Can1p correlates with nonsense suppression of *can1-100*. (A) ^{3}H-labeled arginine uptake in yeast strains with the indicated *UPF*/*NMD* and *CAN1* genotypes. The control yeast strain harboring the *CAN1* allele is PLY148 (36). *WT*, wild type. Error bars indicate standard deviations. (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.

reduction in Can1p synthesis. The data in Fig. 3B 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 observations that wild-type cells harboring the *CAN1* gene, wild-type cells harboring *can1-100*, and *upf1*Δ 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. 2B and 3B 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

A



B

Relative mRNA levels

Strain	YEp	YEp *can1-100*	YCp *can1-100*
WT	28	328	40
*upf1*Δ	100	1000	150
*nmd2*Δ	120	1250	175
*upf3*Δ	150	1000	175

C

Can. Sensitivity (μg/ml)

Strain	YEp	YEp *can1-100*	YCp *can1-100*
WT	300	50	300
*upf1*Δ	25	2	10
*nmd2*Δ	40	3	15
*upf3*Δ	40	3	15

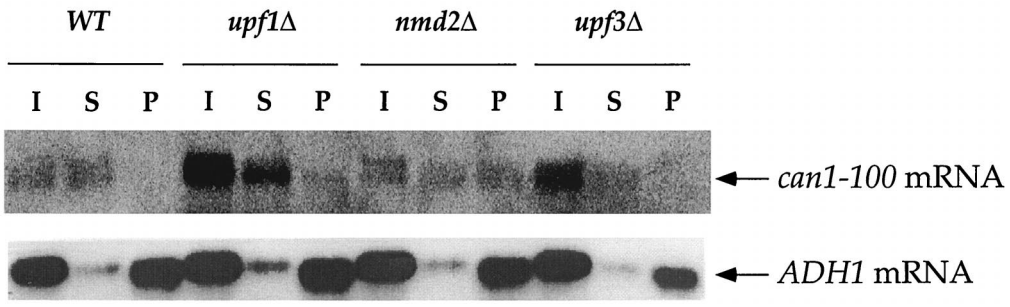
FIG. 4. *can1-100* nonsense suppression is only partially attributable to increased mRNA abundance. (A) Northern analysis of *can1-100* mRNA levels. RNA isolated from yeast strains with the indicated genotypes was analyzed by Northern blotting with probes specific for *can1-100* mRNA and *SCR1* RNA (the 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 *upf1*Δ strain. (C) Canavanine sensitivities of strains harboring single-copy or high-copy-number plasmids. Suppression assays analogous to those shown in Fig. 2 were used to define the canavanine (Can.) sensitivities of cells with different *UPF*/*NMD* genotypes.

*upf1*Δ, *nmd2*Δ, and *upf3*Δ mutants (Fig. 1), 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. 4A and B), and the respective suppression phenotypes (i.e., canavanine sensitivities) of the different strains were determined (Fig. 4C). 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. 4A and B), but this increase did not alter the suppression phenotype of wild-type cells containing either single-copy or high-copy-number vectors without inserts (Fig. 4C, compare *WT*–YCp *can1-100* with *WT*–YEp; also, data not shown). Wild-type cells transformed with the high-copy-number plasmid containing the *can1-100* allele showed a 12-fold increase in *can1-100* mRNA abundance compared to the same cells containing only the vector (Fig. 4A and B, compare *WT*–YEp *can1-100* with *WT*–YEp). Accompanying this increase in mRNA levels was a sixfold increase in sensitivity to canavanine (Fig. 4C).

The same phenomena were exhibited when this experiment was repeated with *upf1*Δ, *nmd2*Δ, and *upf3*Δ mutants. All strains expressing an additional copy of the *can1-100* allele exhibited modest increases in *can1-100* mRNA levels (15 to 50%; Fig. 4A and B) but showed approximately threefold increases in their respective levels of suppression (Fig. 4C, compare YCp *can1-100* with YEp for all three mutants). When the *can1-100* 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. 4A and B) and a comparable increase in the level of nonsense suppression (Fig. 4C). 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 *can1-100* (*WT*–YEp *can1-100*) had two- to threefold higher levels of *can1-100* mRNA than any of the *upf*/*nmd* mutant cells (Fig. 4A and B), yet the level of suppression in the *WT*–YEp *can1-100* strain was still lower than that in any of the mutants (Fig. 4C).

