Article

YRA1 Autoregulation Requires Nuclear Export and Cytoplasmic Edc3p-Mediated Degradation of Its Pre-mRNA

Shuyun Dong,[1] Chunfang Li,[1] Daniel Zenklusen,[2] Robert H. Singer,[2] Allan Jacobson,[1] and Feng He[1,*]
[1] Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, MA 01655, USA
[2] Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461, USA
*Correspondence: feng.he@umassmed.edu
DOI 10.1016/j.molcel.2007.01.012

SUMMARY

Autoregulatory loops often provide precise control of the level of expression of specific genes that encode key regulatory proteins. Here we have defined a pathway by which Yra1p, a yeast mRNA export factor, controls its own expression. We show that *YRA1* exon 1 sequences in *cis* and Yra1p in *trans* inhibit *YRA1* pre-mRNA splicing and commit the pre-mRNA to nuclear export. Mex67p and Crm1p jointly promote *YRA1* pre-mRNA export, and once in the cytoplasm, the pre-mRNA is degraded by a 5′ to 3′ decay mechanism that is dependent on the decapping activator Edc3p and on specific sequences in the *YRA1* intron. These results illustrate how common steps in the nuclear processing, export, and degradation of a transcript can be uniquely combined to control the expression of a specific gene and suggest that Edc3p-mediated decay may have additional regulatory functions in eukaryotic cells.

INTRODUCTION

mRNA degradation, an integral event in gene expression, provides posttranscriptional titration of transcript levels and ensures the elimination of aberrant mRNAs or those that have exited the translation-competent state (Parker and Song, 2004). Two general and functionally redundant mechanisms, the deadenylation-dependent 5′ to 3′ pathway and the exosome-mediated 3′ to 5′ pathway, are responsible for the decay of most mRNAs in yeast (Coller and Parker, 2004). Subsequent to poly(A) shortening, substrates of the 5′ to 3′ pathway are decapped by the Dcp1p/Dcp2p decapping enzyme complex and then digested exonucleolytically by the 5′ to 3′ exoribonuclease, Xrn1p (Coller and Parker, 2004), whereas those subject to 3′ to 5′ decay are deadenylated and then degraded by the

ten-subunit exosome complex of 3′ to 5′ exonucleases (Mitchell et al., 1997). Components of both pathways are also utilized by several cytoplasmic translation-dependent mRNA surveillance mechanisms that target mRNAs containing premature termination codons (nonsense-mediated mRNA decay [NMD]), mRNAs lacking translational termination codons (nonstop decay [NSD]), and mRNAs impaired in translational elongation (no-go decay [NGD]) (Doma and Parker, 2006; Frischmeyer et al., 2002; Jacobson and Peltz, 1996; van Hoof et al., 2002).

Almost all mRNAs subject to 5′ to 3′ decay, whether normal or aberrant, are shunted into the pathway as a consequence of specific decapping activators (Coller and Parker, 2004). For example, the decapping of conventional mRNAs is promoted by Pat1p, Dhh1p, and the Lsm1p-7p complex (Coller and Parker, 2004), and Upf1p, Nmd2p/Upf2p, and Upf3p serve a similar function for NMD substrates (He and Jacobson, 2001). The precise mechanism of action of these decapping activators has yet to be elucidated, but it is thought that they promote both departure from the translation pathway and recruitment of the Dcp1p/Dcp2p complex (Coller and Parker, 2004; Coller and Parker, 2005). Additional potential regulators of decapping have been identified either as high-copy suppressors of in vivo decapping defects caused by mutations in the *DCP1* and *DCP2* genes or as factors that interact with the decapping enzyme (Dunckley et al., 2001; Kshirsagar and Parker, 2004). These regulators include Edc1p, Edc2p, and Edc3p. Interestingly, depletion of each these factors does not affect general decapping rates of wild-type mRNAs or the decapping rates of nonsense-containing mRNAs (Dunckley et al., 2001; Kshirsagar and Parker, 2004), suggesting that these factors may be required for the decapping of specific mRNAs.

Cytoplasmic mRNA decay pathways can also play a role in the degradation of intron-containing pre-mRNAs. Normally, such pre-mRNAs are retained in the nucleus to be processed to mRNAs and are rarely exported to the cytoplasm. Pre-mRNAs that fail to complete splicing or 3′ end formation are usually degraded by the nuclear exosome (Bousquet-Antonelli et al., 2000; Torchet et al., 2002). However, *cis* mutations in the 5′ splice site or the

branchpoint region or *trans* mutations in several splicing factors (including Prp6p, Prp9p, and the U1 snRNA), or the Mlp1p nuclear retention factor, result in the export of pre-mRNAs to the cytoplasm (Galy et al., 2004; Hilleren and Parker, 2003; Legrain and Rosbash, 1989; Rain and Legrain, 1997). As shown by our previous studies, pre-mRNAs that escape the nuclear retention system and enter the cytoplasm are usually recognized by ribosomes and degraded in a translation-dependent manner by the NMD pathway (He et al., 1993; Vilardell et al., 2000).

We have been using microarray analysis to better understand the function of the cytoplasmic mRNA decay pathways (He et al., 2003). Application of this methodology to the NMD and 5′ to 3′ decay pathways identified endogenous substrates of the respective pathways and provided insights into the determinants of substrate status (He et al., 2003). Here, we report that comparable delineation of the substrates of Edc3p identified a specific cytoplasmic decay pathway involved in the degradation of intron-containing *YRA1* pre-mRNA. Subsequent investigation of the means by which *YRA1* pre-mRNA is actively exported to the cytoplasm revealed a complex autoregulatory network that involves splicing inhibition, Crm1p-mediated nuclear export, and Edc3p-mediated decapping of the pre-mRNA.

RESULTS

Deletion of *EDC3* Selectively Stabilizes *YRA1* Pre-mRNA

To assess the role of Edc3p in mRNA decay, we utilized high-density oligonucleotide microarrays to analyze the effect of *EDC3* deletion on global RNA accumulation. Five independent expression profiling experiments with *EDC3* and *edc3Δ* strains indicated that, of 7839 potential transcripts analyzed, only two were differentially expressed (see the Supplemental Data available with this article online), and both showed increased levels in the *edc3Δ* strain. One transcript, from the *RPS28B* gene, codes for a 40S ribosomal protein (Lecompte et al., 2002), and the other, from the *YRA1* gene, codes for an hnRNP-like protein (Yra1p) involved in an early stage of mRNA export (Portman et al., 1997; Strasser and Hurt, 2000). Significantly, the *RPS28B* transcript, identified in a similar screen as the sole differentially expressed mRNA in *edc3Δ* cells, has been shown to be degraded through an Ecd3p-mediated mRNA decay pathway (Badis et al., 2004). Thus, in this study, we focused our analysis on the *YRA1* transcript(s).

The *YRA1* gene contains an intron in the middle of its coding region and has the potential to produce an intron containing pre-mRNA and a mature mRNA. To validate our microarray data and to identify the RNA species affected by deleting *EDC3*, we examined the steady-state levels of the *YRA1* transcripts in *EDC3* and *edc3Δ* strains. As shown in Figure 1A, deletion of *EDC3* had no effect on the level of *YRA1* mRNA but resulted in a 5-fold increase in
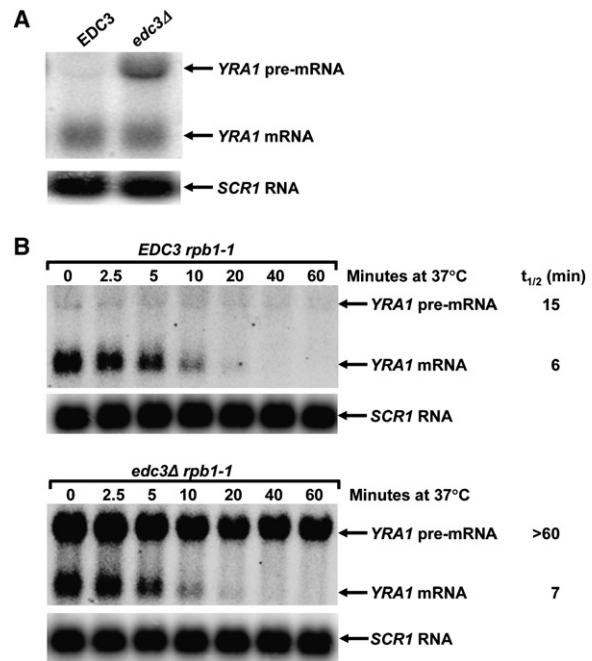

Figure 1. *YRA1* Pre-mRNA Is Selectively Stabilized in *edc3Δ* Cells

(A) Northern analysis of *YRA1* pre-mRNA and mRNA levels in *EDC3* and *edc3Δ* cells.

(B) Determination of *YRA1* pre-mRNA and mRNA half-lives. Cultures of wild-type and *edc3Δ* cells harboring the *rpb1-1* allele were shifted from 25°C to 37°C to inactivate transcription by RNA polymerase II, and samples were taken for northern analysis at different times after the shift.

In (A) and (B), blots were hybridized with probes complementary to *YRA1* and *SCR1* transcripts, with the latter serving as a loading control.

YRA1 pre-mRNA. As controls, we found that deletion of *EDC3* did not affect the levels of the intron-lacking *CYH2* and *PGK1* mRNAs or the intron-containing *CYH2* and *DBP2* pre-mRNAs (data not shown).

To determine whether Edc3p plays a direct role in *YRA1* pre-mRNA degradation, we monitored *YRA1* pre-mRNA decay kinetics subsequent to inhibiting transcription. This analysis revealed that the *YRA1* pre-mRNA has a half-life of >60 min in *edc3Δ* cells and ~15 min in *EDC3* cells (Figure 1B). In contrast, deletion of *EDC3* did not alter the decay rate of the *YRA1* mRNA ($t_{1/2}$ ~6 min). Deletion of *EDC3* also did not alter the decay rates of the *CYH2*, *PGK1*, and *RPS28A* mRNAs (data not shown). Taken together, these results indicate that Edc3p directly controls *YRA1* pre-mRNA degradation.

