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Transcript-Specific Cytoplasmic Degradation of YRA1 Pre-mRNA Mediated by the Yeast EDC3 Protein: A Dissertation

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TRANSCRIPT-SPECIFIC CYTOPLASMIC DEGRADATION OF *YRA1* PRE-mRNA MEDIATED BY THE YEAST EDC3 PROTEIN

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ABSTRACT

mRNA degradation is a fundamental process that controls both the level and the fidelity of gene expression. Using a combination of bioinformatic, genomic, genetic, and molecular biology approaches, we have shown that Edc3p, a yeast mRNA decay factor, controls the stability of the intron-containing *YRA1* pre-mRNA. We found that Edc3p-mediated degradation of *YRA1* pre-mRNA: 1) is a component of a negative feedback loop involved in the autoregulation of *YRA1*, 2) takes place in the cytoplasm, 3) is independent of translation, 4) occurs through a deadenylation-independent decapping and 5′ to 3′ exonucleotic decay mechanism, and 5) is controlled by specific *cis*-acting elements and *trans*-regulatory factors. *Cis*-regulation of *YRA1* pre-mRNA degradation is complicated and precise. Sequences in exon1 inhibit *YRA1* pre-mRNA splicing and/or promote pre-mRNA export in a size-dependent but sequence-independent manner. Sequences in the intron dictate the substrate specificity for Edc3p-mediated decay. Five structurally different but functionally interdependent modules were identified in the *YRA1* intron. Two modules, designated Edc3p-responsive elements (EREs), are required for triggering an Edc3p-response. Three other modules, designated translational repression elements (TREs), are required for repressing translation of *YRA1* pre-mRNA. TREs enhance the efficiency of the response of the EREs to Edc3p by inhibiting

translation-dependent nonsense-mediated mRNA decay (NMD). *Trans*-regulation of *YRA1* pre-mRNA is governed by Yra1p, which inhibits *YRA1* pre-mRNA splicing and commits the pre-mRNA to nuclear export, and the RNP export factors, Mex67p and Crm1p, which jointly promote *YRA1* pre-mRNA export. Mex67p also appears to interact with sequences in the *YRA1* intron to promote translational repression and to enhance the Edc3p response of *YRA1* pre-mRNA. 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.

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LIST OF ABBREVIATIONS

RNAPII (RNA polymerase II)

CTD (C-termial domain of RNAPII)

CBC (cap binding complex)

THO complex: (consist of Hpr1p, Tho2p, Mft1p, and Thp2p)

TREX (transcription and export complex): formed by THO, Yra1p, and Sub2p

TREX-2 (transcription and export complex 2): formed by Sac3p, Thp1, Sus1p, and Cdc31p

NPC (nuclear pore complex)

RES (retention and splicing complex): formed by Snu17p, Bud13p, and Pml1p

RRE (Rev responsive element)

NES (nuclear export signal)

MPMV (Mason Pfizer Monkey Virus)

CTE (constitutive transport element)

ERE (Edc3p responsive element)

TRE (translational repression element)

CHAPTER I

Introduction

This dissertation describes the investigation of a novel, transcript-specific, mRNA-decay mechanism in the yeast, *Saccharomyces serevisiae*. In this mRNA decay mechanism, the intron-containing *YRA1* pre-mRNA is specifically degraded in the cytoplasm through a pathway mediated by Edc3p, a newly identified decapping activator. Importantly, Edc3p-mediated *YRA1* pre-mRNA degradation is a key component of a negative feedback loop that autoregulates *YRA1*, an essential gene encoding a general RNA nuclear export factor.

To introduce these studies, I will first review the nuclear biogenesis and the potential fate of an intron-containing pre-mRNA. I will then review cytoplasmic mRNA decay pathways and the *cis*- and *trans*-regulation of these pathways. Finally, I will summarize previous studies about the *YRA1* gene and its autoregulation mechanism, and then introduce the specific rationale for the studies in my dissertation.

1. Overview of gene expression and quality control

Gene expression is a multistep process. In eukaryotic cells, the expression of nuclear-encoded genes starts with transcription to produce pre-mRNAs. During and after transcription, the newly synthesized pre-mRNAs are processed by the addition of a methyl guanosine cap to the 5′ ends, by splicing to eliminate introns, and by the addition of poly(A) tails to the transcript 3′ ends. The processed, mature mRNAs are then exported to the cytoplasm. Once in the cytoplasm, the mRNAs are translated into proteins and are eventually degraded by an mRNA decay system.

Previously, it was believed that gene expression is a linear pathway, i.e., every step in gene expression is isolated and independent of the others. However, more and more studies indicate that steps in gene expression are actually coordinated (reviewed in Kuersten and Goodwin, 2005; Maciag et al., 2006; Maniatis and Reed, 2002; Orphanides and Reinberg, 2002). First, almost all nuclear events are coupled. Many factors involved in the post-transcriptional events are co-transcriptionally recruited. These recruitments are mediated by the C-terminal domain (CTD) of RNA Polymerase II (RNAPII) (reviewed in Proudfoot et al., 2002). Moreover, some factors previously thought to function in only one specific nuclear event have been found to function in multiple steps, or to interact with proteins implicated in different steps (Reviewed in Aguilera, 2005; Reed, 2003; Stutz and Izaurralde, 2003). Second, cytoplasmic events are coupled, i.e., mRNA translation and degradation are tightly integrated, with degradation of numerous mRNAs dependent on their translation (Coller and Parker, 2004; Hilleren and Parker, 1999; Jacobson and Peltz, 1996; Wilusz and Wilusz, 2004). Global inhibition of translational initiation, elongation or termination can increase or decrease the stability of different mRNAs (FH, SD, C. Li and AJ, manuscript in preparation). Third, nuclear events are also coupled with cytoplasmic events. For example, nuclear splicing is coupled to the nuclear export and cytoplasmic fate of an mRNA through the exon-junction complex (EJC) in mammalian cells (reviewed in Chang et al., 2007; Kuersten and Goodwin, 2005). The extensive nuclear, cytoplasmic, and nuclear-cytoplasmic couplings make gene expression efficient. The work in this thesis provides an example of nuclear-

cytoplasmic coupling in which the expression of a specific gene (*YRA1*) is regulated through the coordination of multiple steps of gene expression.

Since mistakes can occur at each of the many steps of gene expression, cells have evolved quality control mechanisms to ensure the production of functional products. For example, during the biogenesis of functional mRNAs: 1) aberrant pre-mRNAs generated by transcription or processing mistakes are retained in the nucleus and destroyed by the nuclear decay system (Bousquet-Antonelli et al., 2000; Burkard and Butler, 2000; Hilleren et al., 2001; Houseley et al., 2006; Saguez et al., 2005); 2) mRNAs retained in the nucleus due to mutations of mRNA export factors undergo decay by the decay of RNA in nucleus (DRN) system (Das et al., 2003); 3) cytoplasmic mRNAs with translation defects are degraded through cytoplasmic surveillance mechanisms. mRNAs containing premature termination codons, mRNAs lacking translational termination codons, and mRNAs impaired in translational elongation are degraded by the nonsense-mediated decay [NMD], non-stop decay [NSD], and the no-go decay [NGD] pathways, respectively (reviewed in Garneau et al., 2007). All these quality controls ensure that only properly generated mRNA is translated into functional protein.

2. Nuclear pre-mRNA metabolism

2.1 Biosynthesis and modification of nuclear pre-mRNA

Biosynthesis of mRNA starts with transcription. RNAPII, general transcription factors, and specific transcription factors are coordinately assembled onto the DNA to form the transcription machinery and begin synthesizing pre-mRNAs. During and after transcription, nascent transcripts are processed by three biochemically distinct reactions: 5′ capping, splicing, and 3′ end cleavage/polyadenylation.