Additional support for the notion that increased mRNA abundance is not sufficient for *can1-100* nonsense suppression is the finding that single deletions of *UPF1*, *NMD2*, *UPF3*, *DCP1*, or *XRN1* were found to stabilize *can1-100* mRNA to comparable levels (approximately fourfold; Fig. 1A), yet there were substantial differences in the canavanine sensitivities of the respective strains (Fig. 1B and C and 2B). Collectively, the data in Fig. 1 to 4 provide strong support for the notion that the *UPF*/*NMD* genes regulate not only the rates of decay of nonsense-containing mRNAs but also their efficiencies of translation.

A



B

	can1-100		*ADH1*	
	Uncapped	Capped	Uncapped	Capped
WT	0.80	0.20	0.21	0.79
*upf1*Δ	0.72	0.28	0.22	0.78
*nmd2*Δ	0.48	0.52	0.22	0.78
*upf3*Δ	0.64	0.36	0.27	0.73

FIG. 5. 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 *UPF*/*NMD* 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 *ADH1* 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 *ADH1* transcripts. RNA in the S and P fractions of panel A was quantitated by phosphorimaging, and the relative percentages of capped and uncapped transcripts were determined by calculating the fraction each sample represented of its respective total (S + P).

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, *upf1*Δ, *nmd2*Δ, and *upf3*Δ strains. In both wild-type and mutant cells, the *ADH1* mRNA was predominantly capped (Fig. 5). 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 the percentage of capped molecules (Fig. 5). These changes in the ratios of capped to uncapped *can1-100* mRNAs do not correlate with the suppression data of Fig. 1 to 4 and indicate that variations in suppression efficiencies must reflect events unrelated to mRNA cap status. This conclusion is underscored by experiments indicating that *dcp1*Δ and *xm1*Δ, two mutations that have negligible effects on *can1-100* suppression (Fig. 1), lead to the accumulation of mRNAs that are predominantly capped or uncapped, respectively (4, 27, 40; 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 (41; 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 (63).

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 (Fig. 2B), 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. 2, double deletion of *UPF1* and either *NMD2* or *UPF3* resulted in a suppression phenotype identical to that caused by *upf1*Δ alone. This result indicates that combining an *nmd2*Δ or *upf3*Δ mutation with *upf1*Δ does not have an additive effect on nonsense suppression and that the *upf1*Δ phenotype supersedes the *nmd2*Δ and *upf3*Δ phenotypes. Of the double mutants, the *nmd2*Δ *upf3*Δ mutant showed the lowest level of suppression, displaying a phenotype like that of an *nmd2*Δ strain (compare *nmd2*Δ *upf3*Δ to *nmd2*Δ in Fig. 2A). These results indicate that Upf1p is epistatic to Nmd2p and Upf3p and suggest a role for

TABLE 2. Overexpression of *UPF1*, *NMD2*, or *UPF3* in wild-type and *upf*/*nmd* strains[a]

Strain	Canavanine sensitivity (μg/ml)			
	YEp	YEp-*UPF1*	YEp-*NMD2*	YEp-*UPF3*
Wild type	300	300	300	300
*upf1*Δ	25	300	25	25
*nmd2*Δ	35	75	300	35
*upf3*Δ	35–40	75	25	300
*upf1*Δ *nmd2*Δ	25	75	25	25
*upf1*Δ *upf3*Δ	25	75	25	25
*nmd2*Δ *upf3*Δ	35	75	35	35
*upf1*Δ *nmd2*Δ *upf3*Δ	50	125	50	50

