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Stabilization and ribosome association of unspliced pre-mRNAs in a yeast *upf1*⁻ mutant

(mRNA decay/nonsense mutations/translation)

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ABSTRACT **Nonsense-mediated mRNA decay, the accelerated turnover of mRNAs transcribed from genes containing early nonsense mutations, is dependent on the product of the *UPF1* gene in yeast. Mutations that inactivate *UPF1* lead to the selective stabilization of mRNAs containing early nonsense mutations but have no effect on the half-lives of almost all other mRNAs. Since the transcripts of nonsense alleles are not typical cellular constituents, we sought to identify those RNAs that comprise normal substrates of the nonsense-mediated mRNA decay pathway. Many yeast pre-mRNAs contain early in-frame nonsense codons and we considered it possible that a role of this pathway is to accelerate the degradation of pre-mRNAs present in the cytoplasm. Consistent with this hypothesis, we find that, in a strain lacking *UPF1* function, the *CYH2*, *RP51B*, and *MER2* pre-mRNAs are stabilized 2- to 5-fold and are associated with ribosomes. We conclude that a major source of early nonsense codon-containing cytoplasmic transcripts in yeast is pre-mRNAs and that the *UPF1* protein may be part of a cellular system that ensures that potentially deleterious nonsense fragments of polypeptides do not accumulate.**

In eukaryotes and prokaryotes nonsense mutations in a gene can enhance the decay rate of the mRNA transcribed from that gene (1–12), a phenomenon we describe as nonsense-mediated mRNA decay (1). Trans-acting factors that are essential for nonsense-mediated mRNA decay have been identified in experiments that characterized a class of nonsense suppressors in the yeast *Saccharomyces cerevisiae*. Mutants in the *UPF1* gene, originally isolated on the basis of their ability to enhance the suppression of a frameshift mutation that led to premature translational termination (13), selectively stabilize mRNAs containing early nonsense mutations without affecting the decay rates of most other mRNAs (ref. 2; S.W.P., A. H. Brown, and A.J., unpublished data).

The existence of trans-acting factors that promote rapid decay of nonsense-containing mRNAs raises the question of whether such mRNAs are the sole substrates of these factors—i.e., whether the cell has an apparatus to specifically degrade nonsense-containing mRNAs. It seemed unlikely that the normal function of the *UPF1* gene was anticipatory—i.e., solely involved in the degradation of mRNAs derived from nonsense alleles—so we sought to determine whether these factors have additional substrates. Since introns generally lack contiguous open reading frames, and yeast introns are almost always at the 5′ ends of their genes (14), we considered it possible that the *UPF1* gene product (Upf1p) might also be involved in controlling the abundance of yeast pre-mRNAs. If this supposition were correct, the presence of unspliced introns within a pre-mRNA would lead to premature translational termination and accelerated RNA decay in wild-type strains but not in a *upf1*⁻ strain. To test this hypothesis, the half-lives and cytoplasmic localization of three yeast pre-mRNAs and their mRNA products were determined in isogenic *UPF1*⁺ or *upf1*⁻ yeast strains. We find that, in a strain lacking *UPF1* function, the *CYH2*, *RP51B*, and *MER2* pre-mRNAs are stabilized and are associated with ribosomes.

MATERIALS AND METHODS

Yeast Strains and Medium. The pair of isogenic *UPF1*⁺ and *upf1*⁻ yeast strains used in these experiments was derived from SWP154 (*ura3-52 trp1-Δ1 UPF1::ura3 rpb1-1 his4-38 leu2-1*; S.W.P., A. H. Brown, and A.J., unpublished data). A centromere plasmid, YCpPL53 (2), containing the *UPF1* and *TRP1* genes was transformed into SWP154 to yield SWP154(+); the same centromere plasmid harboring the *TRP1* gene but lacking the *UPF1* gene (YCpMS38; ref. 2) was also transformed into SWP154, yielding SWP154(−). (YCpPL53 and YCpMS38 were generously provided by P. Leeds and M. Culbertson.) Cells were grown in SC minimal medium lacking tryptophan (15).