Once a nascent transcript reaches 22-25 nt, a m^7GpppN cap structure is added to the 5′ end of the transcript. This cap structure is recognized by the nuclear cap-binding complex (CBC) which comprises Cbp20p and Cbp80p (Schroeder et al., 2004; Zenklusen et al., 2002). Binding of the cap structure by CBC is believed to stabilize the transcript byblocking the 5′-to-3′ exonucleases (Beelman and Parker, 1995; Das et al., 2003), and stimulate spliceosome assembly on the nascent pe-mRNA (Gornemann et al., 2005). During transcription, a dynamic complex comprising the small nuclear RNAs (snRNAs) U1, U2, U4, U5, and U6, and their associated proteins, is assembled stepwise onto the pre-mRNA to form the spliceosome, a machine that eliminates intron from the pre-mRNA. The spliceosome also affects the process of transcription (reviewed in Maniatis and Reed, 2002; Proudfoot et al., 2002), and recruits mRNA export factors to form an export-competent mRNP (reviewed in Reed, 2003; Reed and Hurt, 2002). Once transcription is complete, the 3′ end of the nascent transcript is endonucleolytically

cleaved at a specific site, and then 70-100 adenosines in yeast, or more than 200 adenosines in mammalian cells, are added to the cleaved 3′ end of the transcript. This poly(A) tail protects the transcript from 3′-to-5′ exonuclease digestion; facilitates export of the mature mRNA from the nucleus to the cytoplasm (Brune et al., 2005; Dunn et al., 2005); and forms a closed loop-structure with the 5′ end of the transcript promoting translation in the cytoplasm.

Together, these three processing reactions are coordinated with transcription and are also tightly coupled to each other as well as to the downstream events of gene expression. The processing of pre-mRNA ultimately results in the formation of a mature, translatable mRNA with protective structures at its 5′ and 3′ ends.

2.2 Export of mRNA

Processed, mature mRNAs need to be exported to the cytoplasm for translation. mRNA export is evolutionarily conserved and tightly coupled to transcription and processing (reviewed in Cullen, 2003b; Jensen et al., 2003; Reed, 2003; Reed and Hurt, 2002; Rodriguez et al., 2004). Currently, two distinct mRNA export pathways have been identified.

The major mRNA export pathway in yeast is diagrammed in Figure 1.1. During transcription elongation, the THO complex composed of Hpr1p, Tho2p, Mft1p, and Thp2p associates with the transcription machinery, facilitating the loading of Sub2p (a DEAD-box RNA-dependent ATPase) and Yra1p (an RNA binding export factor) along

the nascent mRNA molecule. The newly formed TREX complex (transcription and exporting complex) containing THO, Yra1p, and Sub2p is assembled onto the nascent mRNA to form an export-competent mRNP. Mex67p-Mtr2p, an export receptor heterodimer, is then recruited to displace Sub2p, binding Yra1p at the previous Sub2p binding site. This Mex67p-containing mRNP is targeted to the nuclear periphery by the Thp1p-Sac3p complex, a NPC- (nuclear pore complex) associated RNA-binding factor (Rodriguez et al., 2004). It has been reported that Mex67p-Mtr2p can also be recruited by the association of Sus1p, a component of the SAGA histone-acetylase complex involved in transcription initiation, with the NPC-associated Thp1p-Sac3p complex (Rodriguez-Navarro et al., 2004). This alternative Mex67p-Mtr2p recruiting unit, TREX-2, comprises Sac3p, Thp1, Sus1p and Cdc31p. Transient interaction of Mex67p-Mtr2p and a subset of nucleoporins containing repeated phenylalanine-glycine (FG) motifs mediates the translocation of the export-competent mRNP to the NPC. After being transported through the nuclear pores, Mex67p is thought to be removed by Dbp5, a conserved DEAD-box ATPase/RNA helicase associated with the cytoplasmic face of NPCs, from the mRNPs (Lund and Guthrie, 2005). Activation of Dbp5 requires Gle1p, a cytoplasmic nucleoporin, and inositol hexaphosphate (IP6), a signaling molecule (Alcazar-Roman et al., 2006; Kohler and Hurt, 2007; Weirich et al., 2006).

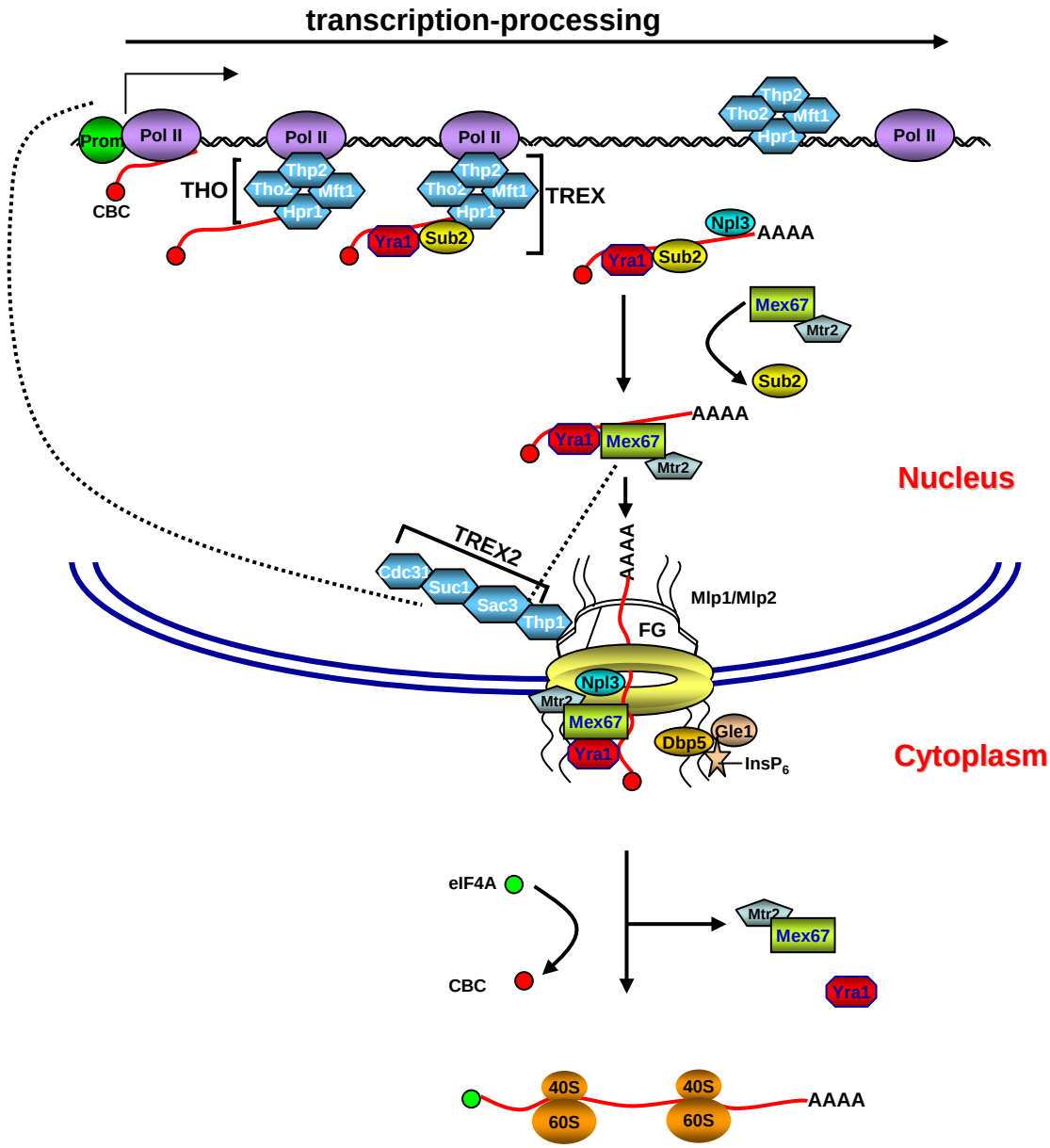

Figure 1.1 Current model of yeast mRNA nuclear export (modified from Kohler and Hurt, 2007; Rodriguez and Stutz, 2004). Details are described in the text.