YRA1 Pre-mRNA Is Degraded through a 5′ to 3′ Decay Mechanism

To elucidate the mechanism of *YRA1* pre-mRNA decay, we analyzed its level in strains containing deletions of genes encoding well-characterized factors involved in deadenylation, the general 5′ to 3′ or 3′ to 5′ decay
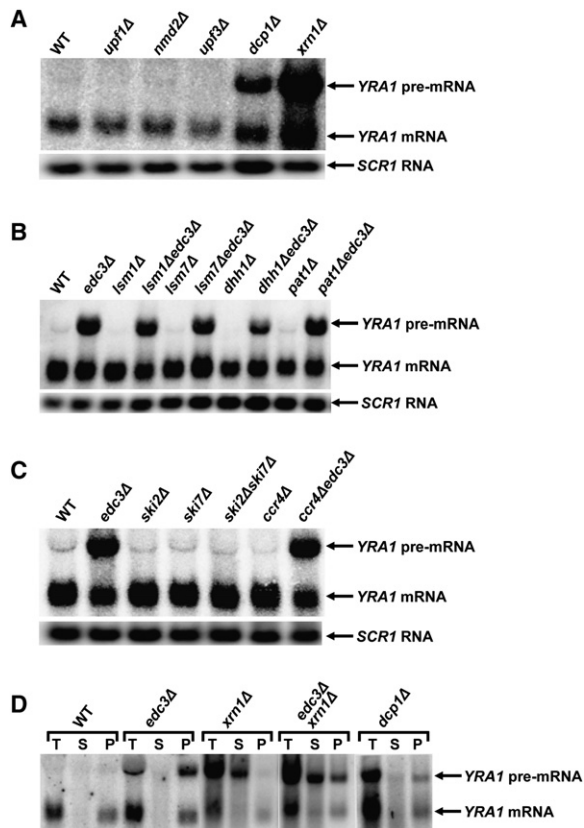
Figure 2. *YRA1* **Pre-mRNA Is Degraded by Deadenylation-Independent Decapping and 5′ to 3′ Exonucleolytic Digestion**

(A–C) Northern analysis of *YRA1* pre-mRNA and mRNA in yeast strains containing deletions of genes encoding (A) the principal NMD factors, a subunit of the decapping enzyme, or the 5′ to 3′ exoribonuclease; (B) decapping activators of the general 5′ to 3′ mRNA decay pathway; or (C) components of the cytoplasmic exosome or the major cytoplasmic deadenylase complex. Blots were hybridized to a *SCR1* probe to serve as a loading control.

(D) Analysis of the 5′ cap status of the *YRA1* pre-mRNA by anti-m⁷G immunoprecipitation and northern blotting. T, total input RNA; S, supernatant (uncapped); P, pellet (capped).

pathways, or the NMD pathway. Among these factors, only deletion of the genes encoding Dcp1p, a component of the decapping enzyme, and Xrn1p, the cytoplasmic 5′ to 3′ exoribonuclease, affected *YRA1* pre-mRNA levels (Figure 2A). Compared to the level in wild-type cells, deletion of these two genes resulted in 5- to 10-fold increases in *YRA1* pre-mRNA levels. In contrast, deletion of the genes encoding all the other factors, including the major cytoplasmic deadenylase, Ccr4p (Tucker et al., 2002); the exosome components, Ski2p and Ski7p; the decapping activators, Pat1p, Dhh1p, Lsm1p, and Lsm7p; and the NMD factors, Upf1p, Nmd2p, and Upf3p, had no effect on *YRA1* pre-mRNA accumulation (Figures 2A–2C). These results indicate that *YRA1* pre-mRNA is degraded by a 5′ to 3′ mechanism that requires decapping by

Dcp1p and Dcp2p and 5′ to 3′ exonucleolytic digestion by Xrn1p.

Edc3p-Mediated *YRA1* Pre-mRNA Degradation Occurs in the Cytoplasm

The observation that *YRA1* pre-mRNA degradation requires Dcp1p and Xrn1p, two cytoplasmic factors, strongly suggests that Edc3p-mediated degradation of *YRA1* pre-mRNA occurs in the cytoplasm. To test this hypothesis, we assessed the subcellular localization and levels of *YRA1* pre-mRNA in wild-type, *edc3Δ*, *dcp1Δ*, and *xrn1Δ* strains by fluorescent in situ hybridization (FISH) analysis. We utilized sets of Cy3- and Cy5-labeled oligonucleotide probes to detect *YRA1* intron and exon sequences, respectively. In wild-type cells, the exon signal was detected in the nucleus and cytoplasm, whereas the intron signal was mainly detected in the nucleus, colocalizing with the exon signal and likely reflecting nascent *YRA1* transcripts (Figures 3A–3C). Compared to the *YRA1* exon and intron signals in wild-type cells, *edc3Δ*, *xrn1Δ*, *dcp1Δ*, and *edc3Δxrn1Δ* cells all showed a significant increase in both the exon and intron signals in the cytoplasm. In addition, the cytoplasmic intron signals in these cells colocalized largely with the exon signals (Figures 3E–3T). Interestingly, *dcp1Δ* cells displayed a local enrichment of *YRA1* pre-mRNA in cytoplasmic dots, suggesting that *YRA1* pre-mRNA may enter P bodies but fail to be degraded due to the lack of Dcp1p (Figures 3Q–3T). These data demonstrate that *YRA1* pre-mRNA degradation occurs after the transcript has been exported to the cytoplasm and that, when degradation is inhibited by deleting *EDC3*, *DCP1*, or *XRN1*, *YRA1* pre-mRNA accumulates in the cytoplasm.

We also analyzed the effects of inactivation or deletion of nuclear decay factors on *YRA1* pre-mRNA accumulation. We found that neither inactivation of the 5′ to 3′ exoribonuclease, Rat1p, an essential component of the nuclear 5′ to 3′ decay pathway (Bousquet-Antonelli et al., 2000), nor deletion of the gene encoding Rrp6p, a component of the nuclear exosome involved in the nuclear 3′ to 5′ decay pathway (Bousquet-Antonelli et al., 2000), affected *YRA1* pre-mRNA levels (Figure S1). Taken together, these results indicate that Edc3p-mediated *YRA1* pre-mRNA degradation occurs in the cytoplasm.

Edc3p Activates but Does Not Catalyze Decapping of the *YRA1* Pre-mRNA

To define the functional role of Edc3p in *YRA1* pre-mRNA decay, we analyzed *YRA1* pre-mRNA cap status. As shown in Figure 2D, *YRA1* pre-mRNAs that accumulate in *edc3Δ* and *dcp1Δ* cells are essentially all in the capped fraction. In contrast, pre-mRNA transcripts that accumulate in *xrn1Δ* cells are essentially all in the uncapped fraction. However, the pre-mRNAs that accumulate in *edc3Δxrn1Δ* cells contain both capped (~40%) and uncapped (~60%) species, demonstrating that deletion of *EDC3* inhibits but does not eliminate decapping and that Edc3p activates but does not catalyze decapping of *YRA1* pre-mRNA.
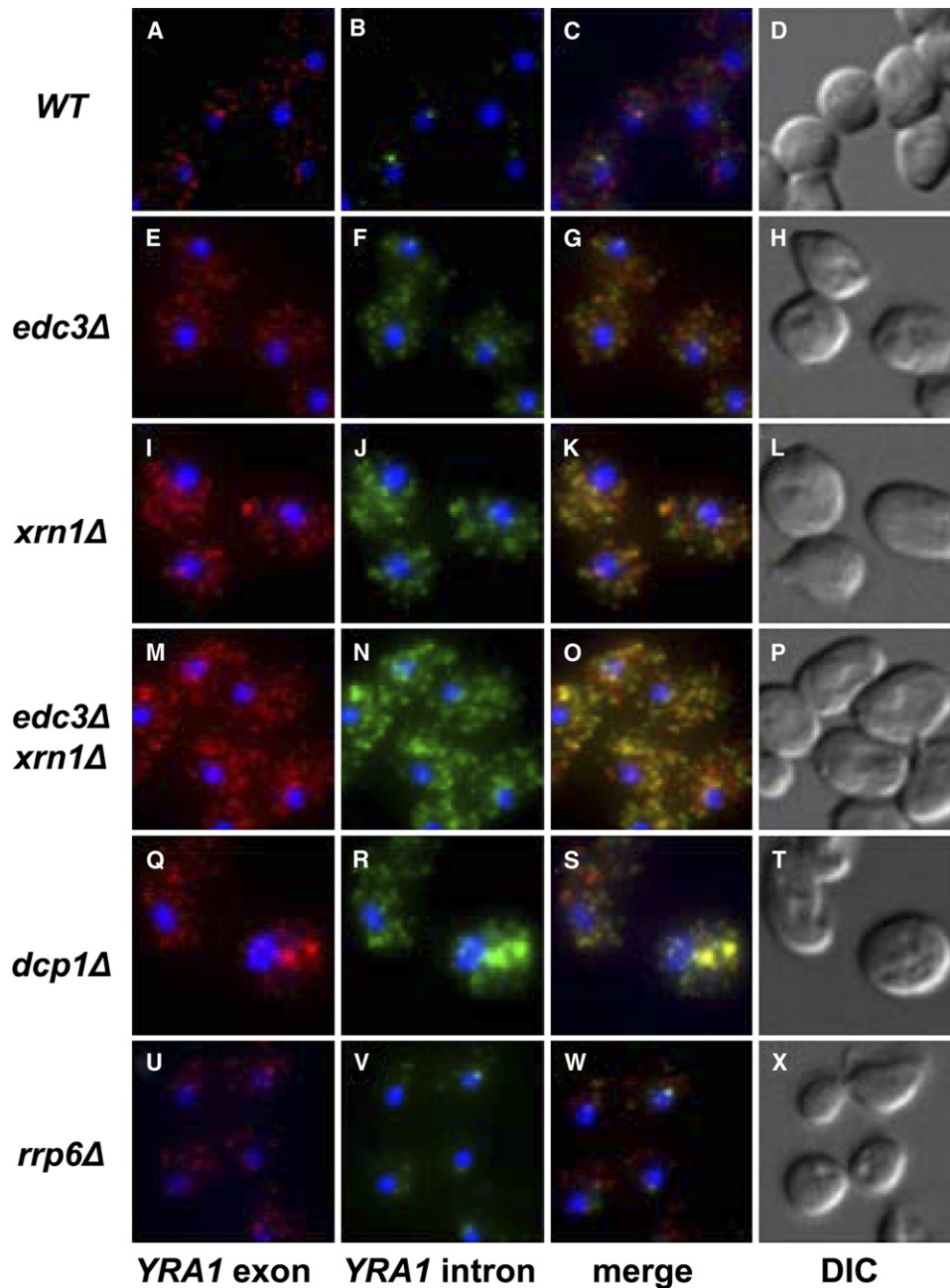
Figure 3. Edc3p-Mediated *YRA1* Pre-mRNA Degradation Occurs in the Cytoplasm

Localization of *YRA1* pre-mRNA in wild-type, *edc3Δ*, *xrn1Δ*, *edc3Δxrn1Δ*, *dcp1Δ*, and *rrp6Δ* strains by in situ hybridization (FISH). Intron probes were labeled with Cy3 (green), and exon probes were labeled with Cy5 (red). DAPI labeling was used to identify the position of the nucleus. The corresponding merged and phase-contrast images are shown on the right side of the figure.