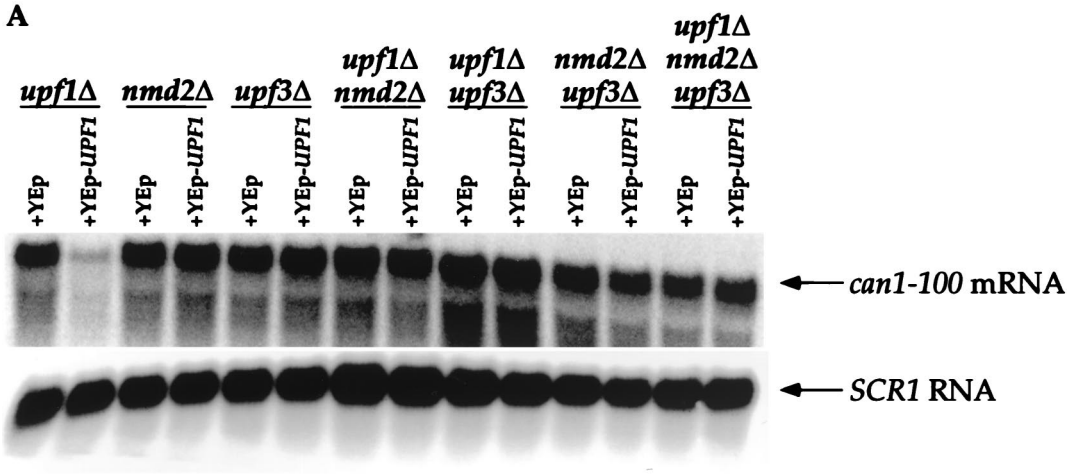
[a] Suppression assays are summarized, with the concentration of canavanine at which each strain began to exhibit the canavanine-sensitive phenotype indicated (canavanine sensitivity). Yeast strains of the indicated genotypes were transformed with the high-copy-number vector alone (YEp; control) or high-copy-number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

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 *upf*/*nmd* mutants tested (sensitivity to 50 μg of canavanine per ml; Fig. 2), 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 (22). 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 2 [YEp column] with Fig. 2B).

Overexpression of *UPF1* in all of the single, double, and triple mutant strains (not including the *upf1*Δ control) was found to lower suppression levels two- to threefold (Table 2, compare YEp and YEp-*UPF1* columns). These results are consistent with the notion that Upf1p can, by itself, enhance termination fidelity and also implicate a regulatory role for



B

Strain	YEp	YEp-*UPF1*
*upf1*Δ	100	25
*nmd2*Δ	92	107
*upf3*Δ	90	100
*upf1*Δ,*nmd2*Δ	69	70
*upf1*Δ,*upf3*Δ	96	107
*nmd2*Δ,*upf3*Δ	87	85
*upf1*Δ,*nmd2*Δ,*upf3*Δ	74	77

FIG. 6. Overexpression of *UPF1* in *upf*/*nmd* mutant strains does not affect *can1-100* mRNA abundance. (A) Northern analysis of *can1-100* mRNA levels. Total RNA isolated from yeast strains with the indicated genotypes was analyzed by Northern blotting as described in the legend to Fig. 4. Each of the mutant strains contained either a high-copy-number *UPF1* plasmid (YEp-*UPF1*) or an empty vector as a control (YEp). (B) Quantitation of *can1-100* steady-state mRNA levels. *can1-100* mRNA levels were determined, standardized to *SCR1* RNA, and normalized to data for the *upf1*Δ strain as described in the legend to Fig. 4.

TABLE 3. Cellular levels of Upf1p, Nmd2p, and Upf3p[a]

Protein or ribosomes	Protein	
	No. of molecules per cell	% Soluble
Upf1p	1,600	0.007
Nmd2p	160	0.0007
Upf3p	80	0.00035
Sup45p	29,600[b]	0.2[b]
Xrn1p	29,000[c]	0.2[c]
Ribosomes	300,000[d]	7

[a] The 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.

[b] Cellular Sup45p levels determined by Gygi et al. (16).

[c] Cellular Xrn1p levels determined by Heyer et al. (25).

[d] Cellular levels of ribosomes determined by Waldron and Lacroute (56).

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 *upf1*Δ *nmd2*Δ strain (Table 2). Overexpression of *NMD2* had comparable effects, except that, in *upf3*Δ cells, it also enhanced suppression to a level comparable to that obtained in *upf1*Δ cells (Table 2). 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 *upf1*Δ strain restored NMD to wild-type levels, resulting in a fourfold decrease in *can1-100* mRNA levels (Fig. 6, compare *upf1*Δ–YEp to *upf1*Δ–YEp-*UPF1*). Accompanying this restoration of decay function was the restoration of the wild-type suppression phenotype (Table 2). 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. 6). 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 (11, 58, 59).