In higher eukaryotic cells, most cellular mRNAs exit the nucleus via the pathway dependent on TAP, the metazoan homolog of Mex67p. However, a small but significant subset of cellular mRNAs exits the nucleus via a pathway specifically requiring CRM1, a nuclear export receptor for proteins that contain a leucine-rich nuclear export signal (reviewed in Hutten and Kehlenbach, 2007). This subset of cellular mRNAs includes: IFN-α mRNA (Kimura et al., 2004), cyclin D1 mRNA (Culjkovic et al., 2006), cyclooxygenase COX-2 mRNA (Jang et al., 2003), mRNAs containing AU-rich elements such as the c-fos mRNA (Brennan et al., 2000; Higashino et al., 2005), mRNAs induced in activated T cells such as the CD83 mRNA (Prechtel et al., 2006; Schutz et al., 2006), and the tra-2 mRNA in *C. elegans* (Kuersten et al., 2004). Unlike the TAP-dependent pathway, the CRM1-dependent pathway is dependent on Ran-GTP. The CRM1-dependent pathway is also responsible for the export of several U snRNAs, all rRNAs, SRP (signal recognition particle) RNA, and exogenous viral unspliced or spliced mRNAs (reviewed in Cullen, 2003b; Hutten and Kehlenbach, 2007; Rodriguez et al., 2004)). In yeast, it is not known whether Crm1p, the homolog of metazoan CRM1, also mediates export of cellular mRNAs. Currently, it is unclear what determines whether an mRNA is exported from the nucleus by the TAP/Mex67p-dependent pathway or the CRM1-dependent pathway. However, since CRM1 itself is not an RNA-binding protein, and all of the CRM1-mediated RNA export is thought to be mediated by adaptor proteins bearing leucine-rich nuclear export signals.

2.3 Regulated pre-mRNA export

Under normal conditions, endogenous pre-mRNAs are retained in the nucleus. Pre-mRNA leakage happens when pre-mRNAs accumulate due to splicing mutations or impairment of splicing factors (Legrain and Rosbash, 1989; Lopez and Seraphin, 1999; Rain and Legrain, 1997). In some circumstances, cells allow export of partially spliced or unspliced pre-mRNAs to the cytoplasm, e.g. unspliced virus RNA is exported from the nucleus to the cytoplasm in the infected host cell. The virus makes use of the nuclear export and translation systems of the host cell for its replication. The spliced virus RNA exits the nucleus by the same mechanism used by spliced cellular mRNAs. However, the unspliced virus RNA uses specific mechanisms to be exported.

In simple retroviruses, e.g., the simian type D retrovirus Mason Pfizer Monkey Virus (MPMV), export of its unspliced genomic RNA relies on the direct interaction of *cis*-acting RNA elements with cellular factors. The constitutive transport element (CTE) in MPMV interacts with TAP/Mex67p and thus results in constitutive export of the intron-containing virus RNA (Bachi et al., 2000; Gruter et al., 1998; Pasquinelli et al., 1997; Saavedra et al., 1997). Interestingly, a CTE is also found in a cellular intron-containing mRNA. An alternatively spliced cellular TAP pre-mRNA contains a CTE and is exported via the CTE-TAP-mediated mechanism (Li et al., 2006).

Unlike simple retroviruses, complex retroviruses make use of the CRM1-dependent mechanism to export their unspliced or incompletely spliced viral RNAs. For example, the HIV-1 retrovirus encodes a CRM1 recruiting protein Rev, a protein

containing a nuclear export signal. Unspliced or incompletely spliced HIV-1 mRNAs bind Rev with a structured RNA sequence present in these RNAs, the Rev responsive element (RRE) (Hadzopoulou-Cladaras et al., 1989; Hammarskjold et al., 1989; Pollard and Malim, 1998). The resulting mRNP complex is exported by virtue of the nuclear export signal in Rev which interacts with the cellular export receptor CRM1 (Fornerod et al., 1997; Neville et al., 1997). In addition to the Rev protein which is encoded by all members of the lentivirus family, there are other adapter proteins that recruit CRM1 to facilitate viral pre-mRNA export, such as the distinct human T-cell leukemia virus Rex protein (Bogerd et al., 1998) and the K-Rev protein encoded by a family of unrelated human endogenous retroviruses termed the HERV-Ks (Yang et al., 1999).

2.4 Quality control of pre-mRNAs

Intron containing pre-mRNAs in yeast can be processed into mRNAs, undergo nuclear degradation or be exported for translation or degradation in the cytoplasm. However, pre-mRNAs are seldom detected in the cytoplasm, because eukaryotic cells have evolved a tight quality control system to prevent their accumulation outside the nucleus. This quality control is accomplished by RNA nuclear retention and the nuclear turnover system. Moreover, pre-mRNAs leaked to the cytoplasm are degraded by the NMD pathway.

2.4.1 Mechanism of nuclear retention of intron containing pre-mRNA

It is still a mystery how the cell can distinguish between intron-containing pre-mRNAs, spliced mRNAs and intron-free mRNAs to ensure the first are retained in the nucleus while the latter two are exported to the cytoplasm. Since all factors involved in nuclear export of mRNAs in yeast bind indiscriminately to both intron-containing and intron-free mRNAs (Strasser and Hurt, 2001), it is unlikely that pre-mRNAs are retained by differential nuclear export mechanisms. Several studies have shown that pre-mRNA retention is linked to splicing, which might kinetically out-compete export of pre-mRNA. In addition, components of the nuclear pore also contribute to active retention of pre-mRNAs.

2.4.1.1 Association of splicing factors with pre-mRNA

An intact 5′ splice site (5′SS) and branchpoint are required for nuclear retention of pre-mRNAs (Legrain and Rosbash, 1989; Rain and Legrain, 1997). Moreover, several splicing factors, including Prp6p, Prp9p, U1 snRNA, and Mud2p are required for pre-mRNA retention (Legrain and Rosbash, 1989; Lopez and Seraphin, 1999; Rain and Legrain, 1997). Mutations in these factors lead to inefficient splicing and leakage of pre-mRNAs into the cytoplasm. However, there is also evidence pointing towards active retention of pre-mRNAs via a splicing factor. Mutants of *MSL5*, a gene coding for the branchpoint-binding protein BBP/SF1, which is a component of the splicing commitment

complex, lead to a strong defect in nuclear retention of pre-mRNAs while having only small effects on splicing *in vivo* and no effect *in vitro* (Rutz and Seraphin, 1999; Rutz and Seraphin, 2000). All these factors, except Prp6, are involved in early steps of intron recognition that precede assembling of the functional spliceosome (Kramer, 1996). Therefore, blocking early steps in splicing either in *cis* or *trans* can impair the nuclear retention of unspliced mRNA.