Edc3p-Mediated Degradation of *YRA1* Pre-mRNA Is a Component of an Autoregulatory Negative Feedback Loop

Previous studies indicated that *YRA1* regulates its own expression through a negative feedback loop and that this autoregulation requires the *YRA1* intron (Preker et al., 2002; Rodriguez-Navarro et al., 2002). Our obser-

vation that Edc3p is required for *YRA1* pre-mRNA degradation raised the possibility that Edc3p plays a role in *YRA1* autoregulation. To test this notion, we examined the effects of increasing *YRA1* gene copy number on the levels of its pre-mRNA, mRNA, and protein in wild-type and *edc3Δ* strains. As shown in Figure 4A, introduction of extra copies of the intron-containing *YRA1*
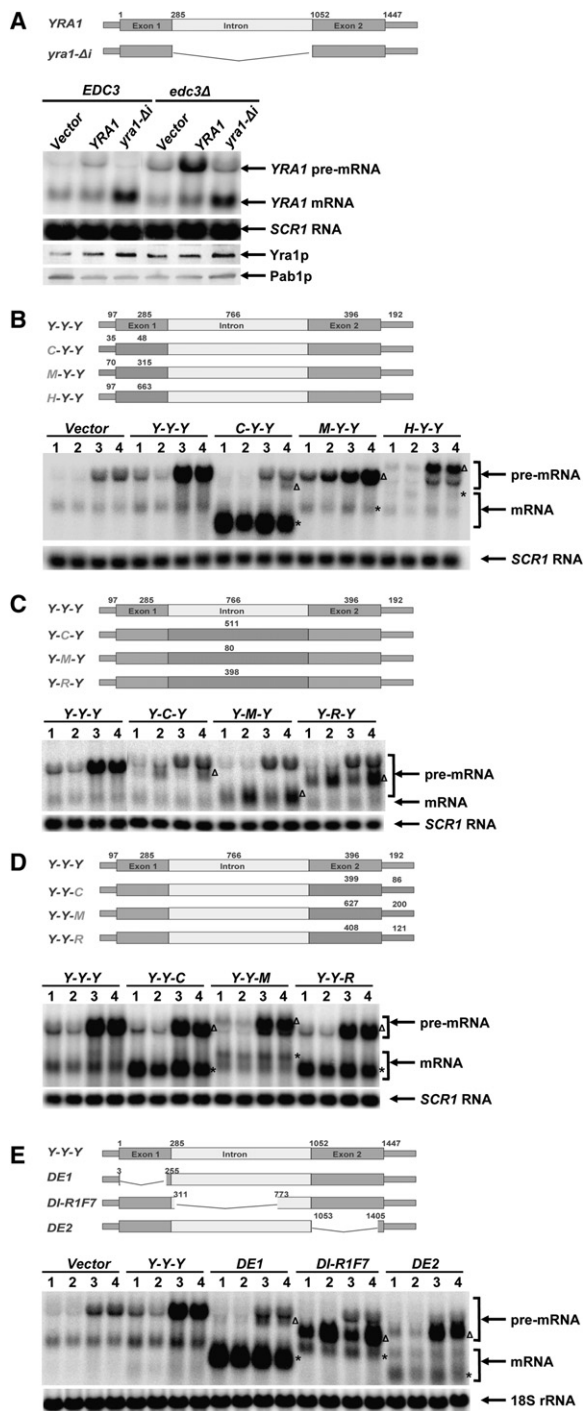
Figure 4. YRA1 Autoregulation Requires Edc3p and Two Distinct Regulatory Elements Localized in Exon 1 and the Intron of YRA1 Pre-mRNA

(A) The effects of overexpressing intron-containing or intron-lacking YRA1 alleles on the levels of YRA1 pre-mRNA, mRNA, and Yra1p in EDC3 and edc3Δ strains.

(B–D) Effects of replacing YRA1 exon 1 (B), intron (C), and exon 2 (D) with different sequences on YRA1 pre-mRNA and mRNA expression. A three-letter code was used to denote the order of exon 1, intron,

allele altered neither YRA1 mRNA levels nor Yra1p levels in EDC3 and edc3Δ strains. Interestingly, introduction of extra copies of YRA1 differentially affected YRA1 pre-mRNA levels, resulting in only a 2-fold increase in the EDC3 strain but a 24-fold increase in the edc3Δ strain. In contrast to the intron-containing YRA1 allele, introduction of extra copies of an intron-lacking YRA1 allele increased the levels of YRA1 mRNA 4-fold and Yra1p 3-fold in both the EDC3 and edc3Δ strains. These results confirm that Yra1p negatively regulates its own level of expression (Preker et al., 2002; Rodriguez-Navarro et al., 2002) and further indicate that Edc3p-mediated YRA1 pre-mRNA degradation is a component of this negative feedback loop. Since Edc3p-mediated YRA1 pre-mRNA degradation occurs in the cytoplasm (see above), this result also suggests that Yra1p regulates its expression by inhibiting YRA1 pre-mRNA splicing and effecting or promoting pre-mRNA nuclear export.

Yra1p Autoregulation Requires Two Functionally Distinct cis-Regulatory Elements

As described above, YRA1 autoregulation likely involves splicing inhibition, nuclear export, and cytoplasmic degradation of YRA1 pre-mRNA. To identify the cis-regulatory elements involved in these functions, we constructed chimeric RNAs encompassing segments of the YRA1 transcript and other non-Edc3p substrate RNAs and examined their decay phenotypes in wild-type, upf1Δ, edc3Δ, and upf1Δedc3Δ strains. We included the upf1Δ strains in this analysis because we speculated that chimeric RNAs that fail to autoregulate are likely to be degraded by the NMD pathway. YRA1 exon 1, intron, and exon 2 sequences were replaced with the corresponding parts of the CYH2, MER2, and RPS51A pre-mRNAs, three transcripts that differ in splicing efficiency (during vegetative growth, the CYH2 and MER2 pre-mRNAs are inefficiently spliced, but the RPS51A pre-mRNA is efficiently spliced; He et al., 1993). In addition, we also replaced YRA1 exon 1 with the HIS3 coding sequence. Analyses of the steady-state levels of these chimeric pre-mRNAs and their spliced products in wild-type, upf1Δ, edc3Δ, and upf1Δedc3Δ strains led to several important observations.

and exon 2 of each chimeric construct, with the first letter of each of these genes specifying its origin. Y, YRA1; C, CYH2; M, MER2; R, RPS51A; and H, HIS3.

(E) Effects of internal deletions in YRA1 exon 1, intron, or exon 2 on YRA1 pre-mRNA and mRNA expression.

In (B)–(E), YCp low-copy plasmids harboring the wild-type YRA1 gene, or a chimeric or deletion allele (depicted above the corresponding blots), were introduced into wild-type (1), upf1Δ (2), edc3Δ (3), or upf1Δ edc3Δ (4) strains, and the levels of the respective pre-mRNAs and mRNAs encoded by these alleles were analyzed by northern blotting. The positions of chimeric or mutant pre-mRNAs and mRNAs are marked by Δ and *, respectively. Northern blots were hybridized to a SCR1 or an 18S rRNA probe to serve as loading controls. Pab1p served as a loading control for the western blot in (A).