Measurement of mRNA and Pre-mRNA Decay Rates. mRNA and pre-mRNA decay rates were measured as described (1, 16). In brief, the use of *rpb1-1* mutants (17) allowed transcription to be inhibited by thermal inactivation of RNA polymerase II. Cells were grown at 24°C and shifted to 36°C, and RNA was isolated from cells at different times after the shift. Using equivalent amounts of RNA from each time point (20 μg), mRNA levels were determined by Northern blotting with DNA or RNA probes labeled to high specific activity. Northern blots were quantitated by direct counting of the β decays present in each RNA band by the use of a Betascope blot analyzer (Betagen, Waltham, MA). Half-lives were determined by plotting the $\log_{10}$ of the percentage of each RNA remaining vs. time at 36°C.

Polysome Analysis. Cytoplasmic extracts were prepared as described (18) in the presence or absence of 40 mM EDTA. Extracts were fractionated on 15–50% sucrose gradients buffered with 50 mM Tris acetate, pH 7.4/50 mM NH_4Cl/12 mM $MgCl_2$/1 mM dithiothreitol. Gradients were centrifuged in an SW41 rotor at 4°C and analyzed by continuous monitoring of A_{254}. RNA was isolated from individual fractions (16, 19) and analyzed by Northern blotting.

RESULTS AND DISCUSSION

The yeast strains used in this study harbor the *rpb1-1* allele (17), a temperature-sensitive mutation in RNA polymerase II

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Table 1. Decay rates of transcripts in *UPF1*$^+$ or *upf1*$^-$ strains

mRNA	*UPF1*$^+$	*upf1*$^-$
	$t_{1/2}$, min	
PGK1	60	60
PGK1(5′-UAG)5.6	3	60
PGK1(5′-UAG)39	5	60
PGK1(3′-UAG)92	60	60
CUP1	18	18
MATα1	5	5
CYH2 mRNA	43	43
CYH2 pre-mRNA	1.5	6
RP51B mRNA	17	17
RP51B pre-mRNA	1.8	4.2
MER2 pre-mRNA	7	34

RNA decay rates were determined in isogenic *UPF1*$^+$ and *upf1*$^-$ strains as described in the legend to Fig. 1. Half-life values presented were accurate to ±15% in multiple determinations. *PGK1* nonsense alleles were constructed by insertion of oligonucleotide linkers into convenient restriction sites (S.W.P., A. H. Brown, and A.J., unpublished data). The locations of the amber (UAG) mutations in the *PGK1* protein coding region are expressed as percentages, where the initiator codon is at 0% and the normal translational terminator is at 100% [e.g., the *PGK1*(5′-UAG)5.6 allele has an amber mutation inserted at 5.6% of the *PGK1* protein coding region].

Table 2. Positions of the first in-frame nonsense codons

Pre-mRNA	Total codons	Intron location	First in-frame nonsense codon*
CYH2	148	17†	19 (UAG)
RP51B	136	1‡	8 (UGA)
MER2	291	106§	132 (UAG)
RP51A	136	1¶	4 (UAA)

Positions of the first in-frame nonsense codons in each of four pre-mRNAs are noted.
*Assuming that translation commences at the normal AUG and proceeds into the intron.
†The 510-nt intron splits codon 17 (25).
‡The 315-nt intron follows the first codon (26).
§The 79-nt intron splits codon 106 (24).
¶The 398-nt intron follows the first codon (27).

that facilitates the measurement of mRNA decay rates. The general methodological approach involves inhibiting transcription with a shift of cell cultures to the nonpermissive temperature (36°C) and subsequent measurements of the relative abundance of selected RNAs at different times after the shift (16). This approach has been used for the measurement of all RNA decay rates reported in this study.

mRNA Destabilization by Nonsense Codons Is Dependent on the Function of the *UPF1* Gene. Nonsense-mediated mRNA decay is position dependent—i.e., 5′-proximal nonsense mu-

