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Gene Set Coregulated by the *Saccharomyces cerevisiae* Nonsense-Mediated mRNA Decay Pathway

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The nonsense-mediated mRNA decay (NMD) pathway has historically been thought of as an RNA surveillance system that degrades mRNAs with premature translation termination codons, but the NMD pathway of *Saccharomyces cerevisiae* has a second role regulating the decay of some wild-type mRNAs. In *S. cerevisiae*, a significant number of wild-type mRNAs are affected when NMD is inactivated. These mRNAs are either wild-type NMD substrates or mRNAs whose abundance increases as an indirect consequence of NMD. A current challenge is to sort the mRNAs that accumulate when NMD is inactivated into direct and indirect targets. We have developed a bioinformatics-based approach to address this challenge. Our approach involves using existing genomic and function databases to identify transcription factors whose mRNAs are elevated in NMD-deficient cells and the genes that they regulate. Using this strategy, we have investigated a coregulated set of genes. We have shown that NMD regulates accumulation of *ADR1* and *GAL4* mRNAs, which encode transcription activators, and that Adr1 is probably a transcription activator of *ATS1*. This regulation is physiologically significant because overexpression of *ADR1* causes a respiratory defect that mimics the defect seen in strains with an inactive NMD pathway. This strategy is significant because it allows us to classify the genes regulated by NMD into functionally related sets, an important step toward understanding the role NMD plays in the normal functioning of yeast cells.

Nonsense-mediated mRNA decay (NMD) is a highly conserved mRNA degradation pathway. Historically, NMD has been thought of as an RNA surveillance system whose role is to identify and rid cells of mRNA with premature termination codons and thus prevents accumulation of potentially harmful truncated proteins. However, more recently, it has become apparent that the NMD pathway of *Saccharomyces cerevisiae* has a second role regulating the decay of wild-type mRNAs.

Genome-wide transcription profiling has revealed that a significant number (estimated to be between 5 and 10%) of wild-type transcripts accumulate in yeast cells when the NMD pathway is inactivated (15, 28). These mRNAs that accumulate can be direct NMD substrates or could accumulate as an indirect consequence of inactivation of NMD. *PPR1* and *URA3* mRNAs represent examples of mRNAs that are directly and indirectly affected by inactivation of the NMD pathway, respectively. PPR1 mRNA is an NMD substrate because it is degraded more rapidly in cells with an active NMD pathway than those in which the NMD pathway has been inactivated (20). It encodes a transcription activator, and the genes activated by Ppr1 are up-regulated in cells with an inactive NMD pathway (18, 27). For example, *URA3* is regulated by Ppr1. *URA3* mRNA accumulates in cells with an inactive NMD pathway; however, *URA3* mRNA has the same half-life in cells with active and inactive NMD pathways (27). Thus, accumulation of *URA3* mRNA is due to increased transcription activation by Ppr1 as an indirect consequence of inactivation of the NMD pathway. To date, a limited number of natural NMD substrates have been identified. In addition to *PPR1* mRNA, 12 wild-type mRNAs that are degraded by the NMD pathway have been identified (15, 20, 37, 41). Given the number of mRNAs that are affected by inactivation of the NMD pathway, it is very likely that additional wild-type mRNAs are direct NMD substrates.

The number of direct versus indirect NMD substrates in *S. cerevisiae* is controversial. Lelivelt and Culbertson (28) measured the half-lives of nine mRNAs whose abundance was increased in NMD mutants. None of these mRNAs has an altered half-life. This suggests that the majority of mRNAs that accumulate in NMD mutants may be indirect targets. He et al. (15) argue, on the other hand, that the majority of mRNAs that accumulate when NMD is inactivated are direct substrates. To resolve this controversy, it is important to identify direct versus indirect NMD substrates. This could be done by determining the mRNA half-lives of all of the potential NMD substrates in wild-type and NMD-deficient cells by using microarrays. However, this approach will miss low-abundance mRNAs, like *PPR1* mRNA, that are below the threshold of detection (28). We have developed a complementary bioinformatics-based approach. Our approach involves using existing genomic and function databases to identify transcription factors whose mRNAs are elevated in NMD-deficient cells and the genes that they regulate. Using this strategy, we have investigated a coregulated set of genes. We have shown that NMD regulates accumulation of *ADR1* mRNA, which encodes a transcription activator of genes for generation of acetyl coenzyme A (CoA) and NADH from nonfermentable substrates (42). Further, we propose that Adr1 also activates expression of *ATS1*.

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TABLE 1. Yeast strains used in this study

Strain	Genotype	Source or reference
W303a	*MAT***a** *ade2-1 ura3-11 his3-11,15 trp1-1 leu2-3,112 can1-100*	S. Wente
AAY320	*MAT***a** *ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 upf1-Δ2 (URA3)*	19
AAY315	*MATα ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 ATS1::URA3*	37
AAY334	*MAT***a** *ura3-1 his3-11,15 trp1-1 leu2-3,112 rpb1-1*	19
AAY335	*MAT***a** *ura3-1 his3-11,15 trp1-1 leu2-3,112 rpb1-1 upf1-Δ2 (URA3)*	19
PJ69-4a	*MAT***a** *trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL7-lacZ*	17
3575	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 adr1::KanMX4*	Research Genetics
BY4741	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Research Genetics
1569	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ppr1::KanMX4*	Research Genetics

MATERIALS AND METHODS

Yeast strains, plasmids, and media. The *S. cerevisiae* strains used in this study are listed in Table 1. Unless otherwise stated, yeast strains were grown and maintained by standard methods (4). Adr1 was repressed in YP medium with 8% glucose (YP–8% glucose) and derepressed in YP medium containing 3% ethanol and 1% D-glucose (YP-ethanol). Adr1p repression and derepression media were made and used as described by Sloan et al. (39). YP medium with 2% galactose (YP–2% galactose) was used for induction of Gal4. YP–2% galactose medium was prepared according to a standard protocol (4). The plasmids for *ADR1* overexpression, pRS314ADR1 (*ADR1 TRP1 CEN6 ARSH4*) and pMW5 (*ADR1 TRP1* 2μ), were generously provided by Elton T. Young. The plasmid for expression of *ADR1* from a *GAL10* promoter, pKD34 (P_{GAL10}-*ADR1*-T_{CYC1} *TRP1* 2μ), was generously provided by Kenneth Dombek. Plasmids were transformed into yeast strains by the high-efficiency Li acetate method (11).

Measurement of mRNA accumulation and half-life. mRNA steady-state levels and half-lives were measured as described by Kebaara et al. (19), with the exception of *ADR1* mRNA expressed from the *GAL10* promoter. The half-life of this mRNA was measured by first growing the cells in YP–2% galactose to activate transcription of the *GAL* promoter and then repressing the *GAL* promoter in YP–2% glucose as described by Parker et al. (34). The PCR primer pairs used for synthesis of DNA used for probe synthesis are in Table 2. Northern blots were PhosphorImaged with a Storm PhosphorImager (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). Analysis of mRNA levels was performed with ImageQuant Software, version 5.1, (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) and Microsoft Excel (Microsoft Corporation, Redmond, WA). mRNA levels were normalized with ScR1 RNA, an RNA polymerase III transcript that is insensitive to NMD (29). mRNA half-lives were determined from graphs of percent mRNA remaining versus time. The graphs were prepared with SigmaPlot software, version 6.10 (SPSS Science, Chicago, IL). Steady-state mRNA levels and mRNA half-lives were averaged from a minimum of three independent experiments.

Bioinformatics. Identification of potential transcription factor binding sites in the *ATS1* promoter region was performed with the Promoter Database of *Saccharomyces cerevisiae* (http://rulai.cshl.edu/SCPD). Genomic DNA sequences for *Saccharomyces paradoxus*, *Saccharomyces mikatae*, and *Saccharomyces bayanus* were obtained from the Saccharomyces Genome Sequencing at the Genome Sequencing Center (http://genomeold.wustl.edu/projects/yeast/). BLASTN was used to find the 5′ end of the *ATS1* open reading frame (ORF) sequences in the *S. paradoxus*, *S. mikatae*, and *S. bayanus* genomic sequences. Five hundred base pairs of sequence upstream of the *ATS1* ORF was recovered from all of the *Saccharomyces* yeast strains and aligned with CLUSTALW (http://www.ebi.ac.uk/clustalw/). The average *n*-fold increase for various mRNAs was obtained from the Nonsense-Mediated mRNA Decay database (http://144.92.19.47/default.htm) (28). A two-sided test was used to calculate *P* values for the all of data in the Lelivelt and Culbertson database with the null hypothesis that the mean transcript abundances of a particular gene are equal in the knockout and wild-type samples and the alternative hypothesis that the transcript abundances are not equal.