First, replacement of *YRA1* exon 1 with the *CYH2* or *MER2* exon 1 or the *HIS3* coding sequence differentially affected pre-mRNA splicing efficiency. Substitution of *YRA1* exon 1 with the shorter *CYH2* exon 1 (285 nt versus 48 nt) dramatically increased the splicing efficiency of the pre-mRNA, as negligible C-Y-Y pre-mRNA signals were detected in the wild-type, *upf1Δ*, *edc3Δ*, and *upf1Δedc3Δ* strains but high levels of mRNA accumulated in these strains (Figure 4B). In contrast, substitution of *YRA1* exon 1 by the comparably sized *MER2* exon 1 (315 nt), or by a longer *HIS3* coding region (633 nt), did not improve the splicing efficiency of the pre-mRNA. Interestingly, like wild-type *YRA1* pre-mRNA, both the M-Y-Y and H-Y-Y pre-mRNAs accumulated to high levels in *edc3Δ* strains (Figure 4B and Figure S4). Second, replacement of the *YRA1* intron with the *CYH2*, *MER2*, or *RPS51A* intron did not alter pre-mRNA splicing efficiency but in each case resulted in a pre-mRNA that is insensitive to Edc3p but sensitive to Upf1p (Figure 4C and Figure S4). Third, replacement of *YRA1* exon 2 with the *CYH2*, *MER2*, or *RPS51A* exon 2 did not alter pre-mRNA splicing efficiency and in each case resulted in a pre-mRNA that behaved the same as the wild-type *YRA1* pre-mRNA (Figure 4D and Figure S4). We also noted that the levels of mRNAs generated from the Y-Y-C and Y-Y-R pre-mRNAs were greatly increased in all four yeast strains (Figure 4D). This is most likely due to increased stability of these chimeric mRNAs. These results indicate that sequences in exon 1 and the intron of the *YRA1* pre-mRNA are required for Yra1p autoregulation and that these sequences have distinct functions. Sequences in exon 1 inhibit *YRA1* pre-mRNA splicing and effect or promote nuclear export of the pre-mRNA, while sequences in the intron dictate the substrate specificity for Edc3p-mediated decay. Consistent with these conclusions, deletion analysis of *YRA1* pre-mRNA showed that (1) a 252 nt internal deletion in exon 1 promoted more efficient splicing of the *YRA1* pre-mRNA, (2) a 462 nt internal deletion in the intron did not significantly improve the splicing efficiency but resulted in a pre-mRNA that is degraded by NMD, and (3) a 352 nt internal deletion in exon 2 did not affect *YRA1* autoregulation and resulted in a pre-mRNA that behaved the same as wild-type *YRA1* pre-mRNA (Figure 4E).
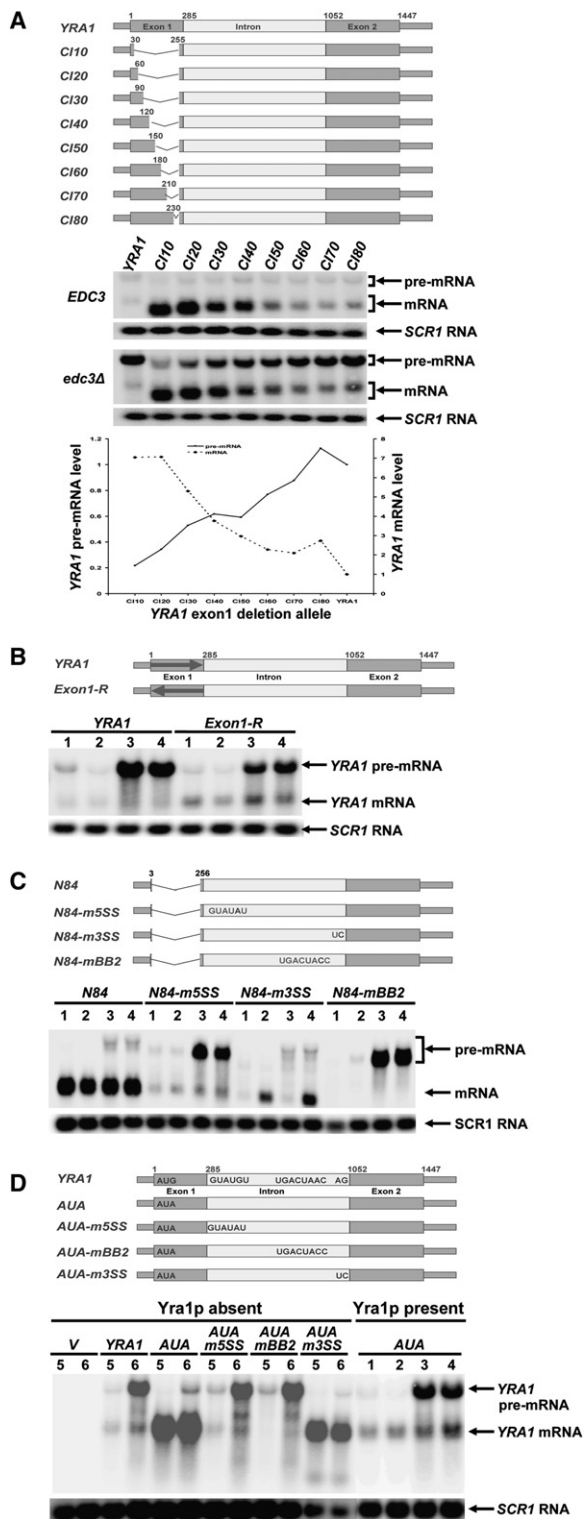
YRA1 Exon 1 Sequences Inhibit Its Pre-mRNA Splicing in a Size-Dependent but Sequence-Independent Manner

Since *YRA1* exon 1 appeared to regulate its pre-mRNA splicing through a size-dependent but sequence-independent mechanism (see above), we analyzed the effects of shortening *YRA1* exon 1 or replacing exon 1 coding sequences with their complementary sequences. This analysis revealed that incremental deletions of exon 1 resulted in incremental increases in *YRA1* mRNA levels in *EDC3* and *edc3Δ* strains (Figure 5A). Importantly, the incremental increases in *YRA1* mRNA levels in both strains were all accompanied by corresponding decreases in *YRA1* pre-

mRNA levels in the *edc3Δ* strain (Figure 5A). These data show that *YRA1* exon 1 functions to inhibit pre-mRNA splicing in *cis*.

Figure 5A also reveals that the splicing efficiency of mutant pre-mRNAs correlates positively with the size of deletions and thus negatively with the size of the remaining exon 1, suggesting that the inhibitory function of exon 1 on *YRA1* pre-mRNA splicing is dictated by its length. Replacing the *YRA1* exon 1 coding sequence with its complementary sequence resulted in a pre-mRNA that behaved very similarly to the wild-type pre-mRNA; i.e., it was inefficiently spliced and degraded by the Edc3p-mediated decay pathway (Figure 5B). Since the complementary sequences of exon 1 function as well as the coding sequences, the ability of *YRA1* exon 1 to inhibit its pre-mRNA splicing must be dictated by its size, but not its primary sequence.

Our analysis of exon 1 deletion mutants in the *edc3Δ* strain also revealed an inverse correlation between the levels of *YRA1* pre-mRNA and mRNA (Figure 5A). This observation, combined with the fact that Edc3p-mediated *YRA1* pre-mRNA degradation occurs in the cytoplasm, suggests that *YRA1* pre-mRNA splicing and nuclear export are functionally linked and compete for common substrates. One explanation for this effect is that exon 1 functions primarily to inhibit pre-mRNA splicing in *cis* and thus results in nuclear export of the pre-mRNA. Alternatively, *YRA1* exon 1 could primarily promote nuclear export of its pre-mRNA and, as a consequence, inhibit pre-mRNA splicing. To distinguish these possibilities, we tested whether *cis* mutations that inhibit splicing can bypass the regulatory function of *YRA1* exon 1. We used *yra1-N84*, a complete loss-of-regulation allele that contains a 252 nt deletion in the coding region of exon 1 and encodes a pre-mRNA that is efficiently spliced, as evidenced by high levels of mRNA but almost no pre-mRNA in wild-type, *upf1Δ*, *edc3Δ*, and *upf1Δedc3Δ* strains (Figure 5C). We found that mutations in the 5' splice site (m5SS) or the branch point region (mBB2), which block splicing prior to the first step of splicing (Jacquier et al., 1985; Parker and Guthrie, 1985), greatly reduced or eliminated splicing of the *yra1-N84* pre-mRNA in wild-type, *upf1Δ*, *edc3Δ*, and *upf1Δedc3Δ* strains (Figure 5C). Remarkably, the m5SS and mBB2 mutations also restored *yra1-N84* pre-mRNA to wild-type pre-mRNA regulation; i.e., the *yra1-N84-m5SS* and *yra1-N84-mBB2* pre-mRNAs accumulated to high levels in *edc3Δ* strains (Figure 5C). In contrast, mutation of the 3' splice site (m3SS), which inhibits the second step of splicing (Rymond et al., 1987), reduced *yra1-N84* pre-mRNA splicing only modestly, as significant levels of *yra1-N84* mRNA accumulated in *upf1Δ* cells (Figure 5C). The modest effect of the m3SS mutation on *yra1-N84* pre-mRNA splicing is likely due to the use of alternative 3' splicing signals in exon 2, and the sensitivity of *yra1-N84* mRNA to *UPF1* probably reflects the creation of a premature stop codon as a consequence of alternative 3' splicing. Significantly, the m3SS mutation did not restore the *yra1-N84* pre-mRNA to wild-type *YRA1*

Figure 5. *YRA1* Exon 1 and Yra1p Are *cis*- and *trans*-Acting Negative Regulators of *YRA1* Pre-mRNA Splicing

(A–D) Effects on the levels of *YRA1* pre-mRNA and mRNA engendered by (A) internal deletions in *YRA1* exon 1; (B) substituting *YRA1* exon 1 coding sequences with their complementary sequences; (C) mutations

pre-mRNA regulation. These data show that *cis* mutations that inhibit the first but not the second step of *YRA1* pre-mRNA splicing completely suppress the defect caused by the N84 deletion and thus result in a bypass of the regulatory function of *YRA1* exon 1. These results indicate that (1) the primary function of *YRA1* exon 1 is to inhibit *YRA1* pre-mRNA splicing, not to promote *YRA1* pre-mRNA nuclear export; and (2) *YRA1* exon 1 most likely exerts its inhibitory function at or before the first step of the splicing pathway.