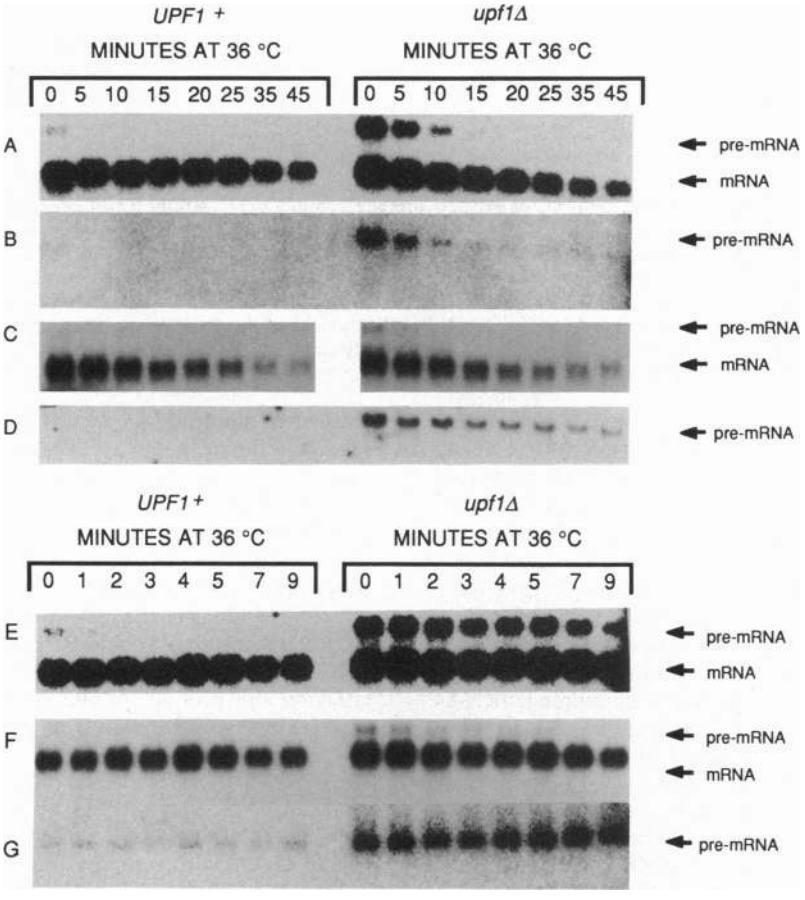

FIG. 1. Decay rates of the *CYH2*, *RP51B*, and *MER2* transcripts were determined (16) by blot analysis of RNAs isolated at different times after transcription was inhibited by a shift from 24°C to 36°C in isogenic *UPF1*$^+$ or *upf1*$^-$ strains. (*A–D*) Measurements were made over a 45-min time course. (*E–G*) Decay rates were determined as in *A–D*, except that the time course was limited to RNAs isolated at early times after transcription was inhibited. (*A* and *E*) Hybridization of the RNA blot with a radioactive *CYH2* DNA probe containing intron and exon sequences [a 485-bp *Acc* I–*Bgl* II fragment spanning *CYH2* nt 357–842 (25)]. (*B*) Rehybridization of the blot shown in *A* with a radioactive *CYH2* DNA probe containing only intron sequences [a 202-bp fragment synthesized by PCR and spanning *CYH2* nt 357–554 (25)]. (*C* and *F*) Hybridization of the RNA blot with a radioactive *RP51B* DNA probe containing intron and exon sequences [a 570-nt *Bam*HI fragment from the Hb *RP51B*/*lacZ* fusion spanning *RP51B* exon 1, intron 1, and 180 nt of exon 2 (generously provided by N. Abovich; ref. 26)]. (*D* and *G*) Hybridization of the RNA blot with a radioactive *MER2* riboprobe containing intron and exon sequences [probe transcribed from a 619-bp *Bgl* II–*Xba* I fragment spanning *MER2* nt 98–716 (24)].