RESULTS

Steady-state *ATS1* mRNA levels are elevated in Upf1p-deficient cells, but the *ATS1* mRNA half-life is the same in *UPF1* and *upf1Δ* cells. *ATS1* mRNA is one of the several hundred mRNAs that accumulate in *S. cerevisiae* when the NMD pathway is inactivated (28). Northern blot analysis confirmed that

TABLE 2. Primer pairs used for probe DNA synthesis

Probe DNA	Primers	Source
ATS1	5′-d(TCT AAT GGG CAA AGG CAA CT) 5′-d(CCA AAC ACG CGA GGT TTT)	Operon, Inc.[a]
CYH2	5′-d(CAC TAA GAC TAG AAA GCA CAG AGG) 5′-d(GTA TTG GTC TCT CTT GTC TTC TGG G)	Operon, Inc.
ScR1	5′-d(GGC TGT AAT GGC TTT CTG GTG G) 5′-d(GGT TCA GGA CAC ACT CCA TCC)	Operon, Inc.
GAL4	YPL248C-F, YPL248C-R	Resgen, Inc.[b]
ADR1	5′-d(GGA TCC TAA TAC GAC TCA CTA TAG GGC TTC TTG TAT TGG CAG) 5′-d(ATG GCT AAC GTA GAA AAA CC)	Operon, Inc.
ADH2	YMR303C-F, YMR303C-R	Resgen, Inc.
GAL1	YBR020W-F, YBR020W-R	Resgen, Inc.

[a] Alameda, CA.
[b] Huntsville, AL.

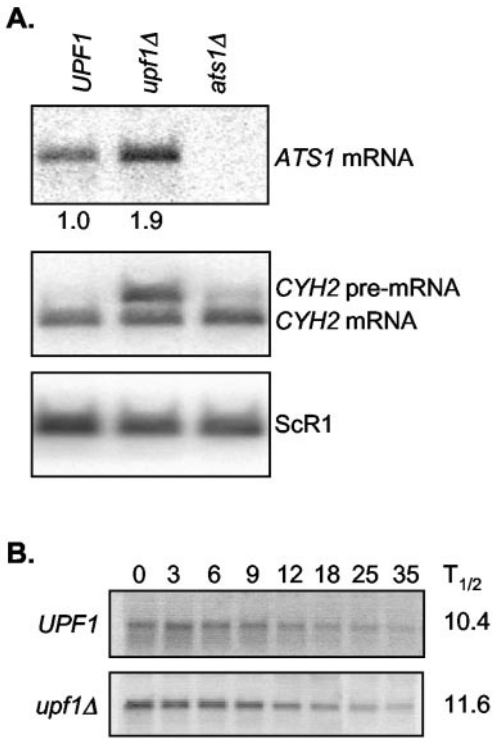

FIG. 1. The increase in *ATS1* mRNA accumulation seen in *upf1*Δ yeast strains relative to *UPF1* yeast strains is an indirect effect of inactivation of the NMD pathway. (A) Representative Northern blot prepared with total RNAs from W303a (*ATS1 UPF1*), AAY320 (*ATS1 upf1*Δ), and AAY315 (*ats1*Δ *UPF1*). The Northern blots were hybridized with radiolabeled *ATS1* (top), *CYH2* (middle), and *ScR1* (bottom) DNA probes. *CYH2* and *ScR1* are controls. *CYH2* is a control for the NMD phenotype of the yeast strains. The *CYH2* probe detects both *CYH2* pre-mRNA and mRNA. *CYH2* pre-mRNA is inefficiently spliced, and consequently a significant amount of this pre-mRNA is exported to the cytoplasm. *CYH2* pre-mRNA has an in-frame stop codon within its intron that targets it for NMD, while *CYH2* mRNA is not an NMD target (14). *ScR1* is an RNA polymerase III transcript that is not degraded by NMD (29). It was used as a loading control. The relative *ATS1* mRNA levels in *UPF1* and *upf1*Δ yeast cells are shown below the corresponding bands in the top part of panel A. (B) Determination of ATS1 mRNA half-life by Northern blot analysis of total RNA harvested from isogenic yeast strains AAY334 (*UPF1*, upper image) and AAY335 (*upf1*Δ, lower image) at the indicated time points (in minutes) following arrest of transcription. Blots were hybridized with radiolabeled *ATS1* DNA and PhosphorImaged. The half-lives ($T_{1/2}$, minutes) are the averages of at least three independent experiments and were determined from a plot of percent mRNA remaining versus time. Percent mRNA remaining was calculated by dividing the number of pixels contained in a particular band by the number of pixels contained in the first time point. The half-life of ATS1 mRNA in each yeast strain is to the right of the PhosphorImages.

ATS1 mRNA levels are elevated in *upf1*Δ cells relative to an isogenic *UPF1* strain (Fig. 1A). *ATS1* mRNA accumulation is 1.9-fold ± 0.3-fold higher in *upf1*Δ cells than in isogenic *UPF1* cells. No hybridization to RNA isolated from an *ATS1*Δ strain was seen. This strain lacks the *ATS1* gene and is thus unable to synthesize *ATS1* mRNA. This confirms that the correct mRNA was detected on the Northern blots. We conclude that NMD affects *ATS1* mRNA accumulation.

The NMD pathway can affect steady-state mRNA levels either directly or indirectly. Directly affected mRNAs are degraded by the NMD pathway. These mRNAs have a shorter half-life in yeast cells with a functional NMD pathway than in yeast cells with mutations in the *UPF* genes. Indirect effects on mRNA accumulation result when transcription of the gene encoding the mRNA is regulated by the product of another mRNA that is affected by the NMD pathway. Indirectly affected mRNAs have the same half-lives in yeast cells with a functional NMD pathway as yeast cells with mutations in the *UPF* genes. To determine if *ATS1* mRNA is a direct or indirect target of NMD, we compared *ATS1* mRNA half-lives in isogenic *UPF1* and *upf1*Δ yeast strains (Fig. 1B). The *ATS1* mRNA half-life is 10.4 ± 2.1 min in *UPF1* cells and 11.6 ± 1.9 min in *upf1*Δ cells. *ATS1* mRNA half-lives are not significantly different in *UPF1* and *upf1*Δ strains. Therefore, *ATS1* mRNA is not a direct substrate of NMD. Rather, it accumulates as an indirect consequence of inactivation of the NMD pathway.

Identification of putative transcription factors that regulate *ATS1* and are affected by NMD. Because *ATS1* mRNA is not degraded by the yeast NMD pathway, we hypothesized that NMD affects the accumulation of an mRNA encoding an *ATS1* transcription regulator. We developed a strategy to identify *ATS1* transcription regulators whose mRNAs are elevated in *upf* mutants relative to *UPF* yeast cells. Our overall strategy was to (i) map putative transcription factor binding sites in the promoter region of *ATS1*, (ii) determine whether the putative transcription factor binding sites are conserved in closely related *Saccharomyces* yeast strains, (iii) identify putative transcription factors of *ATS1* whose mRNAs accumulate in NMD mutants, (iv) test whether *ATS1* is regulated by the candidate transcription factors, and (v) determine if the transcription factor mRNA is directly or indirectly affected by NMD.

Putative transcription factors of *ATS1* were identified by analyzing the 500 bp upstream of the *ATS1* ORF for putative transcription factor binding sites with the Promoter Database of *Saccharomyces cerevisiae* (http://rulai.cshl.edu/SCPD). There are 13 putative binding sites for nine transcription factors in the *ATS1* promoter region (Fig. 2A).