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 the results of previous protein-protein interaction analyses (11, 21, 22, 58) 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 amounts of epitope-tagged Upf1p and Nmd2p 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 1,600 molecules of Upf1p/cell (Table 3). 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 3). 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 (23) (Table 3). 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 *upf1*Δ, *nmd2*Δ, and *upf3*Δ mutations. The *UPF1*, *NMD2*, and *UPF3* genes regulate NMD (7, 20, 22, 34–36, 45). Mutations in any of these genes generally promote the stabilization of nonsense-containing mRNAs by reducing the rate at which the 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 (9, 36, 58, 59) and programmed ribosomal frameshifting (8, 52) 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 (11).

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 *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 were shown to have comparable stabilizing effects on the *can1-100* mRNA but to

produce differential effects on suppression. Strains harboring the *upf1*Δ mutation consistently showed a higher level of nonsense suppression than strains harboring either the *nmd2*Δ or the *upf3*Δ 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 (7, 36, 58, 59). Since these mutations invariably led to increases in the levels of the corresponding mRNAs (35, 36) 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 (36, 47). However, the experiments of Weng et al. (58, 59) 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 a high copy number, inactivated mRNA decay but failed to allow suppression (e.g., DE572AA) (58) and (ii) those which, when expressed in a single copy, promoted normal mRNA decay but allowed suppression to occur (e.g., C84S) (59). 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 (19, 35) and since Upf1p was known to be of relatively low abundance, it was considered likely that the translational effects were targeted not to general initiation or elongation but rather to the premature termination event.

On the basis of the results of Weng et al. (58, 59), 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) *xrn1*Δ- and *dcp1*Δ-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 *upf1*Δ, *nmd2*Δ, or *upf3*Δ mutations; (iii) when *UPF1* 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 increased only 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. 3B).

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 (19, 35), inactivation of the NMD pathway has been shown to promote a modest increase in the translational efficiency of nonsense-containing mRNAs (42). Moreover, recent studies have demonstrated that the *upf1*Δ, *nmd2*Δ, and *upf3*Δ mutations alter the distributions 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 *can1-100* mRNA, possibly because of changes in its extent of capping. However, since deletion of *UPF1*, *NMD2*, or *UPF3* produced differential effects on the amounts of capped and uncapped *can1-100* mRNAs (Fig. 5) that did not correlate with their respective suppression phenotypes, 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 release factors (11) suggests that the former model is more likely.

Upf1p 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 Upf1p as the most critical of the three factors for the maintenance of termination fidelity or suggests that Upf1p and either Nmd2p (i.e., as in the *upf3*Δ strain) or Upf3p (i.e., as in the *nmd2*Δ 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 *nmd2*Δ *upf3*Δ 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, Upf1p 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 Upf1p 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 *upf1*Δ 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. (58,