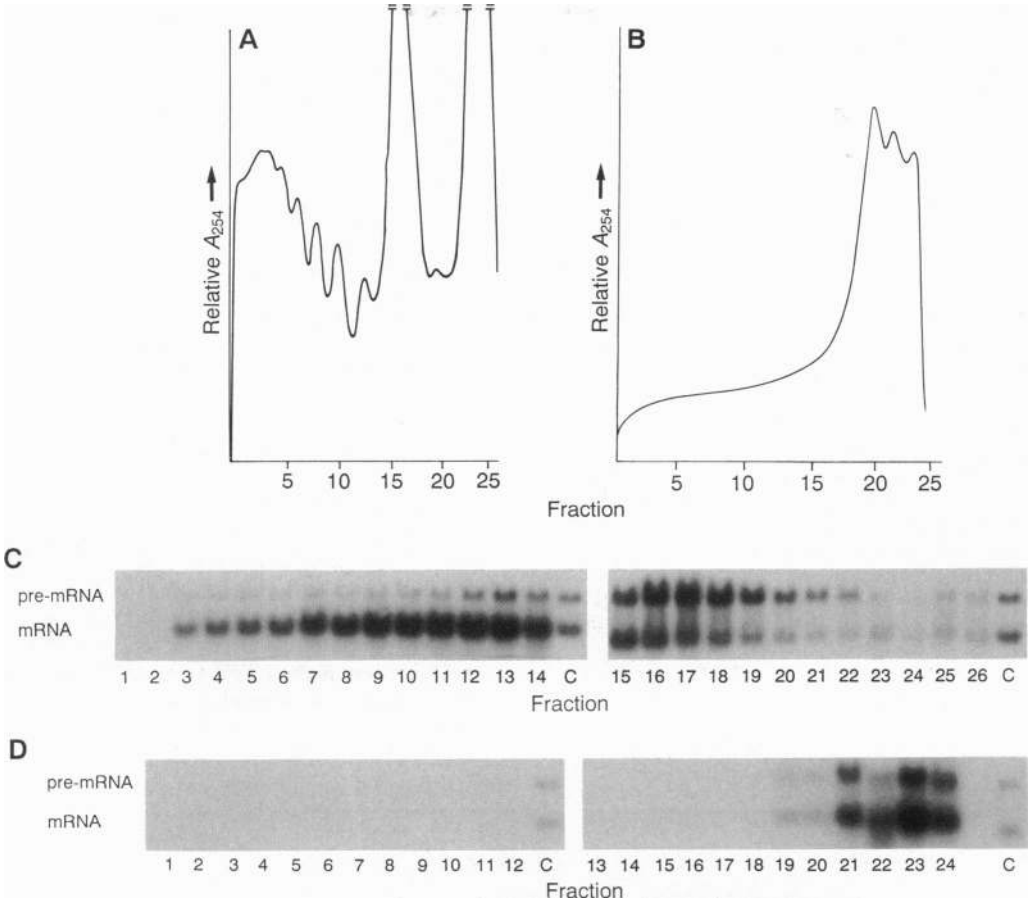

FIG. 2. Polysome profiles and RNA blotting analyses of extracts prepared from a *upf1*$^-$ strain. Yeast cells [strain SWP154(−)] were grown at 24°C in SC minimal medium lacking tryptophan to an OD_{650} of 0.885. Cells were harvested and cytoplasmic extracts were prepared in the presence or absence of 40 mM EDTA. Extracts were fractionated on 15–50% sucrose gradients (28) and RNA was isolated from individual fractions. (*A*) A_{254} absorbance profile of the sample without EDTA. The 80S monosome peak is located between fractions 16 and 17. (*B*) A_{254} absorbance profile of the sample with EDTA. (*C*) RNA blot analysis of the gradient shown in *A*. Samples of equivalent volume were fractionated on two separate gels, blotted, and probed with a radioactive *Acc* I–*Bgl* II fragment of the *CYH2* gene (25). Autoradiographic exposures of the two blots were normalized by the inclusion of an aliquot of unfractionated lysate on each gel (lanes marked C). (*D*) RNA blot analysis of the gradient shown in *B*, analyzed as described for *C*.