Potential transcription factor binding sites can also be identified by alignment of orthologous promoter regions from closely related *Saccharomyces* strains (8, 21). We aligned the sequences of the *ATS1* promoter regions from *S. cerevisiae*, *S. paradoxus*, *S. mikatae*, and *S. bayanus* with ClustalW (http://www.ebi.ac.uk/clustalw/). The binding sites for four transcription factors, Adr1, Gcr1, SCB, and Gal4, are conserved in the *ATS1* promoter (Fig. 2B). Thus, *ATS1* may be regulated by Adr1, Gcr1, SCB, and/or Gal4. Additional conserved sequences were observed, suggesting that *ATS1* has additional transcription factor binding sites. This result is not surprising since the binding sites for only ~40% of the *S. cerevisiae* transcription factors are known (26).

To determine whether Adr1, Gcr1, SCB, or Gal4 might be regulated by NMD, we identified their genes and then found the average *n*-fold increase calculated from high-density oligonucleotide arrays for the corresponding mRNAs (Table 3; 28; http://144.92.19.47/default.htm). The average *n*-fold increase is a measure of mRNA levels in *upf* mutant strains relative to *UPF* yeast strains. The average *GAL4* mRNA increase was 3.51-fold, indicating that this mRNA was elevated in NMD-

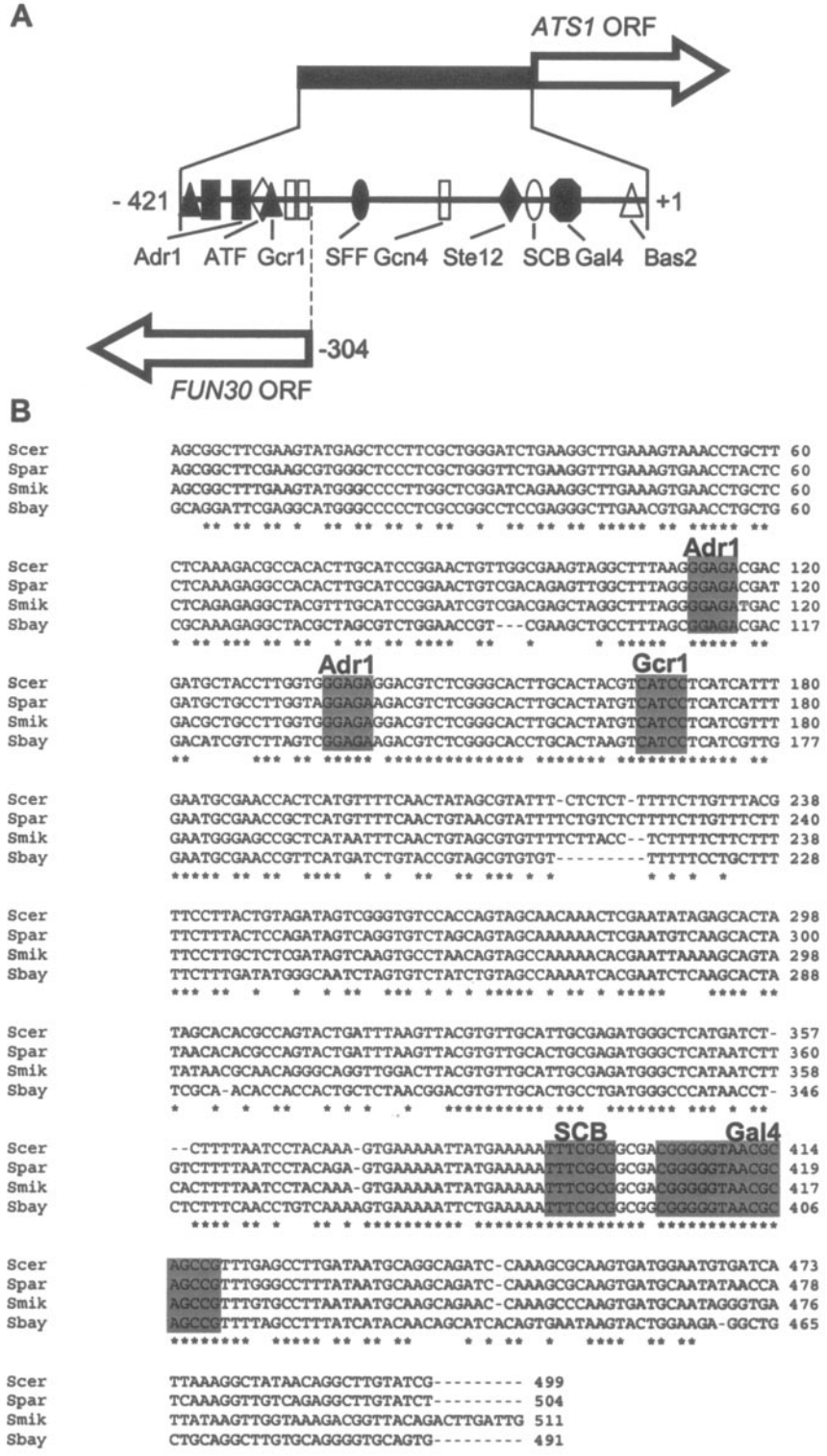

FIG. 2. Potential transcription factor binding sites in the *ATS1* promoter region. (A) Schematic diagram of the putative transcription factor binding sites in the 500 bp upstream of the *ATS1* ORF identified with the Promoter Database of *Saccharomyces cerevisiae* (http://rulai.cshl.edu/SCPD). The FUN30 ORF is on the opposite strand of the ATS1 ORF, and its position relative to putative transcriptional activator binding sites is indicated. (B) Comparison of the *ATS1* promoter regions from *S. cerevisiae*, *S. paradoxus*, *S. mikatae*, and *S. bayanus*. The conserved potential binding sites in the *ATS1* promoter region for Adr1, Gcr1, SCB, and Gal4 are shaded.

TABLE 3. Summary of potential transcription factor binding sites in the *ATS1* promoter region and effect of inactivation of NMD on accumulation of their mRNAs

Transcription factor[a]	Consensus binding site[a]	Location of putative binding site in *ATS1* promoter region[a]	Transcription factor gene(s)[b]	Avg increase[c] (*n*-fold)	Relative mRNA accumulation (*upf1*Δ/*UPF1*)[d]
GCR1	CATCC	−421 to −417, −334 to −330	*GCR1*	1.08	ND[e]
ADR1	TCTCC	−389 to −385, −365 to −361	*ADR1*	1.20	2.6 ± 0.21
SCB	CGCGAAA	−108 to −102	*SW14* *SW16*	0.95 0.96	ND
GAL4	CGGGGGTAACGCAGCCG	−97 to −81	*GAL4*	3.51	2.9 ± 0.21

[a] Potential transcription factor binding sites in *ATS1* were identified by using the Promoter Database of *Saccharomyces cerevisiae* (http://rulai.cshl.edu/SCPD) and conserved in closely related *Saccharomyces* yeast strains.
[b] The genes encoding the putative transcription factors of *ATS1* were identified by using the Saccharomyces Genome Database (http://www.yeastgenome.org/).
[c] The average *n*-fold increase in the corresponding mRNAs from each gene was calculated from high-density oligonucleotide arrays (28; http://144.92.19.47/default.htm).
[d] Relative mRNA accumulation was determined by quantitative Northern blot analysis of RNAs obtained from isogenic W303a (*UPF1*) and AAY320 (*upf1*Δ) yeast strains. Values represent average data ± the standard deviation calculated from a minimum of three independent experiments. All mRNA levels were normalized to ScR1, an RNA polymerase III transcript. In all cases, total RNA from null yeast strains was used as a control to confirm that we quantified the correct band.
[e] ND, not determined.

deficient strains. The average increase in *ADR1* mRNA was 1.20-fold, suggesting that this mRNA might be slightly elevated in NMD-deficient cells (Table 3). The *GCR1* mRNA and the mRNAs encoding the subunits of SCB do not seem to accumulate in NMD mutants (Table 3). Further, the mRNA levels of the genes regulated by Gcr1 and SCB are similar in wild-type and NMD-deficient cells (data not shown). Gcr1 and SCB were not examined further.