2.4.1.2 Active retention of pre-mRNA by the nuclear pore

There is evidence that some nuclear pore proteins help retain unspliced pre-mRNAs in the nucleus. These NPC components include Mlp1p (myosin-like protein 1), Mlp2p (paralogue of Mlp1p), Pml1p (pre-mRNA leakage), and Pml39p (pre-mRNA leakage, 39kDa). Deletion of any one of these factors impairs pre-mRNA retention but has no effect on splicing (Dziembowski et al., 2004; Galy et al., 2004; Palancade et al., 2005; Vinciguerra et al., 2005). Moreover, *PML39* deletion leads to a specific leakage of unspliced mRNAs that is not enhanced by *MLP1* deletion, suggesting that Pml39p is an upstream effector of the Mlp-controlled pre-mRNA retention pathway (Palancade et al., 2005). All reports of the active retention of unspliced pre-mRNAs used reporter systems, and the role of active processes in retaining endogenous pre-mRNA is still unstudied.

2.4.2 Pre-mRNA turnover in the nucleus

Nuclear pre-mRNA, together with mRNAs retained due to defective nuclear export, is degraded by a nuclear turnover system. These nuclear pre-mRNAs and mRNAs can be degraded from the 3′-to-5′ or the 5′-to-3′ directions. In 3′-to-5′ decay, RNA is degraded by the exosome, a complex of at least eleven polypeptides (Bousquet-Antonelli et al., 2000). In 5′-to-3′ decay, RNA degradation involves an unknown decapping activity stimulated by the Lsm2-8p complex (Kufel et al., 2004), followed by exonucleolytic decay by Rat1p, an essential nuclear exoribonuclease, (Johnson, 1997). In addition to direct 5′-to-3′ and 3′-to-5′ decay mechanisms, nuclear pre-mRNAs containing a specific doublestranded structure are cleaved by the nuclear endoribonuclease Rnt1p (Danin-Kreiselman et al., 2003).

Which decay pathway predominates in the nucleus appears to depend on the specific features of the substrate mRNA. Aberrant pre-mRNA generated by transcriptional mistakes or mutations in transcription factors are destroyed by the nuclear exosome (Houseley et al., 2006; Saguez et al., 2005). Uncapped pre-mRNA can undergo decay by the Rat1p-mediated 5′-to-3′ pathway (Saguez et al., 2005). Unspliced pre-mRNA that fail to assemble spliceosomes, splicing intermediates, and transcripts with abnormal 3′ ends or poly(A) tails are degraded by the nuclear exosome (Bousquet-Antonelli et al., 2000; Burkard and Butler, 2000; Hilleren et al., 2001). Downstream fragments from 3′ cleavage of the nascent RNA are degraded by the Rat1p-mediated 5′-to-3′ pathway (Saguez et al., 2005). mRNAs that are restricted to the nucleus because of

export defects can be degraded through both the 5′-to-3′ and the 3′-to-5′ pathways (Das et al., 2003). Finally, pre-mRNA containing appropriate recognition motifs can be cleaved by the endonuclease Rnt1p (Danin-Kreiselman et al., 2003).

Even though most inappropriately processed pre-mRNAs are degraded in the nucleus, 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). Splicing-defective reporter pre-mRNAs and splicing intermediates that are exported to cytoplasm are subsequently degraded through the general 5′-to-3′ pathway by Xrn1p, the cytoplasmic counterpart of Rat1p (Hilleren and Parker, 1999), instead of the NMD pathway.

3. Cytoplasmic mRNA degradation

3.1 Overview of mRNA fate in the cytoplasm and significance of mRNA degradation

Once in the cytoplasm, mRNA can engage the translational apparatus and produce protein; be directly destroyed by a cytoplasmic mRNA decay system without entering the translation pool; and be sequestered into a storage pool for future usage (translation or decay). Translatable cytoplasmic mRNAs are always in a kinetic balance or competition between translation and mRNA decay. Most mRNAs are degraded by a translation-dependent mechanism, but some mRNAs are degraded by translation-independent mechanisms.

The process of mRNA decay has a significant biological role in gene expression. mRNA decay rates are regulated in response to various environmental stimuli, specific hormones, stage of the cell cycle, viral infection, or cell differentiation. Altered control of mRNA decay rates results in aberrant gene expression and human disease, including cancer, immunological disorders and coronary disease (Wilusz et al., 2001). In addition, mRNA decay also assesses the accuracy of mRNA biogenesis and ensures elimination of aberrant transcripts. Therefore, mRNA decay controls both the quantity and quality of mRNAs. In doing so, it affects the final quantity and quality of the protein products. Thus, it is important to understand how the decay rates of different mRNAs are controlled and how aberrant RNAs are recognized and targeted for desctruction.

3.2 Cytoplasmic mRNA decay pathways

3.2.1 General decay pathways

mRNA degradation is a regulated process in eukaryotic cells. Different mRNAs are degraded by distinct mechanisms, and each mechanism requires a specific set of regulators and catalytic enzymes. In the yeast *S. cerevisiae*, most wild-type mRNAs are degraded through two general and functionally redundant mechanisms: the deadenylation-dependent 5´-to-3´ pathway and the exosome dependent 3´-to-5´ pathway (Cao and Parker, 2001; Coller and Parker, 2004; Jacobs Anderson and Parker, 1998) (Figure 1.2A). In both pathways, the initial event is the shortening of the poly(A) tail to

10 to 12 A's (Decker and Parker, 1993; Muhlrad et al., 1995). This deadenylation reaction is carried out by two functionally redundant deadenylases, the Ccr4p/Pop2p/Notp complex and the Pan2p/Pan3p complex (Chen et al., 2002; Daugeron et al., 2001; Tucker et al., 2002; Tucker et al., 2001). Deadenylation is the rate-limiting step in mRNA decay. After poly(A) shortening, the transcript can be degraded either through the 5′-to-3′ or through the 3′-to-5′ mechanism. In the 5′-to-3′ decay pathway, transcripts are decapped by the Dcp1p/Dcp2p complex (Beelman et al., 1996; Dunckley and Parker, 1999). In this reaction, the m^7G capped nucleotide of the mRNA substrate is cleaved to yield the products m^7GDP and a 5′-monophosphorylated mRNA. Subsequently, the 5′-monophosphorylated mRNA is rapidly digested by the 5′-to-3′ exoribonuclease, Xrn1p (Hsu and Stevens, 1993). In the 3′-to-5′ decay pathway, transcripts are further deadenylated and then digested in the 3′-to-5′ direction by the cytoplasmic exosome, a ten-subunit exoribonuclease complex (Mitchell et al., 1997). In addition to the exosome, the 3′-to-5′ decay pathway requires at least two cofactors, Ski7p and the Ski2p/Ski3p/Ski8p complex (Araki et al., 2001; Benard et al., 1999; Jacobs Anderson and Parker, 1998; van Hoof et al., 2000b). The m^7GDP generated by the Dcp1p/Dcp2p decapping enzyme in the 5′-to-3′ decay pathway and the capped oligonucleotides generated by the exosome in the 3′-to-5′ decay pathway are cleaved by the scavenger decapping enzyme (Dcps) to produce m^7GMP (Liu et al., 2002; van Dijk et al., 2003). Inactivation of both general decay pathways leads to a synthetic lethal phenotype (Jacobs Anderson and Parker, 1998), suggesting that mRNA decay is an essential process in yeast.