Yra1p Autoregulates Its Level of Expression by Inhibiting *YRA1* Pre-mRNA Splicing and Committing the Pre-mRNA to Nuclear Export

To further understand the role of Yra1p in its autoregulation, we used the temperature-sensitive (ts) *yra1-1* allele to analyze the effect of Yra1p inactivation on *YRA1* pre-mRNA and mRNA levels in *EDC3* and *edc3Δ* cells. Inactivation of Yra1p resulted in decreased levels of *YRA1* pre-mRNA but increased levels of *YRA1* mRNA in *edc3Δ* cells (Figure S2), a result suggesting that Yra1p regulates its expression by inhibiting *YRA1* pre-mRNA splicing. To test this idea further, we analyzed the effects of eliminating Yra1p. In this experiment, we generated a *yra1-AUA* allele that encodes a G→A substitution (AUG to AUA) in the translation initiation codon. This mutation eliminated Yra1p production and resulted in a complete loss of *YRA1* function, as assessed by western blotting analysis and a genetic complementation assay (data not shown). We cloned the *yra1-AUA* allele, as well as the wild-type *YRA1* gene, into low-copy plasmids and introduced these plasmids into *EDC3* and *edc3Δ* strains that contain chromosomal deletions of both *YRA1* and *YRA2* but harbor *YRA2* on a high-copy plasmid. *YRA2* codes for Yra2p, a yeast homolog of Yra1p that has been previously shown to suppress the lethality of *YRA1* deletion when overexpressed (Zenklusen et al., 2001). Wild-type *YRA1* generated low levels of *YRA1* mRNA in both *EDC3* and *edc3Δ* cells and high levels of *YRA1* pre-mRNA in an *edc3Δ* background (Figure 5D). These data show that introduction of wild-type *YRA1* into *yra1Δ* strains recapitulated

in the 5′ splice site, the 3′ splice site, or the branchpoint region of the *YRA1* intron; and (D) mutations in the translation initiation codon, 5′ splice site, 3′ splice site, and branchpoint region of the *YRA1* intron in the presence or absence of Yra1p. Note that in (B), the replaced complementary sequence does not contain in-frame premature stop codons. In (A)–(D), plasmids carrying the wild-type *YRA1* gene, or one of the mutant *YRA1* alleles (depicted above the corresponding northern blots), were introduced into different yeast strains, and the levels of the respective pre-mRNAs and mRNAs encoded by these alleles were analyzed by northern blotting. The relevant genotypes of yeast strains used in these experiments are indicated (A) or denoted by numbers in (B)–(D): 1, *wild-type*; 2, *upf1Δ*; 3, *edc3Δ*; 4, *upf1Δ edc3Δ*; 5, *EDC3 yra1Δ yra2Δ* (YEplac112-YRA2); and 6, *edc3Δ yra1Δ yra2Δ* (YEplac112-YRA2). In (A), the levels of mutant *YRA1* pre-mRNAs and mRNAs in *edc3Δ* cells were quantified, normalized to the corresponding wild-type pre-mRNA and mRNA levels, and graphed below the northern blot. Blots were hybridized to a *SCR1* probe to serve as a loading control.

YRA1 autoregulation and suggest that overexpression of *YRA2* has little or no effect on *YRA1* autoregulation. Compared to wild-type *YRA1*, the *yra1-AUA* allele generated 6-fold higher levels of *YRA1* mRNA in both *EDC3* and *edc3Δ* cells and generated a 3-fold lower level of *YRA1* pre-mRNA in *edc3Δ* cells (Figure 5D). These data indicate that *YRA1-AUA* pre-mRNA is efficiently spliced in both *yra1Δ* strains, an effect attributable to the absence of Yra1p, not the AUA mutation. We justify the latter conclusion because, when the *yra1-AUA* allele was introduced into *EDC3* and *edc3Δ* strains that contain the endogenous *YRA1* gene, the *yra1-AUA* allele behaved the same as wild-type *YRA1* in that *yra1-AUA* pre-mRNA was inefficiently spliced and a high level of this pre-mRNA accumulated in an *edc3Δ* background (Figure 5D). Taken together, these results indicate that, in the absence of Yra1p, *YRA1* pre-mRNA is efficiently spliced, suggesting that Yra1p regulates its level of expression by inhibiting splicing of its pre-mRNA.

A role for Yra1p in the inhibition of its pre-mRNA splicing raised the question of its primary function in *YRA1* autoregulation. One possibility is that Yra1p primarily promotes *YRA1* pre-mRNA export and, as a consequence, inhibits *YRA1* pre-mRNA splicing. A second possibility is that Yra1p primarily inhibits *YRA1* splicing and, as a consequence, promotes or commits the pre-mRNA to nuclear export. A third possibility is that Yra1p is required for both splicing inhibition and active nuclear export. To distinguish these possibilities, we tested whether *cis* mutations that inhibit *YRA1* pre-mRNA splicing can bypass the regulatory function of Yra1p. Accordingly, we introduced the same m5SS, mBB2, and m3SS mutations described above into the 5′ splice site, the branchpoint region, and the 3′ splice site of the *yra1-AUA* intron. We cloned the resulting alleles into low-copy plasmids that were then introduced into the *EDC3* and *edc3Δ* strains that contain chromosomal deletions of both *YRA1* and *YRA2* but harbor *YRA2* on a high-copy plasmid. Northern analysis revealed that the m5SS and mBB2 mutations fully restored *yra1-AUA* pre-mRNA to wild-type regulation, but the m3SS mutation did not (Figure 5D). These data show that *cis* mutations (m5SS and mBB2) that inhibit the first but not the second step of *YRA1* pre-mRNA splicing completely suppress the *YRA1* autoregulation defect caused by Yra1p elimination, thus bypassing the regulatory function of Yra1p. These results indicate that the primary autoregulatory function of Yra1p is to inhibit *YRA1* pre-mRNA splicing, thus committing the pre-mRNA to nuclear export, and that Yra1p likely inhibits at or before the first step of *YRA1* pre-mRNA splicing.

Autoregulation of *YRA1* Expression Involves Mex67p

Mex67p, a general mRNA export factor in yeast, interacts genetically and physically with Yra1p (Strasser and Hurt, 2000; Zenklusen et al., 2001). To assess whether Mex67p plays a role in *YRA1* autoregulation, we utilized the ts *mex67-5* allele and analyzed the effect of its inactivation on the accumulation of *YRA1* pre-mRNA and

mRNA in *edc3Δ* cells. *mex67-5edc3Δ* and *MEX67edc3Δ* cells grown at 25°C accumulated comparable levels of *YRA1* pre-mRNA and mRNA (Figure 6A, compare t_0 samples). At 37°C, the same strains exhibited dramatically different *YRA1* expression patterns (Figure 6A), including the following: (1) *MEX67edc3Δ* cells had slightly decreased but sustained levels of *YRA1* pre-mRNA during a 24 min time course, but the same transcript decreased rapidly in *mex67-5edc3Δ* cells; (2) *MEX67edc3Δ* cells had significantly lower levels of *YRA1* mRNA at 24 min than at 0 min, whereas *mex67-5edc3Δ* cells accumulated similar mRNA levels at both time points; and (3) at the 12 and 24 min time points, *mex67-5edc3Δ* cells accumulated a novel *YRA1* transcript migrating slightly slower than normal *YRA1* mRNA. Since this RNA species hybridized to an mRNA-specific oligonucleotide that spanned the junction of *YRA1* exons 1 and 2 and also to an oligonuclotide complementary to sequences 92 nt downstream of the mapped canonical poly(A) site (Figure 6B), we conclude that it was comprised of *YRA1* mRNA with an extended 3′UTR. *MEX67edc3Δ* cells also accumulated a *YRA1* RNA species of similar size at 12 and 24 min. However, this RNA hybridized to an intron-specific oligonucleotide, but not to the mRNA-specific oligonucleotide or the 3′UTR oligonucleotide (data not shown), suggesting that it is a 3′ to 5′ decay intermediate of *YRA1* pre-mRNA.

Our observation that, at 37°C, *mex67-5edc3Δ* cells accumulated lower levels of *YRA1* pre-mRNA but higher levels of *YRA1* mRNA than the *MEX67edc3Δ* strain, and that *mex67-5edc3Δ* cells also accumulated *YRA1* mRNA with an extended 3′UTR, suggests that inactivation of Mex67p alters nuclear *YRA1* pre-mRNA metabolism. For example, Mex67p might inhibit *YRA1* pre-mRNA splicing and commit the pre-mRNA to nuclear export such that inactivation of Mex67p would allow a fraction of newly synthesized *YRA1* pre-mRNA normally committed to nuclear export to adopt an alternative fate and proceed to the splicing pathway. Consistent with this interpretation, we found that the effects of inactivating Mex67p on *YRA1* mRNA expression were dependent on ongoing transcription since simultaneous inhibition of transcription with thiolutin and thermal inactivation of Mex67p function resulted in decreased expression of *YRA1* mRNA and eliminated the formation of *YRA1* mRNA with extended 3′UTRs (Figure 6C). Additional control experiments revealed that inactivation of Mex67p did not affect the level of the *CYH2* pre-mRNA, an NMD substrate. At 37°C, *mex67-5edc3Δ* and *MEX67edc3Δ* strains accumulated similar levels of this pre-mRNA as well as its mRNA product (Figure 6A). These results indicate that the effect of Mex67p inactivation on pre-mRNA splicing is specific for *YRA1* pre-mRNA.