***GAL4* and *ADR1* mRNAs accumulate in *upf1*Δ cells relative to *UPF1* cells.** Steady-state *ADR1* and *GAL4* mRNA levels in *upf1*Δ and *UPF1* yeast strains were determined by Northern blot analysis (Fig. 3). Both *GAL4* and *ADR1* mRNAs accumulate to higher levels in *upf1*Δ cells than in *UPF1* cells. Two *GAL4*-specific bands of 2.9 and 1.8 kb were observed on the Northern blots (Fig. 3A). These bands are specific for *GAL4* because they were not present in the lane containing RNA from a *gal4* deletion strain. The expected size of the *GAL4* mRNA is 2.8 kb (25). Thus, the upper band is the expected size of the *GAL4* mRNA and the lower band is a truncated *GAL4* transcript. The lower band was not characterized further. The full-length *GAL4* mRNA accumulation was 2.9-fold ± 0.2-fold higher in *upf1*Δ cells than in *UPF1* cells. *ADR1* mRNA also accumulated to higher levels in *upf1*Δ cells than in *UPF1* cells (Fig. 3B). We found a 2.6-fold ± 0.2-fold higher level of *ADR1* mRNA accumulation in *upf1*Δ cells compared to *UPF1* cells (Fig. 3B). Thus, transcription activation of *ATS1* by Gal4p and/or Adr1p could account for the increased *ATS1* mRNA accumulation in *upf1*Δ cells. For this reason, we tested whether Gal4p and Adr1p regulate *ATS1*.

The NMD-dependent increase in *GAL4* mRNA does not account for the accumulation of *ATS1* mRNA in *upf1*Δ cells. Gal4 is a transcription activator for genes controlling the metabolism of galactose and galactose disaccharides such as lactose (reviewed in reference 6). Gal4-dependent transcription of these genes is activated by galactose and strongly repressed by glucose. Thus, we expect the mRNA levels for Gal4-regulated genes to be higher in YP–2% galactose-grown cells than in YP–2% glucose-grown cells. To test if Gal4 controls *ATS1* transcription, the effects of changes in Gal4 expression on *ATS1* mRNA levels were compared (Fig. 4). We determined steady-state *ATS1* mRNA levels in *UPF1 GAL4* and *upf1*Δ *GAL4* yeast cells grown in YP–2% galactose and YP–2% glucose. Steady-state *ATS1* mRNA levels were 1.0-fold ± 0.0-fold and 0.4-fold ± 0.0-fold in *UPF1 GAL4* yeast cells grown in glucose- and galactose-containing media, respectively. *ATS1* mRNA levels were 2.3-fold ± 0.4-fold higher in *upf1*Δ cells than in *UPF1* cells grown in glucose and 1.9-fold ± 0.2-fold higher in *upf1*Δ cells than in *UPF1* cells grown in galactose (Fig. 4). As a control, we examined *GAL1* mRNA levels in cells grown in YP–2% galactose and YP–2% glucose. *GAL1* is a Gal4-regulated gene (6). As expected, *GAL1* mRNA was undetectable in cells grown in YP–2% glucose and readily detectable in YP–2% galactose. Thus, we conclude that *ATS1* mRNA does not increase under conditions that activate Gal4.

We also tested whether *ATS1* mRNA levels were affected by loss of Gal4 function by examining *ATS1* mRNA levels in *GAL4* and *gal4*Δ yeast strains. *ATS1* mRNA accumulation was 1.0-fold ± 0.0-fold and 0.7-fold ± 0.0-fold in *GAL4* and *gal4*Δ yeast strains, respectively (Fig. 4). Thus, loss of *GAL4* function causes a small decrease in *ATS1* expression.

Based on these results, we conclude that Gal4 is probably not a transcription activator of *ATS1* because *ATS1* expression is not activated under conditions that activate Gal4 and loss of Gal4 results in only a small decrease in *ATS1* expression. Thus, the NMD-dependent increase in *GAL4* mRNA does not account for the accumulation of *ATS1* mRNA in *upf1*Δ cells. We have not examined the basis of the Upf1p-dependent increase in *GAL4* mRNA further.

***Adr1* may be an *ATS1* transcription regulator.** The potential regulation of *ATS1* by Adr1p was tested in two ways. First, steady-state *ATS1* mRNA levels were determined for strains differing only in their *ADR1* gene copy numbers (Fig. 5A). Second, steady-state *ATS1* mRNA levels were determined in yeast strains grown under conditions that repress and derepress Adr1, respectively (Fig. 5B).

If Adr1 regulates *ATS1*, we expect *ATS1* mRNA levels to increase in cells overexpressing *ADR1* and decrease in *adr1*Δ cells because they lack Adr1. The *ADR1* gene copy number was

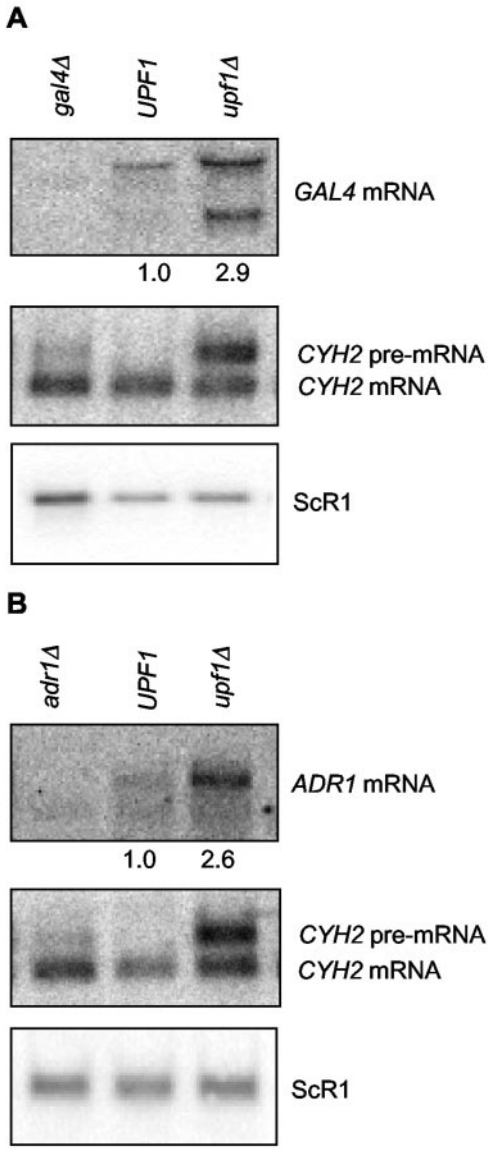

FIG. 3. *GAL4* and *ADR1* mRNAs accumulate in *upf1*Δ cells relative to *UPF1* cells. Representative Northern blots were prepared with total RNAs from W303a (*UPF1*), AAY320 (*upf1*Δ), PJ69-4a (*gal4*Δ), and Research Genetics strain 3573 (*adr1*Δ). The Northern blots were hybridized with radiolabeled *GAL4* or *ADR1* and *CYH2* and *ScR1* DNAs. The relative *GAL4* and *ADR1* mRNA levels in *UPF1* and *upf1*Δ yeast cells are shown below the corresponding bands.