3.2.2 Specific mRNA decay pathways

In addition to these two general decay pathways, three specific decay pathways have also been identified in yeast. These pathways serve, at least in part, as surveillance mechanisms that target unique classes of defective mRNAs, including mRNAs containing premature translation termination codons (nonsense-mediated mRNA decay [NMD]) (Amrani et al., 2006b; Jacobson and Peltz, 2000) (Figure 1.2B), mRNAs lacking translation termination codons (non-stop decay [NSD]) (Frischmeyer et al., 2002; van Hoof et al., 2002) (Figure 1.2C), and mRNAs impaired in translation elongation (no-go decay [NGD]) (Doma and Parker, 2006) (Figure 1.2D). Substrates of the NMD pathway include mRNAs transcribed from nonsense alleles or bearing transcription, splicing or editing errors; mRNAs with upstream open reading frames; intron-containing pre-mRNAs that have entered the cytoplasm; mRNAs with initiator AUGs in a poor context ("leaky scanning"); and mRNAs with extended 3′ untranslated reagens (3′ UTRs) (He et al., 2003). While these pathways each use a unique set of specific factors in a translation-dependent manner to identify their target mRNAs, they also employ the enzymes of the general 5′-to-3′ or 3′-to-5′ decay pathways to carry out mRNA degradation. In the NMD pathway, mRNA degradation requires three regulatory factors, Upf1p, Nmd2p (Upf2p) and Upf3 (Cui et al., 1995; He et al., 1997; He and Jacobson, 1995; Lee and Culbertson, 1995; Leeds et al., 1992), and generally proceeds from decapping by the Dcp1p/Dcp2p complex to Xrn1p-catalyzed 5′-to-3′ decay without prior poly(A) tail shortening (Cao

and Parker, 2003; Muhlrad and Parker, 1994). In the NSD pathway, mRNAs are degraded directly from the 3′-end to the 5′-end by the exosome with the aid of Ski7p and the Ski2p/Ski3p/Ski8p complex, bypassing the deadenylation step (Frischmeyer et al., 2002; Inada and Aiba, 2005; van Hoof et al., 2002). In the NGD pathway, degradation of transcripts requires Dom34p and Hbs1p and initiates endonucleolytically. The resulting 5′ and 3′ fragments are then degraded by the exosome and Xrn1p, respectively (Doma and Parker, 2006).

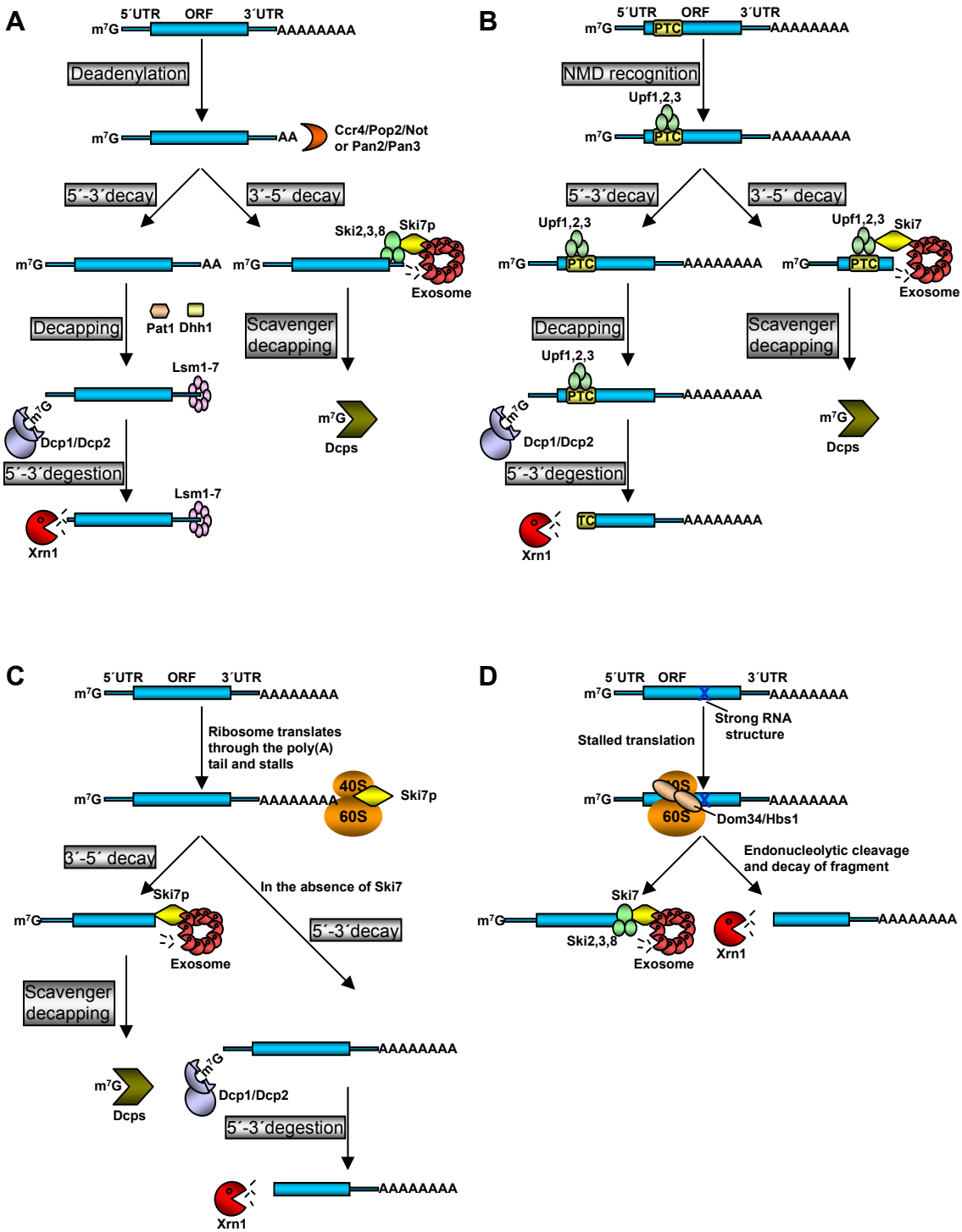

Figure 1.2 Yeast mRNA decay pathways. A. General mRNA decay pathway. B. Nonsense-mediated decay pathway. C. Non-stop decay pathway. D. No-go decay pathway. (modified from Garneau and Wilusz, 2007). Details are described in the text.

4. Regulation of mRNA decay

4.1 *Cis*-acting elements

The substrate status of a transcript for the different decay pathways can be regulated by specific *cis*-acting elements and their respective *trans*-regulatory RNA-binding factors. The former include sequences accelerating or decelerating the decay rate of the host mRNAs. These sequences are defined as "destabilizer elements" and "stabilizer elements", respectively.

4.1.1 Destabilizer elements

Cis-acting sequences that accelerate the decay rates of mRNAs are referred to as "destabilizer elements". They have been identified by their ability to promote rapid decay when transferred to an appropriate location within inherently stable mRNAs, and they have also been identified by mutational analysis. They are present in unstable yeast mRNAs including *MFA2*, *MATα1*, *HIS3*, *STE3*, *SPO13*, *PPR1*, *SDH1, HIS3, Ip, OLE1* and *HTB1* (Caponigro and Parker, 1996; Cereghino et al., 1995; Gonzalez and Martin, 1996; Heaton et al., 1992; Herrick and Jacobson, 1992; LaGrandeur and Parker, 1999; Muhlrad and Parker, 1992; Parker and Jacobson, 1990). These elements are located in the 5′ UTR, the coding region, or the 3′ UTR. Strikingly, in all cases examined, destabilizer elements promote both rapid deadenylation and rapid decapping of the host mRNAs.

They are also proposed to decrease translation initiation (Cereghino et al., 1995; Gonzalez and Martin, 1996; Scheffler et al., 1998).

Most destabilizer elements have weak consensus sequences. However, a group of destabilizer elements containing 50-150 nt sequences rich in adenosines and uridines have been identified. The sequence elements are termed AREs (AU-rich elements) and are located in the 3′ UTRs of many but not all mRNAs. AREs are found in mammalian mRNAs encoding cytokines, proto-oncogenes and growth factors (Khabar, 2005), and also in yeast mRNAs (Vasudevan and Peltz, 2001). Although AREs are identified by their capacity to promote degradation of the mRNA, the presence of an ARE can also lead to the stabilization of an mRNA, depending on the cellular context and the precise stimulus (reviewed in Barreau et al., 2005). In addition, AREs have been reported to be able to regulate translation and mRNA export without affecting the abundance of the mRNA (Espel, 2005; Kontoyiannis et al., 1999; Vasudevan and Steitz, 2007).