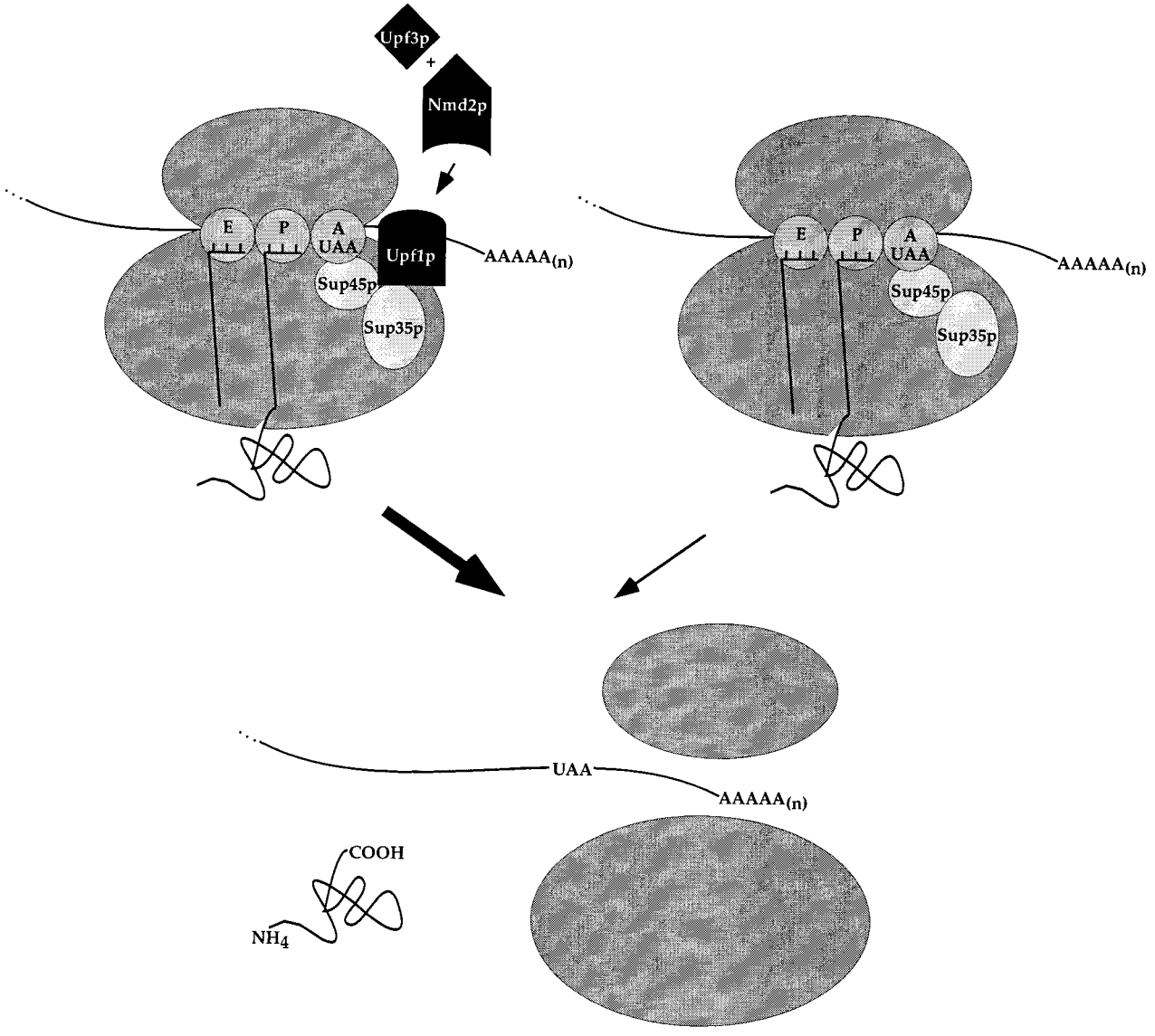


FIG. 7. Model for functional relationships of Upf1p, Nmd2p, and Upf3p in translation termination. Upf1p is depicted as a positive regulator of the efficiency of translation termination mediated by Sup35p and Sup45p. The activity of Upf1p is postulated to be dependent on the function of both Nmd2p and 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 gray ovals).

59) showed that specific *upf1* alleles could separate the translation and turnover functions of Upf1p, i.e., some alleles resulted in normal mRNA decay but impaired termination fidelity, whereas others resulted in the opposite phenotype. Curiously, these phenotypes are not reproduced in the *can1-100* system. Strains with *upf1* alleles shown to result in normal decay but impaired fidelity (e.g., C84S) behaved like the wild-type strain in the *can1-100* system, and strains with alleles resulting in inactive mRNA decay but functional fidelity (e.g., DE572AA) behaved just like *upf1*Δ 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 overexpression of 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 *can1-100* nonsense suppression without having any significant effect on *can1-100* 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 function for Nmd2p and Upf3p (since the overexpression of Upf1p has the ability to enhance fidelity even in the absence of Nmd2p or Upf3p).

The overexpression of *NMD2* in a *upf3*Δ 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 (21, 22), 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 (3, 37, 46), 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 1,600, 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 (3, 20–22, 38, 46, 58, 59), 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 ribosomes recognizing newly synthesized mRNAs or their termination codons. An association with ribosomes actively recognizing termination codons would be consistent with recent studies demonstrating that Upf1p interacts with the peptide release factors Sup35p and Sup45p (11).

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 function (Fig. 7). 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 the most abundant of the three proteins involved in NMD. Further, homologs of Upf1p have been identified in other organisms, including *Caenorhabditis elegans* (44, 49) and humans (2, 48), indicating evolutionary conservation of this factor. It is possible that the role of Upf1p 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, Upf1p may play a more elaborate role in 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 Upf1p (R. Ganesan, F. He, and A. Jacobson, unpublished data; He and Jacobson, unpublished).

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