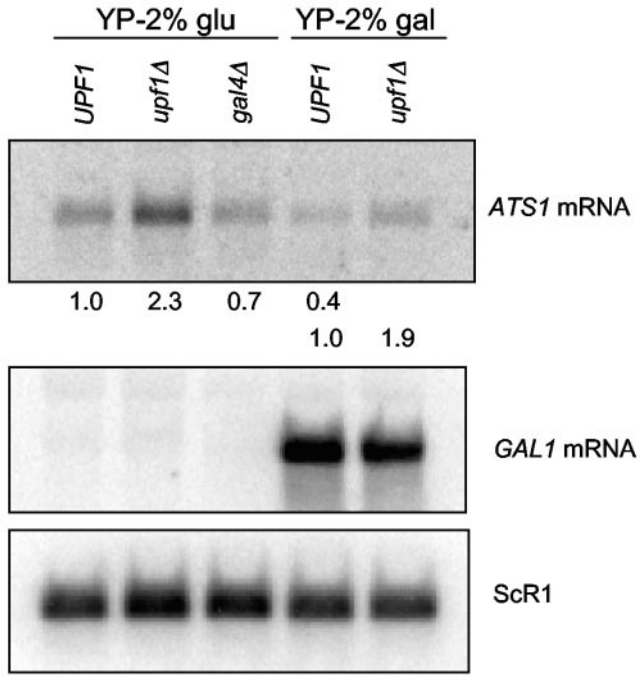

FIG. 4. *ATS1* expression is not increased under conditions that activate Gal4p. Shown is a representative Northern blot prepared with total RNAs from W303a (*UPF1*) and AAY320 (*upf1*Δ) grown in YP with 2% glucose (YP-2% glu) or 2% galactose (YP-2% gal) and from PJ69-4a (*gal4*Δ) grown in glucose. The Northern blots were hybridized with radiolabeled *ATS1*, *GAL1*, and *ScR1* probes. The relative *ATS1* mRNA levels are shown below the corresponding bands in the top panel.

increased by transforming W303a (*ADR1*) with an *ADR1* centromeric plasmid (Fig. 5A). As a control, *ADR1* mRNA accumulation was determined. *ADR1* mRNA accumulation is 4.1-fold ± 0.1-fold higher in cells with additional copies of the *ADR1* gene on a centromeric plasmid than in an isogenic *ADR1* yeast strain which only expressed *ADR1* from its normal chromosomal location (Fig. 5A). Thus, we see an increase in *ADR1* expression when the *ADR1* gene copy number increases. *ATS1* mRNA accumulation is 4.0-fold ± 1.5-fold higher in cells transformed with the *ADR1* gene on a centromeric plasmid than in an isogenic yeast strain expressing only the chromosomal copy of *ADR1* (Fig. 5A). The effect of loss of Adr1 function on steady-state *ATS1* mRNA levels were determined by measuring *ATS1* mRNA levels in isogenic *ADR1* and *adr1*Δ cells grown under Adr1-derepressing conditions (YP–3% ethanol–1% D-glucose; Fig. 5B). The relative *ATS1* mRNA abundance was 0.65-fold ± 0.05-fold lower in *adr1*Δ cells than in the isogenic *ADR1* cells.

If Adr1 regulates *ATS1*, we expect *ATS1* mRNA levels to be higher under conditions that derepress Adr1 and lower under conditions that repress Adr1. Sloan et al. (39) showed that Adr1 is derepressed in cells in ethanol (YP–3% ethanol–1% D-glucose) and is repressed in cells grown in high glucose (YP–8% glucose; note that standard yeast growth medium contains 2% glucose). As a control for repression and derepression of Adr1, we examined *ADH2* mRNA accumulation in cells grown under derepressing and repression conditions, respectively (Fig. 5B). Adr1 positively regulates *ADH2* by binding its promoter (10). *ADH2* mRNA levels were difficult to detect in RNA prepared from cells grown under repressing conditions and readily detectable in RNA prepared from cells grown under derepressing conditions (Fig. 5B). The accumulation of *ATS1* mRNA in *ADR1* cells grown under derepressing and repressing conditions was measured by quantitative Northern blot analysis (Fig. 5B). *ATS1* mRNA accumulation is 6.8-fold ± 2.0-fold higher in *ADR1* cells grown under derepressing conditions than in *ADR1* cells grown under repressing conditions.

Thus, *ATS1* mRNA levels increase when *ADR1* expression

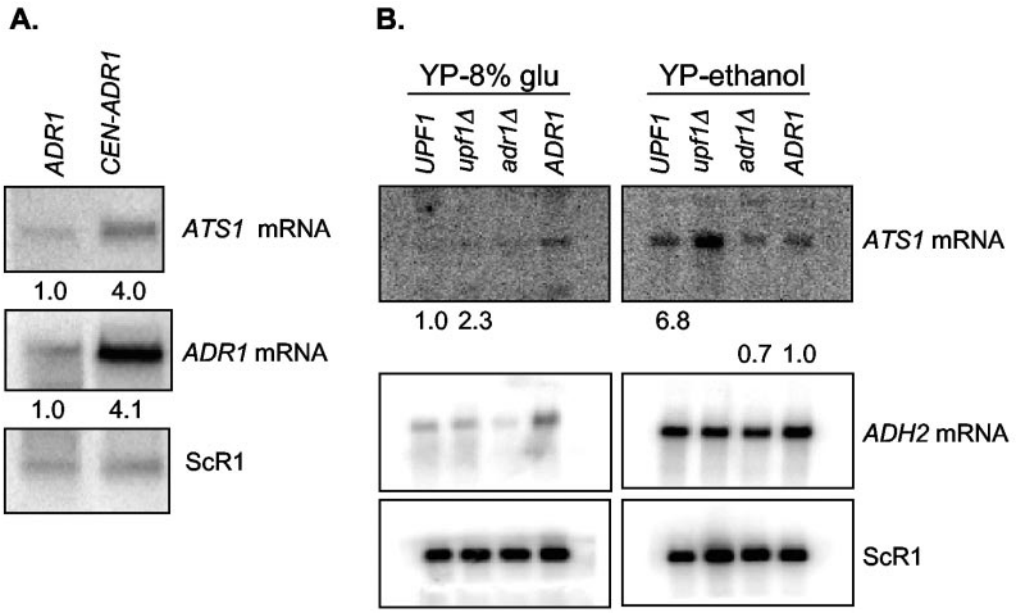

FIG. 5. Adr1 may be a transcription activator of *ATS1*. (A) Representative Northern blot prepared with total RNA from W303a transformed with pRS314 (*ADR1*) and pRS314ADR1 (*CEN-ADR1*). The Northern blots were hybridized with radiolabeled *ATS1*, *ADR1*, and *ScR1* DNA probes. (B) Representative Northern blot prepared with total RNAs from W303a (*UPF1*), AAY320 (*upf1*Δ), 3575 (*adr1*Δ), and BY4741 (*ADR1*). The left column of images are the result of hybridization to Northern-blotted total RNA extracted from cells cultured under repressing conditions (YP–8% glucose [YP-8% glu]). The right column has images of hybridization to Northern blotted total RNA extracted from cells cultured under derepressing conditions (YP–3% ethanol–1% D-glucose [YP-ethanol]). The Northern blots were hybridized with radiolabeled *ATS1*, *ADH2*, and *ScR1* DNA probes. Adr1 is a positive regulator of *ADH2*, so *ADH2* mRNA levels are a control for repression and derepression of Adr1. The relative mRNA levels are shown below the corresponding bands.

increases and when Adr1 is derepressed. *ATS1* mRNA levels decrease when Adr1 is absent or when it is repressed. These results are consistent with Adr1 being a transcription activator of *ATS1*.

Many of the mRNAs encoded by Adr1-regulated genes may accumulate to higher levels in *upf*Δ cells than in *UPF* cells. Adr1 has been shown to bind the promoters of 14 genes (42, 43). We examined the average *n*-fold increase calculated from high-density oligonucleotide arrays for the corresponding mRNAs (Table 4; 28, http://144.92.19.47/default.htm). Eight of the mRNAs have an average increase of 1.3-fold or greater, suggesting that these mRNA might be slightly elevated in NMD-deficient cells (Table 4). Four genes have an average increase of equal to or less than 1.15-fold. The average *n*-fold increase was not available for two of the genes. We confirmed that the mRNA for one Adr1-regulated gene, *CTA1*, accumulates by quantitative Northern blotting. *CTA1* mRNA accumulation is 2.9-fold ± 0.5-fold higher in *upf1*Δ cells compared to that in *UPF1* cells.