tations promote mRNA destabilization, whereas 3′-proximal nonsense mutations have little or no effect on mRNA decay rates. This is exemplified by the decay rates of mutant yeast *PGK1* mRNAs in which nonsense codons are encountered after translation of 5.6%, 39%, or 92% of the *PGK1* codon region. Whereas wild-type *PGK1* mRNA and a *PGK1* mRNA with a nonsense codon at 92% of the coding region have half-lives of 60 min, *PGK1* transcripts with nonsense mutations at 5.6% or 39% of the coding region have half-lives of 3–5 min (Table 1, *UPF1*$^+$ column). Similar position effects have been observed with other nonsense-containing mRNAs (refs. 1, 2, and 7; S.W.P., A. H. Brown, and A.J., unpublished data). This destabilizing effect of "early" nonsense codons is dependent on the function of the *UPF1* gene. For example, in a *UPF1* deletion mutant, the half-lives of mRNAs from *PGK1* alleles harboring 5′-proximal nonsense mutations are stabilized, whereas the half-lives of mRNAs encoded by the wild-type *PGK1* gene, by *PGK1* alleles containing 3′-proximal nonsense mutations, or by other wild-type genes, including those that encode inherently unstable mRNAs, are unaffected (2) (Table 1; S.W.P., A. H. Brown, and A.J., unpublished data).

Decay Rates of Intron-Containing Pre-mRNAs Are Markedly Reduced in a *upf1*$^-$ Mutant. In general, introns lack open reading frames. Thus, translation of pre-mRNAs that enter the cytoplasm should be prematurely terminated and the respective pre-mRNAs rapidly degraded by the nonsense-mediated decay pathway. To evaluate this possibility, we have analyzed two pre-mRNAs that are inefficiently spliced and one whose splicing is regulated. Measurements of the steady-state ratio of pre-mRNA to mRNA for different intron-containing yeast genes indicate that different primary transcripts vary significantly in the efficiency with which they are spliced (20, 21). Based on this criterion, the *CYH2* and *RP51B* pre-mRNAs are inefficiently spliced (21–23). Splicing of a third pre-mRNA, encoded by the *MER2* gene, is regulated by the product of the *MER1* gene that is normally inactive in vegetatively growing yeast cells (24). Starting from the normal translation initiation sites, ribosomes translating these pre-mRNAs would encounter the first in-frame nonsense triplet at codon 19 in *CYH2* pre-mRNA, codon 8 in *RP51B* pre-mRNA, and codon 132 in *MER2* pre-mRNA (Table 2). Using the effect of nonsense codons on the *PGK1* mRNA as a paradigm (Table 1; ref. 1; S.W.P., A. H. Brown, and A.J., unpublished data), the initial nonsense codons in all three pre-mRNAs should be sufficiently early to trigger nonsense-mediated mRNA decay.

To test whether the *UPF1* gene product is involved in regulating the abundance of intron-containing RNAs, the half-lives of the *CYH2*, *RP51B*, and *MER2* pre-mRNAs were measured in isogenic *UPF1*$^+$ or *upf1*$^-$ yeast strains. As controls, half-lives of the *CYH2* and *RP51B* mRNAs were