AREs regulate stability of the mRNAs by binding to ARE-binding proteins (ARE-BPs). ARE-BPs have some binding specificity for target AREs, but they are also typically functionally redundant, or additive, or even sometimes antagonistic. For example, simultaneously decreasing two ARE-BPs (KSRP and BRF1) increases, additively, the stability of a reporter mRNA containing an ARE (Gherzi et al., 2004). On the contrary, ARE-BPs TTP and HuR compete for the same target ARE in that the former decreases and the latter increases stability of the reporter mRNA (Ming et al., 2001). ARE and ARE-BP complexes regulate the stability of target mRNAs in the following ways: 1) AREs directly interact with the exosome to trigger the 3′-to-5′ decay of the

mRNA (Anderson et al., 2006). 2) Several ARE-BPs, e.g. AUF1, TTP, and KSRP, interact with exosome and are required for exosome-mediated 3′-to-5′ decay (Chen et al., 2001; Gherzi et al., 2004). 3) ARE-BPs, e.g. KSRP and RHAU, bind both to PARN deadenylase and to the exosome to enhance 3′-to-5′ decay of their target mRNAs (Gherzi et al., 2004; Tran et al., 2004). 4) The ARE-BP, TTP, and its homolog BRF-1 associate with hDcp1, CCR4 deadenylase, and the exosome to activate both 3′-to-5′ and 5′-to-3′ decay of ARE-containing RNAs (Lykke-Andersen and Wagner, 2005). 5) In microRNA-guided mRNA degradation, TTP associates with Argonaute proteins to recruit the miR-16 microRNA to the ARE in TNF-α mRNA (Jing et al., 2005).

In addition to AREs, a destabilizer element containing UG-rich sequences has been identified in yeast. This UG-rich element is recognized by Puf proteins, a family of RNA-binding proteins, which promote the accelerated decay of the substrate mRNAs by directly binding Pop2p to recruit the deadenylase (Goldstrohm et al., 2006; Goldstrohm et al., 2007).

4.1.2 Stabilizer elements

Unlike destabilizer elements, just a few examples of "stabilizer elements" that block rapid mRNA decay have been identified. The coding region and the context of the start codon of the *PGK1* mRNA in yeast, and pyrimidine-rich elements in the 3′ UTR of α-globin, β-globin, and α-collagen mRNAs in human are required to make these RNAs stable (Garneau et al., 2007; LaGrandeur and Parker, 1999). In addition, a 68-nt region found 3′

of the final uORF4 of GCN4 mRNA can make a nonsense-containing mRNA resistant to NMD (Ruiz-Echevarria et al., 1998). A sequence identified in the 5′ UTR of *YAP1* mRNA appears to bind the poly(U)-binding protein (Pub1p) to inactivate NMD (Ruiz-Echevarria and Peltz, 2000).

4.2 mRNA decay enzymes and their regulatory factors

In addition to *cis*-control, degradation of mRNAs also requires a set of specific *trans*-acting factors. These *trans*-acting factors encompass catalytic enzymes and their associated regulatory proteins. They function together to remove the protective structures of mRNAs including the poly(A) tail and the cap structure and then rapidly destroy the transcript.

4.2.1 The mRNA deadenylating enzymes

Deadenylation is the first and the rate-limiting step in the two general 5′-to-3′ and 3′-to-5′ mRNA decay pathways. Three deadenylase complexes have been identified in eukaryotic cells: the CCR4/POP2/NOT complex, the PAN complex, and the PARN trimer complex.

The major deadenylase is the CCR4/POP2/NOT complex. It contains two nucleases, Ccr4p and Pop2p, and several accessory proteins, including Not1p-Not5p, Caf4p, Caf16p, Caf40p, and Caf130p (Denis and Chen, 2003; Tucker et al., 2001). This

deadenylase is conserved in yeast and metazoans (Albert et al., 2000; Temme et al., 2004). Ccr4p seems to be the predominant catalytic subunit of this deadenylase complex. It is a member of the Mg^{2+}-dependent Exo III family of nucleases (Dlakic, 2000). Mutations in the predicted catalytic residues in its C-terminal nuclease domain abolish Ccr4p activity in yeast, human, and frog cells (Baggs and Green, 2003; Chen et al., 2002; Tucker et al., 2002). In addition, a conserved leucine-rich repeat in Ccr4p is essential for binding the Pop2p subunit of the complex and also affects the exonuclease activity (Clark et al., 2004). The Pop2p subunit in this complex has also been shown to have deadenylase activity (Daugeron et al., 2001; Thore et al., 2003). Pop2p is a member of the RNase D family of exonucleases (Daugeron et al., 2001; Moser et al., 1997). The structure of yeast Pop2p closely resembles that of the DnaQ proofreading exonuclease (Thore et al., 2003). The function of Pop2 might be different under different conditions. For example, knockout (in yeast) or knockdown (in cultured fly cells) of Pop2p show deadenylation defects (Daugeron et al., 2001; Temme et al., 2004; Tucker et al., 2001). However, yeast strains containing full-length Pop2p with point mutations in its active site show no deadenylation phenotypic changes, and the catalytic activity of the recombined protein complex was not affected *in vitro* (Chen et al., 2002; Tucker et al., 2002; Viswanathan et al., 2004). These observations suggest that Pop2p might enhance the activity of Ccr4p under normal conditions and be responsible for deadenylation in some circumstances. The CCR4/POP2/NOT complex is mainly in the cytoplasm (Cougot et al., 2004; Huh et al., 2003; Temme et al., 2004; Tucker et al., 2002), and its activity is inhibited by Pab1p, the major poly(A)-binding protein (Tucker et al., 2002; Viswanathan et al., 2003).

The PAN complex consists of Pan2p and Pan3p and is also conserved from yeast to metazoans (Boeck et al., 1996; Brown et al., 1996; Uchida et al., 2004). Pan2p is a member of an RNase D family of exonucleases (Moser et al., 1997; Zuo and Deutscher, 2001). It is a Mg^{2+}-dependent enzyme, liberating 5′-AMP from the 3′ end of mRNAs and is believed to be the catalytic subunit of the complex (Lowell et al., 1992; Uchida et al., 2004). Pan3p appears to enhance and regulate Pan2p activity by directly interacting with Pab1p for general mRNAs or with Dun1p for a specific transcript (*RAD5* mRNA) (Hammet et al., 2002; Mangus et al., 2003; Sachs and Deardorff, 1992; Uchida et al., 2004). Pan3p also interacts with the mRNA export receptor Mex67p. This interaction might deliver the PAN complex to the nascent mRNP for initial deadenylation of the poly (A) tail (Ito et al., 2001). The PAN complex is not the main deadenylase and its main role is thought to be an initial shortening of poly(A) tails (Brown and Sachs, 1998; Yamashita et al., 2005). It functions for residual deadenylation when the predominant deadenylase is deleted (Tucker et al., 2001). The PAN complex is in the cytoplasm in yeast (Huh et al., 2003) and human cells (Uchida et al., 2004). Unlike the CCR4/POP2/NOT complex, the activity of the PAN complex requires that the poly(A)-binding protein (Pab1p in yeast, PABPC in metazoans) first binds to the poly(A) tail of the substrate transcript.