***ADR1* mRNA accumulation depends on the proteins required for NMD.** Initiation of nonsense mRNA decay depends on Upf1p, Upf2p, and Upf3p. Single, double, and triple deletions of the *UPF* genes have essentially identical effects on nonsense mRNA accumulation (3, 13). *ADR1* mRNA accumulation depends on Upf1p (Fig. 3). We tested whether ADR1 mRNA accumulation also depends on Upf2p and Upf3p by measuring steady-state *ADR1* mRNA levels in *UPF*, *upf1*Δ, *upf2*Δ, and *upf3*Δ yeast strains (Fig. 6A). The steady-state *ADR1* mRNA levels were 2.1-fold ± 0.1-fold and 1.8-fold ± 0.4-fold higher in *upf2*Δ and *upf3*Δ yeast cells relative to those in *UPF* yeast cells. The increase in steady-state *ADR1* mRNA levels observed in *upf2*Δ and *upf3*Δ yeast cells is similar to the increase in steady-state *ADR1* mRNA levels observed in *upf1*Δ yeast cells (2.1 ± 0.4). Thus, *ADR1* mRNA accumulation is dependent on Upf2p and Upf3p, as well as Upf1p.

Nonsense mRNAs are degraded by deadenylation-independent decapping, followed by 5′→3′ decay (32). Dcp1p and Xrn1p are required for decapping and 5′→3′ decay, respectively (5, 32). To determine if decapping and 5′→3′ decay are also required for *ADR1* mRNA decay, we examined steady-state *ADR1* mRNA levels in isogenic wild-type, *dcp1*Δ, and *xrn1*Δ yeast strains (Fig. 6B). Steady-state *ADR1* mRNA levels were 5.2-fold ± 2.4-fold and 4.9-fold ± 2.9-fold higher in *dcp1*Δ and *xrn1*Δ cells, respectively, relative to wild-type cells. Thus, *ADR1* mRNA accumulation also depends on these same decay activities because *ADR1* mRNA accumulates in a decapping mutant and a 5′→3′ exoribonuclease mutant.

Accumulation of *ADR1* mRNA following arrest of RNA polymerase II transcription. *ADR1* mRNA has two features that could target this mRNA for NMD. First, the *ADR1* start codon is located in a suboptimal context for initiation of translation and it is followed by an out-of-frame AUG in the optimal context at +83 with respect to the first base of the ORF. Second, *ADR1* mRNA has an unusually long 3′ untranslated region (UTR) (420, 590, and 810 nucleotides; 7). A suboptimal start codon context predisposes an mRNA for leaky scanning of the ribosome past the translation initiation codon. NMD is then triggered when termination occurs following initiation of translation at a downstream, out-of-frame AUG (41). The *S. cerevisiae* optimal start codon context is ANNAUGPuPuPu,

TABLE 4. Average *n*-fold increases in mRNAs transcribed from promoters previously shown to bind Adr1 (42)[a]

Standard ADR1-regulated gene name (alias[es])	Systematic name	Gene product	Avg *n*-fold increase (*P* value)[a]
ADH2	YMR303C	Alcohol dehydrogenase II	1.06 (0.55)
ACS1 (*FUN44*)	YAL054C	Acetyl-CoA synthetase	1.39 (0.64)
GUT1	YHL032C	Glycerol kinase	1.5 (0.22)
FDH2	YPL275W[b] YPL276W[b]	NAD^+-dependent formate dehydrogenase	1.3 (0.42) 1.82 (0.49)
CIT3	YPR001W	Citrate synthase	1.98 (0.40)
ICL2	YPR006C	2-Methylisocitrate lyase	1.31 (0.64)
ALD4	YOR374W	Aldehyde dehydrogenase	1.14 (0.86)
POX1 (*FOX1*)	YGL205W	Fatty acyl CoA oxidase	NA
CTA1	YDR256C	Catalase A	1.86 (0.23)
POT1 (*FOX3*, *POX3*)	YIL160C	3-Oxoacyl-CoA thiolase	1.53 (0.62)
ADY2 (*ATO1*)	YCR010C	Transmembrane protein	1.15 (0.57)
GIP2	YER054C	Glc7-interacting protein	1.44 (0.73)
ETR1 (*MRF1*, *MRF1′*)	YBR026C	2-Enoyl thioester reductase	0.95 (0.91)
	YIL057C	Hypothetical ORF	NA
ADR1	YDR216W		1.20 (0.83)

[a] Average *n*-fold increase are from Lelivelt and Culbertson (28). NA, not available. For comparison, the average fold increase and *P* value for *ADR1* mRNA are shown.
[b] YPL275W and YPL276W comprise a continuous ORF in some *S. cerevisiae* strains but not in genomic reference strain S288C.

where N is any base and Pu is an A or a G. The *ADR1* start codon context is ACUAUGGCT. Further, the downstream out-of-frame AUG at +83 in the optimal context for initiation of translation. The average UTR length (5′ plus 3′ UTRs) of yeast mRNAs is 256 nucleotides (16). mRNAs with unusually long 3′ UTRs are substrates for NMD (2, 33). Furthermore, the *ADR1* 3′ UTR contains at least three potential downstream sequence elements (DSEs). DSEs are thought to function with premature translation termination to target mRNAs for NMD (35). Termination of translation upstream of a DSE targets an mRNA for NMD, while termination of translation downstream of a DSE does not. We hypothesized that leaky scanning and/or the unusually long *ADR1* 3′ UTR could make this mRNA an NMD substrate.

To test the possibility that ADR1 mRNA could be a direct target of the NMD pathway, we determined *ADR1* mRNA half-lives in *upf1*Δ and *UPF1* yeast cells. *ADR1* mRNA levels were determined following inhibition of RNA polymerase II. RNA polymerase II was inhibited by shifting *rpb1-1* cells to the nonpermissive temperature or with thiolutin. *rpb1-1* is a temperature-sensitive allele that encodes an RNA polymerase II subunit. As a control, we examined *CYH2* pre-mRNA levels following inhibition of transcription (Fig. 7B; data not shown). Both treatments effectively arrested transcription, judging by the decrease in *CYH2* pre-mRNA levels following arrest. Further, the CYH2 pre-mRNA levels decreased faster in the *UPF1* cells than in the *upf1*Δ cells. This is consistent with previously published work (14).

The pattern of *ADR1* mRNA levels following arrest of transcription is unusual. Initially, an increase in *ADR1* mRNA levels lasting approximately 10 min in *upf1*Δ cells and approximately 15 min in *UPF1* cells was observed. The amount of *ADR1* mRNA then decreases with time. The half-lives are 42.3 ± 12.9 and 9.3 ± 4.0 min in *UPF1* and *upf1*Δ cells, respectively. Interestingly, the pattern of *ADR1* mRNA levels in these experiments was independent of the method used to arrest transcription because the pattern of *ADR1* mRNA levels was the same when transcription was arrested with thiolutin (data not shown). Thus, *ADR1* mRNA appears to actually be degraded faster in *upf1*Δ cells than in *UPF1* cells following arrest of transcription by shifting *rpb1-1* cells to the nonpermissive temperature or by treatment with thiolutin.

Transient inhibition of general mRNA transcription by either genetic or chemical means induces a general stress response (12). As a part of this response, the mRNA levels for a subset of heat shock genes increase. Consistent with this, Adr1 is activated by growth in ethanol, which also induces stress responses (1). To begin to examine the basis for *ADR1* mRNA accumulation in *upf1*Δ cells, we determined the half-life of *ADR1* mRNA expressed from pKD34, which carries P_{GAL10}-*ADR1*-T_{CYC1} in *upf1*Δ and *UPF1* yeast cells (Fig. 7C). The half-lives of the *ADR1* mRNA expressed from this construct