Thermal inactivation of Mex67p resulted in almost complete disappearance of *YRA1* pre-mRNA in *mex67-5-edc3Δ* cells in 24 min (Figure 6A). While this rapid disappearance is at least partially attributable to a loss of Mex67p inhibition of *YRA1* pre-mRNA splicing, other mechanisms must be operative. Since, in steady-state,
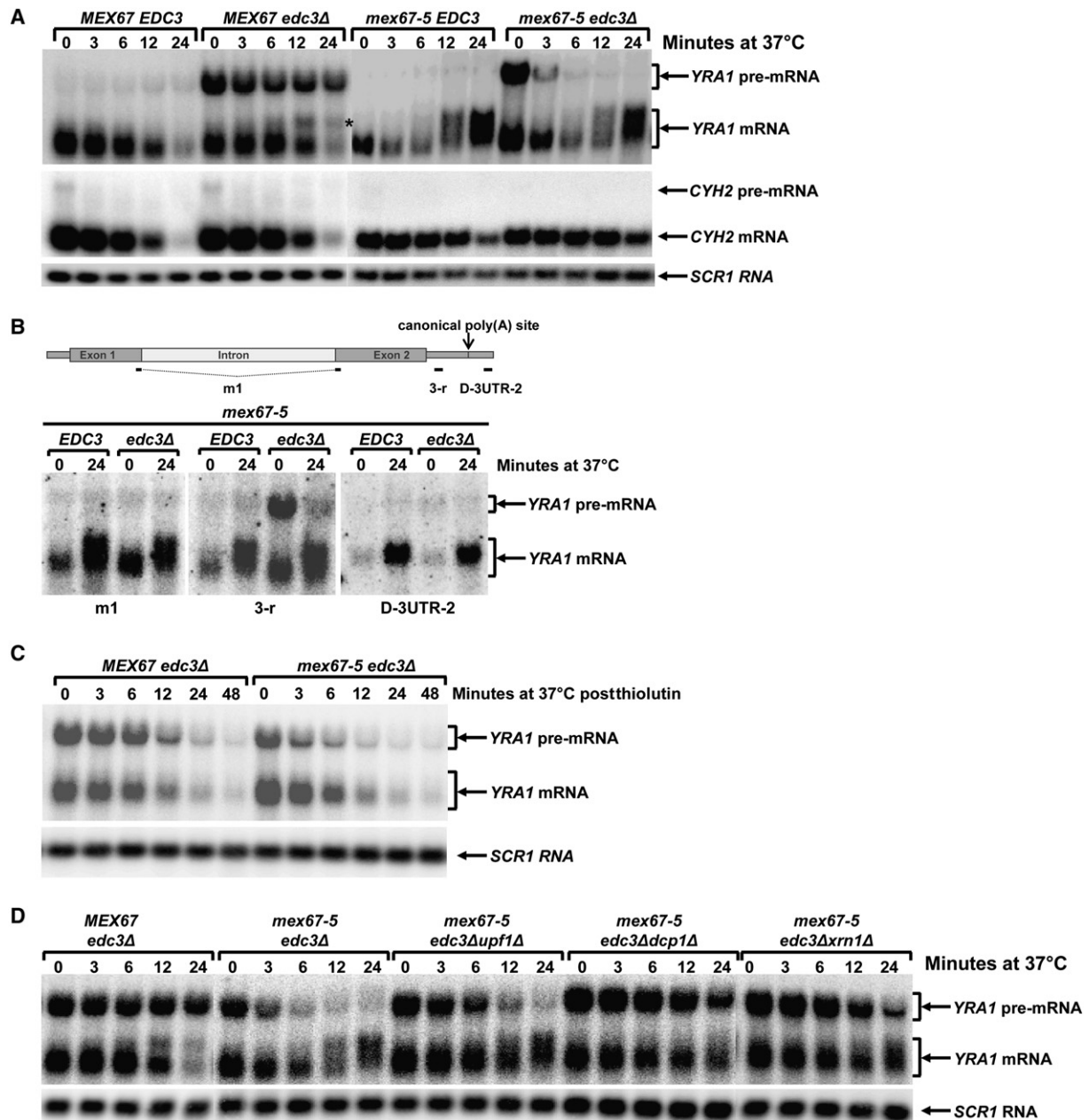
Figure 6. *YRA1* Autoregulation Requires the Function of Mex67p

(A) Inactivation of Mex67p alters the levels of the *YRA1*, but not the *CYH2* pre-mRNA and mRNA, and also results in the accumulation of an atypical *YRA1* RNA species.

(B) Inactivation of Mex67p promotes accumulation of *YRA1* mRNA with an extended 3′UTR. RNA samples from time points 0 and 24 min of the *Mex67-5 EDC3* and *Mex67-5 edc3Δ* strains shown in (A) were analyzed with a *YRA1* oligonucleotide probe that spans exon 1 and exon 2 (left), an oligonucleotide probe downstream of the *YRA1* translation termination codon (middle), or an oligonucleotide probe downstream of the canonical poly(A) addition site (right).

(C) The effects of Mex67p inactivation on *YRA1* mRNA level and the accumulation of *YRA1* mRNA with an extended 3′UTR require ongoing transcription.

(D) Deletion of *UPF1*, *DCP1*, or *XRN1* restores *YRA1* pre-mRNA levels at 37°C in *mex67-5 edc3Δ* cells.

In (A), (C), and (D), yeast strains of the indicated genotypes were grown at 25°C and then shifted to 37°C, and the levels of *YRA1* or *CYH2* transcripts were analyzed by northern blotting. In (C), thiolutin was added to cell cultures just before the temperature shift. A putative 3′ to 5′ decay intermediate of *YRA1* pre-mRNA detected in the *MEX67edc3Δ* strain in (A) is marked by *. Blots were hybridized to a *SCR1* probe to serve as a loading control.
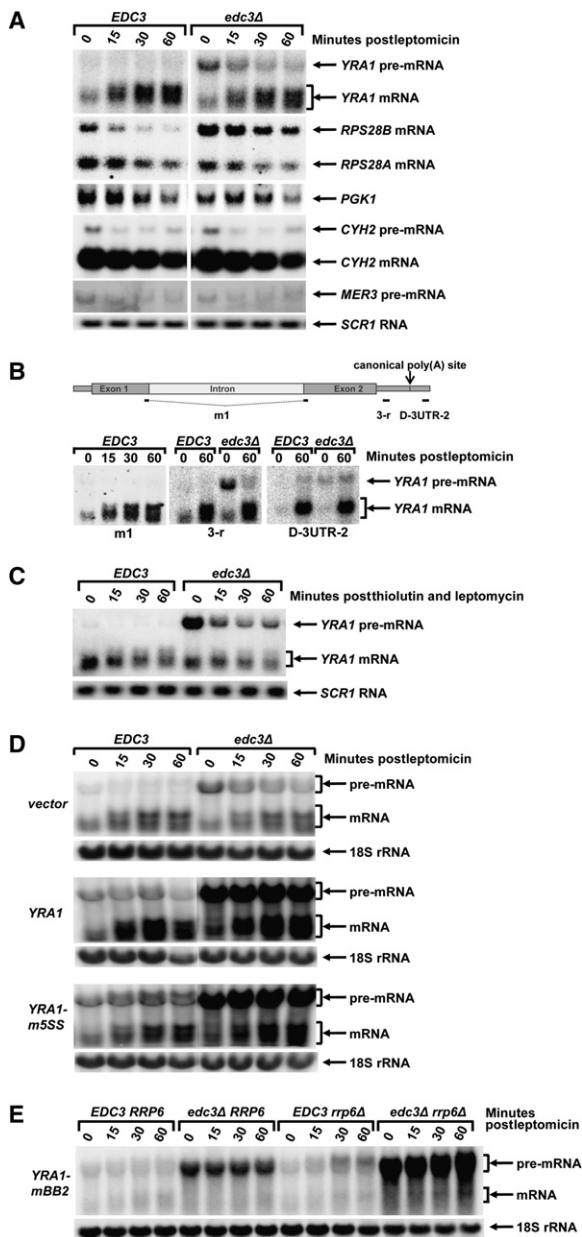
Figure 7. Crm1p Mediates *YRA1* Pre-mRNP Export to the Cytoplasm

(A) Inhibition of Crm1p function increases expression of *YRA1* mRNA but slightly decreases expression of other control RNAs including the *CYH2* and *MER3* pre-mRNAs and the *RPS28A*, *RPS28B*, and *PGK1* mRNAs.

(B) Inhibition of Crm1p promotes accumulation of *YRA1* pre-mRNA and mRNA with extended 3′UTRs. RNA samples from the indicated time points of the *EDC3* and *edc3Δ* strains shown in (A) were analyzed with the same set of oligonucleotide probes used in Figure 6B.

(C and D) Leptomycin-promoted increases in *YRA1* mRNA levels and the accumulation of *YRA1* mRNA with an extended 3′UTR requires ongoing transcription (C) and *YRA1* pre-mRNA splicing (D).

(E) *YRA1* pre-mRNA defective in splicing becomes a target of the nuclear exosome when Crm1p function is inhibited.

In (A), (C), (D), and (E), yeast strains of the indicated genotypes (all harboring the *CRM1-T539C* allele) were treated with leptomycin, and the

YRA1 pre-mRNA is predominantly cytoplasmic in *edc3Δ* cells (Figure 3A) and since, in a *MEX67edc3Δ* background, *YRA1* pre-mRNA has a half-life of ≥60 min (Figure 1B), the rapid disappearance of *YRA1* pre-mRNA in *mex67-5edc3Δ* cells must be due principally to accelerated cytoplasmic decay of the pre-mRNA. In turn, this suggests that Mex67p is a component of the cytoplasmic *YRA1* pre-mRNP and functions to repress *YRA1* pre-mRNA translation and thus inhibit NMD. To test this hypothesis, we assessed whether deletion of *UPF1*, *DCP1*, or *XRN1* could restore the steady-state levels of *YRA1* pre-mRNA in *mex67-5edc3Δ* cells at 37°C. Indeed, Figure 6D shows that deletion of *UPF1* restored *YRA1* pre-mRNA levels at early time points and that deletion of *DCP1 or XRN1* restored *YRA1* pre-mRNA levels at almost all time points. These results indicate that inactivation of Mex67p also triggers rapid cytoplasmic degradation of *YRA1* pre-mRNA by NMD. Our observation that deletion of *UPF1* results in a partial restoration and deletion of *DCP1* or *XRN1* results in a complete restoration of *YRA1* pre-mRNA levels at 37°C in *mex67-5 edc3Δ* cells suggests that, when NMD is inactivated, the *YRA1* pre-mRNA is degraded by an alternative mechanism, most likely the general 5′ to 3′ decay pathway, as we have observed previously for other nonsense-containing mRNAs (He et al., 2003). Taken together, the data of Figure 6 show that inactivation of Mex67p promotes *YRA1* pre-mRNA splicing and triggers rapid cytoplasmic degradation of the pre-mRNA by NMD.