The third deadenylase complex is the PARN trimer (poly(A)-specific ribonuclease). Unlike the other two deadenylases, this deadenylase has only been identified in vertebrates, *C. elegans,* and *S. pombe* (Astrom et al., 1992; Korner and Wahle, 1997), but not in *S. serevisiae* or *Drosophila*. It is a member of the DEDD nuclease family (Zuo and Deutscher, 2001). PARN also releases 5′-AMP from the 3′ end

of target transcripts in a Mg^{2+}-dependent manner. Mutations in predicted catalytic residues in PARN inhibit enzyme activity both *in vitro* and *in* vivo (Lai et al., 2003; Moser et al., 1997; Ren et al., 2002). Like the CCR4/POP2/NOT complex, PARN is inhibited by PABPC (Korner and Wahle, 1997; Ren et al., 2002). PARN, but not the CCR4/POP2/NOT complex, is the major deadenylase in mammalian mRNA decay systems *in vitro* (Gao et al., 2000) and is required for default deadenylation in *X. laevis* oocytes (Korner et al., 1998). In addition, PARN seems to be required for the rapid deadenylation induced by TTP, an ARE-binding protein, and can affect the process of NMD (Lai et al., 2003; Lejeune et al., 2003). These observations suggest that PARN may be an important mRNA deadenylase in vertebrate cells.

4.2.2 Decapping factors

4.2.2.1 The mRNA decapping enzymes

Decapping is a critical step in both the general 5′-to-3′ and the NMD pathways in yeast (Coller and Parker, 2004). Decapping has also been implicated in mammalian NMD and ARE-mediated decay (Chen and Shyu, 2003; Fenger-Gron et al., 2005; Lejeune et al., 2003; Yamashita et al., 2005), and in microRNA-guided decay in *Drosophila* (Behm-Ansmant et al., 2006; Rehwinkel et al., 2005).

Genetic and biochemical experiments indicate that mRNA decapping in yeast is carried out by the Dcp1p/Dcp2p complex (Beelman et al., 1996; Dunckley and Parker,

1999; LaGrandeur and Parker, 1998; Steiger et al., 2003). Recent experimental evidence suggests that mRNA decapping in human cells is carried out by a structurally related complex (containing hDcp1p and hDcp2p) possessing one additional factor, Hedls (Fenger-Gron et al., 2005). Genetic, biochemical, and structural data indicate that Dcp2p is the catalytic subunit in both yeast and human cells (Beelman et al., 1996; Cohen et al., 2005; Dunckley and Parker, 1999; LaGrandeur and Parker, 1998; Lykke-Andersen, 2002; Piccirillo et al., 2003; Steiger et al., 2003; Van Dijk et al., 2002; Wang et al., 2002). Dcp2 proteins from different species all contain a conserved Nudix (nucleoside diphosphate linked moiety X) motif in their N-terminal domains, but are divergent in their C-terminal domains (Beelman et al., 1996; Cohen et al., 2005; Dunckley and Parker, 1999; LaGrandeur and Parker, 1998; Lykke-Andersen, 2002; Piccirillo et al., 2003; Steiger et al., 2003; Van Dijk et al., 2002; Wang et al., 2002). *In vitro*, the N-terminal domains of *S. cerevisiae, S. pombe*, and human Dcp2p cleave the cap structure at the 5′ end of mRNA (She et al., 2006; Van Dijk et al., 2002; Wang et al., 2002), releasing m^7GDP and 5′-monophosphorylated RNA, a preferred substrate for the Xrn1p 5′-to-3′ exoribonuclease. In contrast to the scavenger decapping enzyme, human and yeast Dcp2p do not bind to a free cap structure and prefer capped, longer mRNA substrates (Piccirillo et al., 2003; Steiger et al., 2003). Structural analysis reveals that the N-terminal domain of *S. pombe* Dcp2p contains two sub-domains, an all-helical N-terminal sub-domain required to bind Dcp1p, and a C-terminal sub-domain that is a classic Nudix fold of pyrophosphate hydrolytic enzymes (She et al., 2006). The Dcp1p subunit (including both isoforms [hDcp1a and hDcp1b] in humans) (Lykke-Andersen, 2002) is likely to perform an

essential regulatory function in the decapping complex. Deletion of *DCP1* results in a complete block to mRNA decapping *in vivo* (Beelman et al., 1996), and recombinant Dcp1p greatly stimulates the decapping activity of Dcp2p *in vitro* (Steiger et al., 2003). Initial bioinformatics analysis of hDcp1p and more recent high-resolution structural analysis of yeast Dcp1p show that Dcp1p is a member of a new protein family that contains an EVH1/WH1 (Ena/VASP homology 1/Wiskott-Aldrich syndrome protein homology 1) domain (Callebaut, 2002; She et al., 2004).

The precise function of Dcp1p in the process of mRNA decapping is currently unknown. The presence of an EVH1/WH1 domain and the observation that this domain is a protein-protein interaction module (Callebaut, 2002) suggest that Dcp1p may function at least in part to link the decapping enzyme to other decapping regulators. Interestingly, Dcp1p and Dcp2p from several different species have also been localized to special cytoplasmic foci called P-bodies (Cougot et al., 2004; Ingelfinger et al., 2002; Sheth and Parker, 2003). In yeast and mammalian cells, mRNA decay intermediates of the 5′-to-3′ decay pathway are also found in P-bodies, suggesting that some mRNA decapping could occur in these cytoplasmic structures (Cougot et al., 2004; Sheth and Parker, 2003).

4.2.2.2 Activators of mRNA decapping

Decapping is an irreversible step in mRNA decay that leads to rapid exonucleolytic degradation of the body of a transcript. Genetic data in yeast indicate that mRNA decapping is a highly regulated process (Coller and Parker, 2004). In addition to the

Dcp1p/Dcp2p decapping enzyme, mRNA decapping *in vivo* requires specific regulatory factors that are required for efficient decapping, but not for decapping per se. Accordingly, these factors are generally referred to as activators of mRNA decapping (Coller and Parker, 2004).

Several classes of mRNA decapping activators have been identified in yeast. One class includes Pat1p (Bonnerot et al., 2000; Wyers et al., 2000), a protein of unknown biochemical properties, Dhh1p, a member of the DEAD helicase family (Coller et al., 2001; Fischer and Weis, 2002), and the Lsm1p-Lsm7 proteins, a putative RNA-binding complex (Bouveret et al., 2000; Tharun et al., 2000; Tharun and Parker, 2001). These factors are all activators of the general 5′-to-3′ decay pathway. Inactivation or deletion of any of these factors results in the accumulation of deadenylated, capped transcripts, but does not eliminate the accumulation of 5′-to-3′ decay intermediates for both stable and unstable mRNAs (Bonnerot et al., 2000; Bouveret et al., 2000; Tharun et al., 2000; Tharun and Parker, 2001). The precise functions of these activators are still largely unknown, but the following observations have provided some insight: 1) these factors interact with each other and with the decapping enzyme complex (Bouveret et al., 2000; Tharun et al., 2000; Tharun and Parker, 2001), 2) these factors exhibit genetic interaction with translation initiation factors (Bonnerot et al., 2000; Wyers et al., 2000), 3) Pat1p, eIF4E, and Pab1p can co-exist in an mRNP complex and Pat1p, Lsm1-7p, Dcp1p, and Dcp2p can co-exist in a different mRNP complex (Bouveret et al., 2000; Tharun et al., 2000; Tharun and Parker, 2001), 4) two homologs of Dhh1p (the Me31b protein in *Drosophila* and the Xp54 protein in *Xenopus*) function in maternal mRNA storage

(Ladomery et al., 1997; Nakamura et al., 2001), and 5) mRNA decapping and translation are in competition (see below). Coller and Parker proposed that these factors activate mRNA decapping by promoting the exit of an mRNA from translation and the subsequent assembly of a decapping complex (Coller and Parker, 2004).