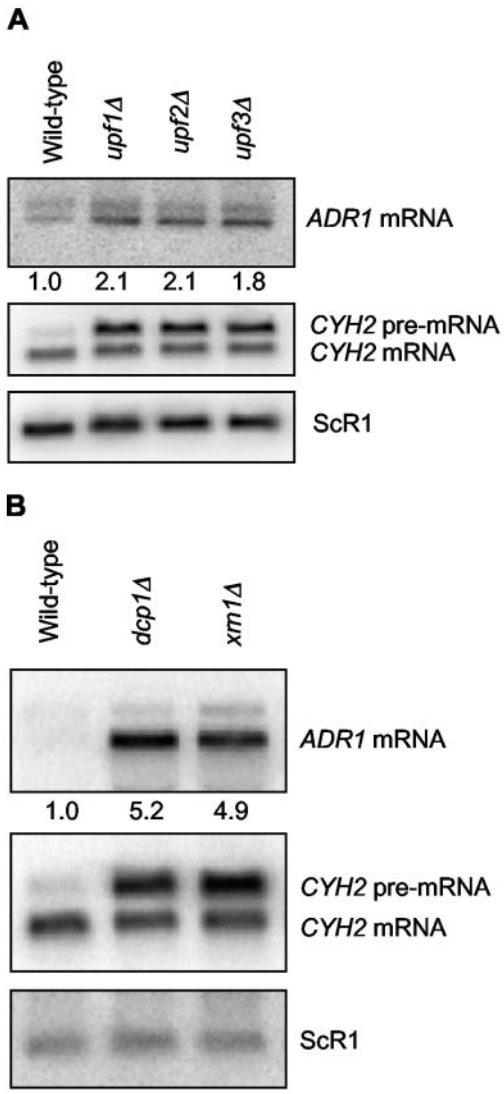

FIG. 6. Wild-type *ADR1* mRNA also accumulates in *upf2*Δ, *upf3*Δ, *dcp1*Δ, and *xrn1*Δ cells. (A) Steady-state *ADR1* mRNA levels in HFY1200 (*UPF1 UPF2 UPF3*; wild type), HFY870 (*upf1*Δ), HFY1300 (*upf2*Δ), and HFY861 (*upf3*Δ) yeast cells grown in YAPD. (B) Steady-state *ADR1* mRNA levels in HFY1200 (wild type), HFY1067 (*dcp1*Δ), and HFY1081 (*xrn1*Δ) cells grown in YAPD. The Northern blots were prepared with total RNA and hybridized with *ADR1*, *CYH2*, and *ScR1* DNA probes. The relative *ADR1* mRNA levels are shown below the corresponding bands.

are 3.8 ± 0.1 and 3.7 ± 0.1 min in *upf1*Δ and *UPF1* yeast cells, respectively. We can eliminate leaky scanning as a mechanism for targeting *ADR1* mRNA for NMD because this mRNA has the same half-life in *upf1*Δ and *UPF1* yeast cells and it contains the *ADR1* translation initiation codon in its native context. However, we cannot distinguish targeting of *ADR1* mRNA for NMD by a long 3′ UTR from an NMD-dependent change in *ADR1* transcription because this mRNA lacks the long *ADR1* 3′ UTR (P_{GAL10}-*ADR1*-T_{CYC1} has 34 bp of sequence downstream of the *ADR1* ORF, followed by the *CYC1* terminator).

Overexpression of *ADR1* results in respiratory impairment. Adr1 regulates genes involved in aerobic oxidation of nonfermentable carbon sources, including lactate (42). *UPF1*, *UPF2*, and *UPF3* are required for full respiratory competence (9). *upf* mutants have a respiratory impairment because they grow poorly on medium containing lactate as a nonfermentable carbon energy source at 18°C. This correspondence suggests that altered Adr1 expression could account for the poor growth of *upf* mutants on lactate-containing medium. We tested this possibility by plating yeast cells overexpressing *ADR1* on *CEN* and 2μ plasmids. As shown in Fig. 8, W303a cells transformed with pMW5 (2μ-*ADR1*) grow slower on complete minimal medium lacking tryptophan and containing lactate than W303a cells transformed with pRS314. The reduced growth rate of W303a(pMW5) was specific for lactate because these transformants grew at the same rate as W303a(pRS314) on complete minimal medium lacking tryptophan and containing glucose. The reduced growth rate of W303a(pMW5) was specific for overexpression of *ADR1* because lactate sensitivity was not seen in Research Genetics strain 3575 (*adr1*Δ). Interestingly, the *upf1*Δ strain (AAY320) grew as well as the isogenic *UPF1* strain (W303a) in these experiments.

DISCUSSION

We have used a bioinformatics-based strategy to investigate a coregulated gene set. We have shown that NMD regulates accumulation of *ADR1* mRNA, which encodes a transcription activator, and that Adr1 is probably a transcription activator of *ATS1*. The NMD-dependent regulation of *ADR1* mRNA is physiologically significant because overexpression of *ADR1* causes respiratory impairment. This strategy is significant because it allows us to classify the genes regulated by NMD into functionally related sets, an important step toward understanding the role NMD plays in the normal functioning of yeast cells. Further, this is a unique way to identify genes regulated by transcription factors.

Adr1 is a transcription activator, and three lines of evidence indicate that it activates expression of *ATS1*. (i) *ATS1* has conserved Adr1 binding sites in its promoter region (Fig. 2), (ii) *ATS1* mRNA levels correlate with *ADR1* expression levels (Fig. 5A), and (iii) conditions that affect Adr1 activity have a corresponding effect on *ATS1* mRNA levels (Fig. 5B). Our results are consistent with a global localization analysis in which intergenic microarrays were probed with DNA from chromatin bound by Adr1. Hybridization to the intergenic region of *ATS1* is 1.544-fold by Adr1-bound DNA relative to background. This binding ratio may underestimate Adr1 binding to the *ATS1* promoter because the putative Adr1 binding sites in the promoter region overlap the *FUN30* ORF. Thus, only the Adr1-bound chromatin fragments that extend into the intergenic region between the *ATS1* and *FUN30* ORFs would hybridize to the DNA on the intergenic microarray. Based on our results and the results of Tachibana et al. (40), we propose that Adr1 activates *ATS1* expression and that activation accounts, at least in part, for the *UPF1*-dependent effect on *ATS1* mRNA accumulation.

Why might Adr1 regulate *ATS1*? In previous studies, Adr1 was shown to bind the promoters for 14 different genes involved in the generation of acetyl-CoA and NADH from nonfermentable substrates (42, 43; Table 4). We showed that Ats1p interacts with Nap1p, a cytoplasmic protein that regu-