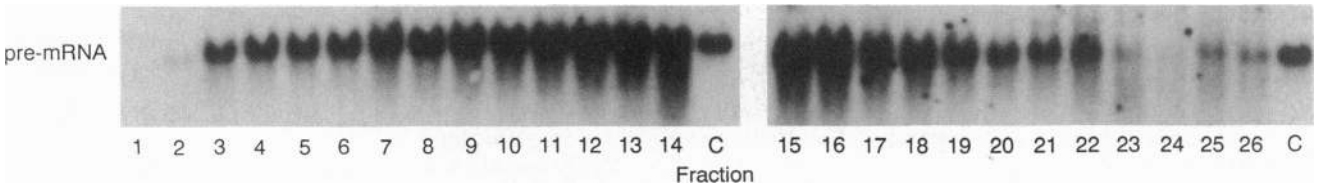

FIG. 3. RNA blotting analysis of the polysomal distribution of the *MER2* pre-mRNA. The blot shown in Fig. 2*C* was stripped and rehybridized with a riboprobe specific for the *MER2* pre-mRNA. The *MER2* riboprobe is described in the legend to Fig. 1.

also measured. The results of these experiments are shown in Fig. 1 and summarized in Table 1. For *CYH2*, the decay rate and steady-state level of its mRNA were equivalent in either *UPF1*$^+$ or *upf1*$^-$ strains ($t_{1/2}$ = 43 min; Fig. 1 *A* and *E*; Table 1). However, turnover and accumulation of the *CYH2* pre-mRNA differed in *UPF1*$^+$ and *upf1*$^-$ strains. Compared to its decay rate in a *UPF1*$^+$ strain ($t_{1/2}$ = 1.5 min), the *CYH2* pre-mRNA was stabilized 4-fold in a *upf1*$^-$ strain ($t_{1/2}$ = 6 min) and showed a comparable increase in steady-state level (Fig. 1 *A* and *E*; Table 1). Confirmation that the upper band of Fig. 1 *A* and *E* was the *CYH2* pre-mRNA was obtained by demonstrating that a *CYH2* intron probe hybridizes only to this band and not to the *CYH2* mRNA (Fig. 1*B*). The half-life and steady-state level of the *RP51B* mRNA were also unaffected by the presence or absence of a functional *UPF1* gene ($t_{1/2}$ = 17 min; Fig. 1 *C* and *F*; Table 1), but its pre-mRNA and the pre-mRNA encoded by the *MER2* gene were stabilized, and showed increased accumulation, in a *upf1*$^-$ strain. Compared to their decay rates in *UPF1*$^+$ cells, the *RP51B* and *MER2* pre-mRNAs were stabilized 2-fold and 5-fold, respectively, in *upf1*$^-$ cells (Fig. 1 *C, D, F,* and *G*; Table 1). Thus, three different pre-mRNAs are all degraded via the nonsense-mediated mRNA decay pathway. Interestingly, the *MER2* pre-mRNA was 6-fold more stable than the *CYH2* or *RP51B* pre-mRNAs in a *upf1*$^-$ strain (Table 1), suggesting that the *CYH2* and *RP51B* introns may harbor instability sequences that are absent from the *MER2* pre-mRNA.

Intron-Containing Pre-mRNAs Associate with Ribosomes. Our previous results suggest that nonsense-mediated mRNA decay in yeast is a cytoplasmic process requiring ongoing translation (1). One prediction that follows from this conclusion is that unspliced transcripts are associated with ribosomes. To test this premise, we determined whether the *CYH2, RP51B*, and *MER2* pre-mRNAs were polysome-associated. Post-mitochondrial supernatants from a *upf1*$^-$ strain, prepared in the presence of cycloheximide and heparin (to inhibit fortuitous association of RNAs with ribosomes), were fractionated on sucrose gradients and the positions of the respective pre-mRNAs and mRNAs were determined by RNA blotting (Figs. 2 and 3). The *CYH2* mRNA was predominantly associated with polysome fractions containing between 2 and 4 ribosomes per transcript (fractions 7–14, Fig. 2*C*). The *CYH2* pre-mRNA was also predominantly polysome-associated, but the average number of ribosomes per transcript was one (fractions 16–18, Fig. 2*C*), consistent with premature translational termination due to the presence of the 5′-proximal intron sequence shortly after the methionine initiation codon (Table 2; ref. 25). In a control experiment, in which the cytoplasmic extract was first treated with EDTA to dissociate ribosomes from mRNA, the *CYH2* pre-mRNA and mRNA were located in the lighter, ribonucleoprotein (RNP) fractions of the gradient (Fig. 2 *B* and *D*, fractions 21–24). Reprobing of the same blots shown in Fig. 2 *C* and *D* for the *RP51B* and *MER2* transcripts revealed that (*i*) the *RP51B* mRNA and pre-mRNA sedimented in virtually the same positions in both gradients as the analogous *CYH2* transcripts (data not shown), (*ii*) in the presence of EDTA, the *MER2* pre-mRNA was associated with the same RNP fractions that contained the *CYH2* and *RP51B* transcripts (data not shown), and (*iii*) in the absence of EDTA, the *MER2* pre-mRNA was predominantly associated with 1 to 4 ribosomes (Fig. 3, fractions 7–18)—i.e., it formed polysomes significantly larger than those formed by the *CYH2* and *RP51B* pre-mRNAs. The similarity in size of the polysomes formed by the *CYH2* and *RP51B* pre-mRNAs and the larger size of the polysomes formed by the *MER2* pre-mRNA are all consistent with the relative positions of the first nonsense codons within the respective transcripts (Table 2).