YRA1 Pre-mRNPs Are Exported to the Cytoplasm by the Crm1p-Mediated Export Pathway

Genome-wide two-hybrid analyses have identified an interaction between Edc3p and Crm1p (Ito et al., 2001), a result that we have confirmed (Figure S3). Since the human Crm1p homolog is implicated in the nuclear export of unspliced or incompletely spliced HIV RNAs in mammalian cells (Cullen, 2003), the yeast Edc3p:Crm1p interaction suggested the possibility that Crm1p is involved in *YRA1* pre-mRNA nuclear export. To assess the physiological relevance of the Edc3p:Crm1p interaction, we used yeast strains harboring the leptomycin-sensitive *CRM1-T539C* allele and analyzed the effect of inhibition of Crm1p function by leptomycin treatment. In both *EDC3* and *edc3Δ* cells, leptomycin treatment resulted in increased accumulation of *YRA1* mRNA and the appearance of a novel *YRA1* mRNA with an extended 3′UTR (Figures 7A and 7B). The increased accumulation of *YRA1* mRNA and the appearance of *YRA1* mRNA with

effects on *YRA1* pre-mRNA and mRNA were analyzed by northern blotting. In (C), thiolutin and leptomycin were simultaneously added to *EDC3* and *edc3Δ* cells. In (D), a YCp plasmid harboring the *YRA1* gene or its *yra1-m5SS* allele, as well as the empty vector, was introduced into *EDC3* and *edc3Δ* strains. In (E), a YCp plasmid harboring the *yra1-mBB2* allele was introduced into yeast strains of the indicated genotypes. Blots were hybridized to a *SCR1* or an 18S rRNA probe to serve as loading controls.

an extended 3′UTR in both *EDC3* and *edc3Δ* cells after leptomycin treatment are likely to reflect one of the consequences of inhibition of Crm1p-mediated *YRA1* pre-mRNA nuclear export. A simple interpretation of these results is that, when Crm1p function is inhibited, a fraction of newly synthesized *YRA1* pre-mRNA that is normally committed to nuclear export adopts an alternative fate and proceeds to the splicing pathway. Consistent with this interpretation, we found that the effect of leptomycin treatment on *YRA1* mRNA expression is dependent on both ongoing transcription and splicing of *YRA1* pre-mRNA (Figures 7C–7E).

Leptomycin treatment also resulted in the accumulation of longer *YRA1* pre-mRNA transcripts in both *EDC3* and *edc3Δ* cells (Figure 7E). These longer pre-mRNA species hybridize with an oligonuclotide probe downstream of the mapped canonical poly(A) site (Figure 7B), indicating that these transcripts have an extended 3′UTR. The accumulation of mRNAs with extended 3′UTRs has been observed in many yeast mRNA export mutant strains and is a general characteristic of mRNA nuclear export defects (Forrester et al., 1992; Hammell et al., 2002). Interestingly, as observed for other mRNAs, *YRA1* pre-mRNAs with extended 3′UTRs are also the target of the nuclear exosome surveillance system (Torchet et al., 2002). When Crm1p is functional, elimination of the exosome component Rrp6p had no effect on levels of the pre-mRNA transcripts encoded by either endogenous wild-type *YRA1* or by the exogenous *yra1-mBB2* allele in both *EDC3* and *edc3Δ* backgrounds (Figure S1 and Figure 7E, t$_0$). In contrast, when Crm1p function is inhibited by leptomycin, deletion of *RRP6* resulted in increased accumulation of *YRA1* and *yra1-mBB2* pre-mRNA transcripts with an extended 3′UTR in both *EDC3* and *edc3Δ* backgrounds (Figure 7E). These results show that inhibition of Crm1p function leads a fraction of newly synthesized *YRA1* pre-mRNAs that normally commit to nuclear export to adopt yet another alternative fate and be degraded by the nuclear exosome.

In both *EDC3* and *edc3Δ* backgrounds, inhibition of Crm1p function by leptomycin treatment did not alter the metabolism of several other intron-containing pre-mRNAs and intron-lacking mRNAs (Figure 7A). These include the intron-containing *CYH2* and *MER3* pre-mRNAs that are substrates of the NMD pathway; the *RPS28B* mRNA, a second substrate of the Edc3p-mediated decay pathway; and the *PGK1* and *RPS28A* mRNAs, substrates of the general 5′ to 3′ and 3′ to 5′ decay pathways. We thus conclude that *YRA1* pre-mRNPs are transported to the cytoplasm by the Crm1p-mediated export pathway and that, when this pathway is inhibited, the pre-mRNA transcripts are trapped in the nucleus and either spliced to generate mRNA or degraded by the nuclear exosome. In contrast to inactivation of Mex67p, inhibition of Crm1p function by leptomycin did not alter the *YRA1* pre-mRNA decay rate in *edc3Δ* cells (compare Figures 7C and 1B), indicating that Crm1p function is not required for the cytoplasmic degradation of *YRA1* pre-mRNA.

DISCUSSION

YRA1 Pre-mRNA Is a Cytoplasmic Substrate for Edc3p-Mediated 5′ to 3′ Decay

Intron-containing pre-mRNAs are generally processed or degraded within the nucleus and rarely enter the cytoplasm (Bousquet-Antonelli et al., 2000; Legrain and Rosbash, 1989). In the limited instances that such export occurs, these transcripts are often degraded by the translation-dependent NMD pathway (He et al., 1993, 2003; Vilardell et al., 2000). As shown here, the intron-containing *YRA1* pre-mRNA is a notable exception to this rule. A sizeable fraction of *YRA1* pre-mRNA can be exported to the cytoplasm and subsequently degraded via an NMD-independent 5′ to 3′ mechanism that requires the Dcp1p/Dcp2p decapping enzyme, the Xrn1p exoribonuclease, and a specific regulator, Edc3p.

The atypical localization of this decay pathway is surprising but is consistent with the results of several independent approaches. Cytoplasmic localization of Edc3p-mediated *YRA1* pre-mRNA degradation follows from (1) the involvement of the Dcp1p/Dcp2p complex and Xrn1p, two cytoplasmic ribonucleases (Figure 2); (2) the in situ detection of *YRA1* pre-mRNA in the cytoplasm of *edc3Δ*, *dcp1Δ*, or *xrn1Δ* cells (Figure 3); (3) the resistance of *YRA1* pre-mRNA degradation to inactivation or depletion of the nuclear exonucleases, Rat1p or Rrp6p; and (4) the unchanged levels of *YRA1* mRNA in *edc3Δ* cells (Figure 1), a result to be contrasted to the increased levels of mature mRNAs that generally follow the inhibition of intron-containing pre-mRNA degradation in the nucleus (Bousquet-Antonelli et al., 2000; Danin-Kreiselman et al., 2003).

Edc3p Activates Decapping of *YRA1* Pre-mRNA

Our results indicate that Edc3p activates but does not catalyze decapping of *YRA1* pre-mRNA. In light of the physical interactions between Edc3p and Dcp1p or Dcp2p (Gavin et al., 2002; Ho et al., 2002; Ito et al., 2001; Uetz et al., 2000), this activation may reflect Edc3p recruitment of the decapping enzyme or one of its subunits. In the general 5′ to 3′ decay pathway, decapping requires the functions of Dhh1p, Pat1p, and the Lsm1-7p complex (Coller and Parker, 2004), and in the NMD pathway, decapping requires the functions of Upf1p, Nmd2p/Upf2p, and Upf3p (He and Jacobson, 2001). Like Edc3p, all of these factors function as decapping activators (Coller and Parker, 2004). Edc3p-mediated decapping has several unique features, of which the most notable is its limited number of substrate transcripts. At present, we cannot exclude the possibility that Edc3p targets a much larger set of mRNAs that are underrepresented in conventional microarray analyses, e.g., transcripts lacking poly(A) tails. Another unique feature of Edc3p-mediated decapping is that it requires specific *cis*-regulatory elements in its target transcripts. For *YRA1* pre-mRNA, we mapped the *cis* element to its intron (Figure 4), and in *RPS28B* mRNA, the *cis* element has been localized to its 3′UTR (Badis et al.,

2004). These sequence requirements indicate that Edc3p substrate specificity is likely dictated by a specific structure and/or composition of the target mRNPs. While specific mRNP structures may also be required for other decapping activators, these structures do not appear to be mRNA specific (Coller and Parker, 2004).

While our data and those of a previous study (Badis et al., 2004) demonstrate that decapping activation by Edc3p is transcript specific, several observations also suggest that Edc3p may have a general function in decapping: (1) Edc3p appears to exist in a complex with Dcp1p and Dcp2p in vivo (Gavin et al., 2002; Ho et al., 2002), (2) Edc3p and Dcp1p have comparable numbers of molecules per cell (Huh et al., 2003), and (3) simultaneous inactivation of Edc3p and mutation of *DCP1* or *DCP2* promote synthetic or additive effects on both cell growth and decapping of reporter mRNAs (Kshirsagar and Parker, 2004). Given that mRNA decapping is likely to be a multistep process in vivo (Coller and Parker, 2004), Edc3p may function at a step that is not rate limiting for general mRNA decapping (Kshirsagar and Parker, 2004) or may have a function that is redundant with another component of the decapping complex.

YRA1 Autoregulation: Yra1p Inhibits Pre-mRNA Splicing and Commits the Transcript to Nuclear Export

Our observation that introduction of extra copies of the intron-containing *YRA1* gene into *EDC3* or *edc3Δ* strains altered neither the *YRA1* mRNA level nor the Yra1p level in these cells but yielded a >10-fold higher level of *YRA1* pre-mRNA in *edc3Δ* cells (Figure 4) confirms that *YRA1* autoregulates its own expression through a negative feedback loop (Preker et al., 2002; Rodriguez-Navarro et al., 2002) and further indicates that Edc3p-mediated *YRA1* pre-mRNA degradation is a component of *YRA1* autoregulation.

Further experiments defined the functional role of Yra1p in its autoregulation. Since inactivation or depletion of Yra1p resulted in increased accumulation of *YRA1* mRNA in *EDC3* or *edc3Δ* cells but diminished accumulation of *YRA1* pre-mRNA in *edc3Δ* cells, Yra1p must inhibit *YRA1* pre-mRNA splicing (Figure S2 and Figure 5). Moreover, since *cis* mutations (m5SS and mBB2) that inhibit the first but not the second step (m3SS) of *YRA1* pre-mRNA splicing completely suppress the autoregulation defect caused by Yra1p depletion (Figure 5), Yra1p must inhibit its pre-mRNA splicing at or before the first step of the splicing reaction. These observations also indicate that the sole function of Yra1p in its autoregulation is to inhibit *YRA1* pre-mRNA splicing and that Yra1p per se is not required for nuclear export of its pre-mRNA. Combined with our observation that *yra1-AUA* pre-mRNA is efficiently exported to the cytoplasm in the presence of Yra1p but is efficiently spliced in the nucleus in the absence of Yra1p (Figure 5), these findings also suggest that Yra1p inhibits *YRA1* pre-mRNA splicing by committing the transcript to nuclear export. A simple interpreta-

tion of these data is that *YRA1* pre-mRNA splicing and nuclear export compete for common substrates and that, by enhancing nuclear export of its pre-mRNA, Yra1p inhibits its splicing.