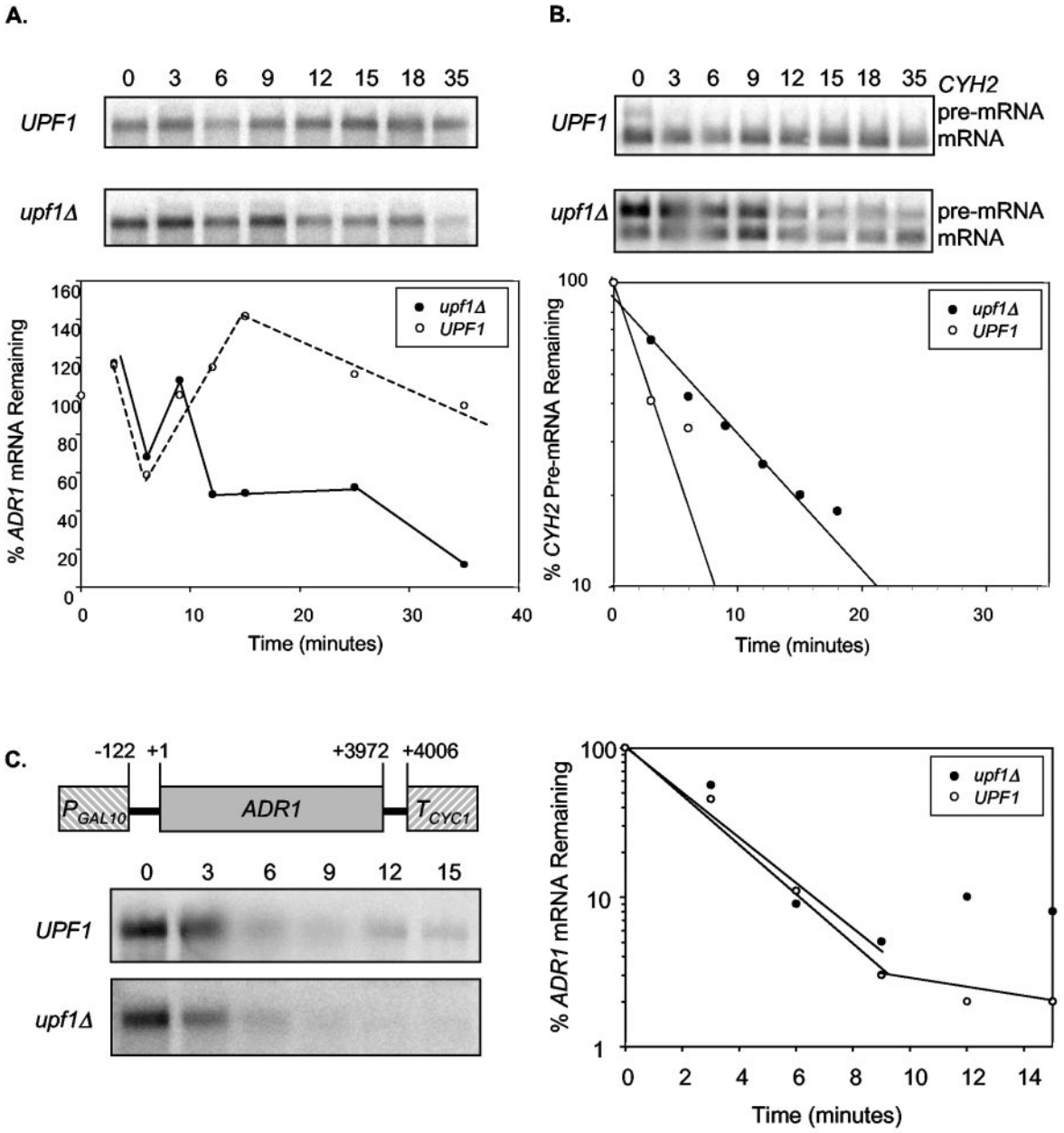

FIG. 7. *ADR1* mRNA accumulation following arrest of transcription. Northern blots were prepared with total RNAs harvested from isogenic yeast strains AAY334 (*UPF1*) and AAY335 (*upf1*Δ) at the indicated time points (minutes) after inhibition of RNA polymerase II. AAY334 and AAY335 carry *rpb1-1*, a temperature-sensitive allele coding for a component of RNA polymerase II. Transcription arrests rapidly in these strains when they are shifted to the nonpermissive temperature. (A, B) PhosphorImages of a representative Northern blot hybridized with radiolabeled *ADR1* DNA (A) and then stripped and reprobed with radiolabeled *CYH2* DNA (B). Essentially identical results were obtained in three independent experiments where transcription was arrested by a temperature shift and in one experiment in which thiolutin was used to arrest transcription. Inhibition of RNA polymerase II was effective in these experiments because *CYH2* pre-mRNA levels decreased as expected following transcription arrest (B). (C) PhosphorImages of a representative Northern blot prepared with RNAs from W303a and AAY320 transformed with pKD34, which carries P_{GAL10}-*ADR1*-T_{CYC1}, and hybridized with radiolabeled *ADR1* DNA. Percent mRNA remaining at each time point following inhibition of transcription was calculated by dividing the amount of probe hybridized to a particular band (corrected for loading with ScR1) by the amount of probe hybridized to the band at time zero. The percent mRNA remaining versus time after transcription inhibition was plotted with SigmaPlot.

lates the activity of the Cdc28p/Clb2p complex (38). Based on these results, we proposed that the interaction between Ats1p and Nap1p coordinates the microtubule state with the cell cycle. Cell size changes during growth in different environments. For instance, cells grown in ethanol are larger than glucose-grown cells (24). The increase in cell size seen in ethanol-grown cells is due to a delay in the cell cycle that is partially mediated by the tyrosine kinase Swe1, a negative regulator of the Cdc28-Clb complexes (24). Since Ats1p regulates the activity of the same complex, we propose that Adr1

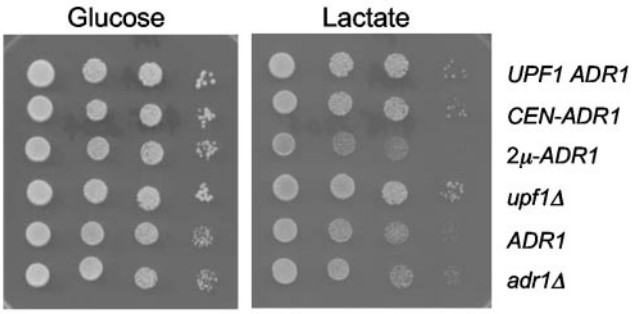

FIG. 8. Overexpression of *ADR1* causes respiratory impairment. The yeast strains used were W303a (*UPF1 ADR1*) transformed with pRS314 (vector control), pRS314ADR1 (*CEN-ADR1*), and pMW5 (2μ-*ADR1*); AAY320 (*upf1*Δ) transformed with pRS314; BY4741 (*ADR1*); and Research Genetics strain 3575 (*adr1*Δ in BY4741). The strains were grown to an optical density at 260 nm of 0.4 to 0.6; diluted 10^0, 10^{-1}, 10^{-2}, and 10^{-3}; spotted onto complete minimal medium lacking tryptophan and containing either glucose (left panel) or lactate (right panel); and incubated at 18°C.

regulates *ATS1* as part of a mechanism to coordinate the cell cycle with the metabolic status of the cell. If this is the case, we predict that changes in *ATS1* expression will have an effect on cell size. Consistent with this prediction, deletion of *ATS1* results in larger cells (23, 38).

Here we have shown that *ADR1* mRNA accumulation is regulated by NMD (Fig. 6). The NMD-dependent regulation of *ADR1* mRNA is physiologically relevant because overexpression of *ADR1* mRNA may partially contribute to the respiratory impairment of *upf* mutants. We show that overexpression of *ADR1* from a 2μ plasmid causes respiratory impairment (Fig. 8). Overexpression of *ADR1* may only partially explain the respiratory impairment of *upf* mutants because *ADR1* mRNA accumulation is 2.6-fold ± 0.2-fold in a *upf1*Δ strain (Fig. 3B) and we did not see respiratory impairment until *ADR1* was overexpressed from a 2μ plasmid where *ADR1* mRNA levels were elevated 9.9-fold ± 3.8-fold (data not shown). Alternatively, deletion of the *upf* genes in the strains used by de Pinto et al. (9) may have a more significant impact on *ADR1* expression than in the W303a background. We have previously observed strain-dependent differences in the accumulation of mRNAs degraded by the NMD pathway (19). Future experiments will focus on determining whether *ADR1* regulation by NMD is direct or indirect.

Our strategy complements existing microarray analyses of the effects of inactivation of NMD on global mRNA abundance in two ways. First, it allows us to sort the mRNAs affected by NMD into physiologically relevant, coregulated gene sets. Second, it uncovers low-abundance mRNAs at or below the threshold of detection on microarrays. For example, *PPR1* mRNA is near the threshold of detection on microarrays (28). Our approach is applicable to other coregulated gene sets. Our strategy is limited to transcription factors with known DNA binding sites. As binding sites are identified for additional transcription factors, we expect to be able to identify additional NMD-regulated gene sets.

Several lines of evidence suggest that a role for NMD in the regulation of decay of select wild-type mRNAs is not unique to *S. cerevisiae*. Upf1 is essential for mammalian embryonic viability (30). NMD-deficient mouse embryos do not develop; instead, they are resorbed shortly after implantation and NMD-deficient blastocysts isolated at 3.5 days postcoitum commit apoptosis after a brief period of growth. And in *Caenorhabditis elegans*, NMD deficiency causes minor morphogenic abnormalities of the genitalia and reduced brood size (36). These effects probably reflect the failure both to rid the cells of mRNAs with premature termination codons and to down regulate natural substrates. As is seen in yeast, a significant percentage (4.9%) of physiologic transcripts are up-regulated mammalian cells depleted of Upf1 or Upf2 accumulate (31). A representative subset of the up-regulated transcripts with potential structural features that could cause premature termination of translation had longer half-lives in Upf1-depleted cells. Furthermore, recently Kim et al. (22) showed that mammalian *Arf* mRNA decay is dependent on Upf1 and Stau1, but not Upf2 or Upf3X. Stau1 binds the 3′ UTR of *Arf1* mRNA and reduces its abundance. This suggests at least that mammalian Upf1 also functions in decay of wild-type mRNAs that lack an apparent premature termination codon, a substrate reminiscent of the yeast *PPR1* mRNA.

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