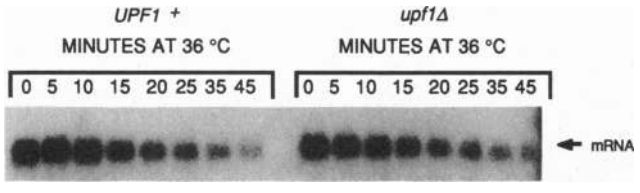

FIG. 4. Decay of the *RP51A* mRNA was measured as described in the legend to Fig. 1. The blot was hybridized with a radioactive 235-nt *Hin*cII fragment from RP51A exon 2 [spanning RP51A nt 470–704 (27)].

Pre-mRNAs and the Function of the *UPF1* Protein. The data of Figs. 1–3 suggest that, in yeast, cytoplasmic pre-mRNAs comprise a major class of substrate for the nonsense-mediated mRNA decay pathway. Crucial to this conclusion is evidence that the phenomena in question are actually cytoplasmic and not nuclear. Degradation of a pre-mRNA fraction by a cytoplasmic decay pathway is supported by the observation that, in the absence of EDTA, the sedimentation of the *CYH2, RP51B*, and *MER2* pre-mRNAs in sucrose gradients varies as a function of the position of the first nonsense codon (Figs. 2 and 3; Table 2). Since all three pre-mRNAs contain a single intron, the sizes of their respective spliceosomes would not be expected to differ significantly. Moreover, evidence against a general role for *UPF1* in pre-mRNA splicing is provided by experiments that indicate that mRNA steady-state levels are unaltered in *upf1*$^-$ strains (see Fig. 1) and that the effects of a *UPF1* mutation on pre-mRNA accumulation and turnover are limited to a subset of pre-mRNAs. For example, in *upf1*$^-$ strains, there is no detectable accumulation of the *RP51A* pre-mRNA [Fig. 4; *RP51A* pre-mRNA also contains an early nonsense codon (Table 2)] or the pre-mRNAs encoded by the *ACT1* and *CRY1* genes (data not shown). Unlike the transcripts of the *CYH2, RP51B*, and *MER2* genes, these three pre-mRNAs are all efficiently spliced (20–24, 29), suggesting that the "escape" of pre-mRNAs from the spliceosome assembly/nuclear retention system into the cytoplasm may vary inversely as a function of splicing efficiency.

If a major source of nonsense-containing cytoplasmic transcripts in yeast are pre-mRNAs, then the prevalence of introns at the 5′ ends of yeast genes (14) may be due, in part, to the existence of a cellular mechanism that ensures rapid degradation of those pre-mRNAs. The *UPF1* gene product would thus function as part of the machinery that degrades these transcripts to reduce the generation of potentially deleterious nonsense fragments.

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