Autoregulatory Roles of the Intron and Exon 1

We have identified two functionally distinct *cis*-regulatory elements that are involved in *YRA1* autoregulation: exon 1 inhibits *YRA1* pre-mRNA splicing, and the intron is required for Edc3p-mediated *YRA1* pre-mRNA degradation (Figures 4 and 5). Unexpectedly, splicing inhibition by exon 1 was found to be dictated by its length, not by specific sequences, and *cis* mutations that inhibit the first but not the second step of *YRA1* pre-mRNA splicing completely suppress the autoregulation defect caused by the N84 deletion of exon 1 (Figure 5). These findings indicate that exon 1 exerts its inhibitory function at or before the first step of the splicing reaction, that the sole autoregulatory role of exon 1 is to inhibit *YRA1* pre-mRNA splicing, and that exon 1 per se is not required for nuclear export of *YRA1* pre-mRNA. Further, the shared autoregulatory roles, as well as the identical patterns of genetic interactions with *cis*-acting mutations in *YRA1* pre-mRNA, strongly suggest that Yra1p's inhibitory function on *YRA1* pre-mRNA splicing is mediated through exon 1.

The primacy of size, not sequence, for the splicing inhibition function of *YRA1* exon 1 also indicates that the position of the *YRA1* intron, rather than the intron itself, is the major determinant in *YRA1* autoregulation. This contrasts with a previous conclusion that intron size is the major determinant in *YRA1* autoregulation (Preker and Guthrie, 2006). Although this discrepancy may reflect differences in experimental designs and assays for splicing efficiency, our conclusion is strongly supported by the following observations. First, internal deletions in exon 1 can result in more than 8-fold increases in the level of *YRA1* mRNA (Figure 5A), but internal deletions in the intron had little (<2-fold) or no comparable effect (Figure 4E and data not shown). Second, replacing the *YRA1* intron with any of five other introns (from the *CYH2*, *MER2*, *RPS51A*, *RPL25*, or *UBC8* transcripts) differing in size and splicing efficiency had little or no effect on *YRA1* mRNA levels (Figures 4B and 4C; Rodriguez-Navarro et al., 2002). Third, an intron position effect on splicing has also been seen with pre-mRNAs derived from the *MER2* gene and from an *ACT1* reporter gene (Klinz and Gallwitz, 1985; Nandabalan and Roeder, 1995).

Our observation that the inhibitory function of *YRA1* exon 1 on *YRA1* pre-mRNA splicing is dependent on its length, but not specific sequences, suggested a kinetic component to *YRA1* autoregulation. Given that Yra1p normally functions in an early stage of mRNA export and that this function is linked to polymerase II transcription elongation through the THO complex (Strasser et al., 2002), this kinetic component is likely to reflect a time window in which Yra1p is recruited to the polymerase II elongation complex and loaded onto a nascent mRNP. Such loading may be dictated by both the rate of elongation and the

nuclear Yra1p concentration, and we suggest that, for intron-containing pre-mRNAs, cotranscriptional packaging of Yra1p onto a nascent mRNP likely promotes the recruitment of the mRNA export receptor Mex67p and thus inhibits spliceosome assembly. For *YRA1* autoregulation, high Yra1p levels would thus favor the packaging of Yra1p into *YRA1* pre-mRNPs, commit the pre-mRNA to nuclear export, and, as a consequence, inhibit pre-mRNA splicing. Given that the vast majority of yeast introns are located at the 5′ ends of their genes (Spingola et al., 1999), tight control of nuclear Yra1p levels could be an important mechanism that prevents premature nuclear export of intron-containing pre-mRNAs.

Roles of Mex67p and Crm1p in *YRA1* Autoregulation
Our data indicate that, in addition to Yra1p and Edc3p, *YRA1* autoregulation also requires the functions of two general nuclear export factors, Mex67p and Crm1p. Since the inactivation of Mex67p or Crm1p function both resulted in decreased accumulation of *YRA1* pre-mRNA in *edc3Δ* cells, increased accumulation of normal *YRA1* mRNA, and the appearance of *YRA1* mRNA with an extended 3′UTR in *EDC3* and *edc3Δ* cells, it appears that Mex67p and Crm1p jointly promote *YRA1* pre-mRNA nuclear export. Given that Mex67p is a general mRNA export receptor (Hieronymus and Silver, 2003; Hurt et al., 2000) and that Crm1p function is usually not required for mRNA export (Neville and Rosbash, 1999), the *YRA1* pre-mRNA nuclear export requirement for both factors indicates that the *YRA1* pre-RNP is exported to the cytoplasm by a mechanism that is distinct from the general Mex67p-mediated mRNA export pathway. Although the precise functions and the functional order of Mex67p and Crm1p in *YRA1* pre-mRNA export are currently not clear, the facts that the function of Yra1p is linked to transcription elongation and that Yra1p physically interacts with Mex67p suggest that Mex67p is likely recruited earlier than Crm1p to *YRA1* pre-mRNP. One possibility is that Yra1p first recruits Mex67p to nascent *YRA1* pre-mRNP. However, the general mRNA export function of Mex67p is subsequently inhibited in this *YRA1* pre-mRNP, and Mex67p, in turn, serves as a unique adaptor to access the Crm1p-mediated export pathway. Consistent with this possibility is the observation that the Mex67p C-terminal domain contains a Rev-like NES and exhibits NES activity (Strasser et al., 2000).

Our observation that inactivation of Mex67p, but not Crm1p, triggers rapid degradation of *YRA1* pre-mRNA by NMD in *edc3Δ* cells indicates that Mex67p has a second function in *YRA1* autoregulation, i.e., it may be a component of the cytoplasmic *YRA1* pre-mRNP and impart Edc3p substrate specificity. Since inactivation of Mex67p leads to rapid degradation of *YRA1* pre-mRNA by NMD and activation of NMD is dependent on translation, these observations argue that Mex67p functions at least in part by repressing *YRA1* pre-mRNA translation. The possibility that this mRNA export factor has a general role in mRNA translation is consistent with observations

that (1) Mex67p shuttles between the nucleus and the cytoplasm (Segref et al., 1997); (2) at steady-state, a significant fraction of Mex67p is localized to the cytoplasm and associated with polyribosomes (Segref et al., 1997; Windgassen et al., 2004); and (3) the *mex67-5* mutation causes aberrant accumulation of Mex67p in the cytoplasm (Segref et al., 1997).

A Model for *YRA1* Autoregulation
To integrate Yra1p-mediated splicing inhibition, Crm1p-mediated nuclear export, and Edc3p-mediated cytoplasmic degradation of *YRA1* pre-mRNA, we propose the following model for *YRA1* autoregulation. When nuclear Yra1p levels are low, *YRA1* is transcribed into pre-mRNA that, in turn, is spliced to mRNA. The mature *YRA1* mRNA is exported to the cytoplasm and translated to generate Yra1p and the latter imported into the nucleus, recruited to elongating Pol II, and then packaged into nascent mRNPs to serve a function in general mRNA export. However, when nuclear Yra1p levels reach a threshold, Yra1p is likely recruited to the *YRA1* gene during an early phase of transcription elongation and packaged into nascent *YRA1* pre-mRNPs. Yra1p then recruits Mex67p, inhibiting pre-mRNA splicing and committing the pre-mRNA to export. The pre-mRNA would then be exported to the cytoplasm by the Crm1p-dependent pathway where it would be degraded by the Edc3p-dependent pathway.

The molecular mechanism uncovered here for *YRA1* autoregulation is reminiscent of Rev-mediated unspliced pre-mRNA nuclear export that is required for HIV replication (Cullen, 2003). This observation suggests that Rev may target the same cellular component or pathway as Yra1p to promote nuclear pre-mRNA export. The existence of a specific cytoplasmic pathway dedicated to *YRA1* pre-mRNA degradation also raises the question of the biological function of this pathway. In contrast to the general decay pathways that control mRNA levels, and the quality control pathways that eliminate aberrant transcripts, the Edc3p-mediated decay pathway may serve a regulatory function, e.g., it may utilize Crm1p-mediated *YRA1* pre-mRNA export to regulate the level of a key nuclear factor or coordinate mRNA decay activity with other cellular processes.

EXPERIMENTAL PROCEDURES

Yeast Strains, Plasmids, Oligonucleotides, Cell Growth Conditions, Microarray Analysis, and Yeast Two-Hybrid Analysis
Yeast strains, plasmids, oligonucleotides, cell growth conditions, microarray analysis, and yeast two-hybrid analysis are described in the Supplemental Data.

RNA Analysis
RNA isolation and northern blotting procedures were as described previously (He and Jacobson, 1995). Transcript-specific signals were quantified with a Storm PhosphorImager and normalized to levels of the 18S rRNA or *SCR1* RNA, a stable RNA polymerase III transcript. RNA immunoprecipitations with monoclonal

anti-2,2,7-trimethylguanosine antibodies (Calbiochem) were performed as described previously (He and Jacobson, 2001).

Protein Analysis

Preparation of whole-cell extracts and western blotting procedures were as described previously (Maderazo et al., 2000). Blots were probed with polyclonal antibodies targeted to Yra1p (a generous gift from Dr. Francoise Stutz, University of Geneve) or Pab1p, with the latter polypeptide serving as a loading control.

In Situ Hybridization

RNA-FISH analysis was carried out by using Cy3 and Cy5 fluorochrome-conjugated oligonucletides as described at http://www.singerlab.org/protocols/insitu_yeast.htm.

Supplemental Data

Supplemental Data include Supplemental Results, Supplemental Experimental Procedures, Supplemental References, four figures, and five tables and can be found with this article online at http://www.molecule.org/cgi/content/full/25/4/559/DC1/.

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