The second class of decapping activators includes the three highly conserved NMD factors, Upf1p, Nmd2p/Upf2p, and Upf3p (Jacobson and Peltz, 2000). Upf1p, a 109 kD protein, is a member of RNA helicase superfamily I and contains N-terminal Zn^{++}-finger/RING domains and a C-terminal helicase domain (Altamura et al., 1992; Kadlec et al., 2006; Koonin, 1992; Leeds et al., 1991). Purified Upf1p has RNA-binding, as well as RNA-dependent ATPase and RNA unwinding activities (Bhattacharya et al., 2000; Czaplinski et al., 1995). Nmd2p/Upf2p is an acidic 127 kD protein with multiple MIF4G (middle portion of eIF4G) domains (Cui et al., 1995; He and Jacobson, 1995; Kadlec et al., 2004). Upf3p is a basic 45 kD protein with an RNP domain and NLS- and NES-like sequences (Lee and Culbertson, 1995). Structural and biochemical analysis of the human Upf2/Upf3b protein complex demonstrates that the complex, but not Upf3b alone, has RNA-binding activity (Kadlec et al., 2004). Deletion of Upf1p, Nmd2p/Upf2p, or Upf3p results in the selective accumulation of capped nonsense-containing mRNAs, but does not eliminate decapping of these mRNAs (He and Jacobson, 2001). The precise functions of Upf1p, Nmd2p, and Upf3p in the decapping of nonsense-containing mRNAs remain largely unknown, but important clues have been derived from the following observations: 1) the Upf/Nmd factors interact with each other and Upf1p also interacts with the Dcp2p subunit of the decapping enzyme (He et al., 1996; He et al., 1997; He and

Jacobson, 1995), 2) these factors play a direct role in translation termination and physically interact with the Sup35p and Sup45p release factors (Czaplinski et al., 1998), 3) the Upf/Nmd factors are polyribosome-associated (Atkin et al., 1995; Atkin et al., 1997; Mangus and Jacobson, 1999), and 4) *in vitro*, premature termination is aberrant and this aberrant event is linked to Upf1p function (Amrani et al., 2004). Collectively, these data imply that the Upf/Nmd factors are recruited to ribosomes by recognizing premature translation termination codons and that they subsequently recruit and/or activate the decapping enzyme on nonsense-containing mRNA substrates.

The third class of decapping activators includes Edc1p, Edc2p, and Edc3p (Coller and Parker, 2004). These factors 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). Edc1p and Edc2p are small, RNA-binding proteins and, *in vitro*, both proteins stimulate the decapping activity of the Dcp1p/Dcp2p decapping enzyme (Schwartz et al., 2003; Steiger et al., 2003). Edc3p is an Lsm-like protein with a long C-terminal extension (Albrecht and Lengauer, 2004; Anantharaman and Aravind, 2004). Edc3p is conserved in eukaryotes and contains an N-terminal Lsm domain, a middle FDF domain that is enriched in polar and charged residues, and a C-terminal YjeF-N domain of unknown function that is also found in prokaryotic proteins (Albrecht and Lengauer, 2004; Anantharaman and Aravind, 2004). Genome-wide proteomic analyses show that Edc3p co-purifies with the Dcp1p/Dcp2p decapping enzyme and two-hybrid analysis indicates that Edcp3p interacts with Dcp1p, Dcp2p, and Dhh1p (Gavin et al., 2002; Ho et al., 2002;

Ito et al., 2001; Uetz et al., 2000). The Lsm domain of fly EDC3 mediates DCP1 binding and P-body targeting (Tritschler et al., 2007). Interestingly, depletion of Edc1p, Edc2p, or Edc3p 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. It was found that Edc3p directly controls *RPS28B* mRNA degradation through a deadenylation-independent decapping mechanism that requires the Dcp1p/Dcp2p decapping enzyme and a *cis*-regulatory element located in the 3′ UTR of *RPS28B* mRNA (Badis et al., 2004). As described below, our genome-wide microarray analysis together with other experiments showed that *YRA1* pre-mRNA is another substrate of Edc3p.

4.2.2.3 Interrelationships between translation and mRNA decapping

The cap structure is not only a substrate for the decapping enzyme (Piccirillo et al., 2003; Steiger et al., 2003; Van Dijk et al., 2002), but also a binding site for eIF4E, a factor that promotes translation initiation (von der Haar et al., 2004). These findings suggest that, *in vivo*, mRNA translation and decapping are in competition. One implication of the competition model is that factors that promote translation will act as inhibitors of mRNA decapping. Consistent with this notion, mutations in genes encoding eIF4E, eIF4G, and eIF4A that strongly inhibit translation initiation increase the rate of decapping of both stable and unstable mRNAs (Schwartz and Parker, 1999; Schwartz and Parker, 2000); depletion of the poly(A)-binding protein, Pab1p, which also has an important role in

translation initiation, promotes deadenylation-independent decapping (Caponigro and Parker, 1995); purified eIF4E can directly inhibit the activity of the decapping enzyme *in vitro* (Schwartz and Parker, 2000); and a temperature-sensitive allele of eIF4E (*cdc33-42*) can suppress the *in vivo* decapping defect caused by a *dcp1* partial loss-of-function allele (Schwartz and Parker, 2000). A second implication of the competition model is that some decapping activators function, at least in part, to repress mRNA translation. This idea is supported by the finding that recognition of a premature stop codon by Upf1p represses translation of nonsense-containing mRNAs. More recently, it has been shown that Pat1p and Dhh1p, two decapping activators of the general 5′-to-3′ decay pathway, are independently required for general translation repression under glucose or amino acid deprivation (Coller and Parker, 2005). In addition, Dhh1p and its human homolog can repress mRNA translation *in vitro* (Coller and Parker, 2005).

4.2.3 Factors involved in the 3′-to-5′ decay pathways

4.2.3.1 Exosome

The catalytic exosome and some regulators are required in the general 3′-to-5′ decay pathway, non-stop decay pathway and nonsense-mediated 3′-to-5′ decay pathway. The yeast exosome has nuclear and cytoplasmic forms sharing at least 10 essential components (Allmang et al., 1999b; Mitchell et al., 1997). Among these components, Rrp4p, Ski6p (Rrp41p) and Rrp44p (Dis3p) have 3′-to-5′ exoribonuclease activity *in*

vitro, and the other members (Rrp40p, Rrp42p, Rrp43p, Rrp45p, Rrp46p and Mtr3p) except Csl4p (Ski4p) have high sequence homology to the *Escherichia coli* 3′-to-5′ exoribonucleases, RNase PH and PNPase (for reviews see (Mitchell and Tollervey, 2000; van Hoof and Parker, 1999)). The nuclear exosome has additional subunits: Rrp6p, a protein also possessing 3′-to-5′ exoribonuclease activity (Allmang et al., 1999b; Briggs et al., 1998; Burkard and Butler, 2000), Rrp47p (Mitchell et al., 2003), a putative nucleic-acid-binding protein, as well as Mtr4p/Dob1p, a putative RNA helicase (van Hoof et al., 2000a). The cytoplasmic exosome degrades mRNAs, whereas the nuclear exosome processes small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs) and rRNAs, in addition to degrading aberrant nuclear RNA precursors of many types, including pre-mRNAs, pre-tRNAs and pre-rRNAs or mRNAs restrained in the nucleus (Allmang et al., 1999a; Jacobs Anderson and Parker, 1998; Mitchell et al., 1997).

4.2.3.2 SKI factors

Four activators contribute to exosome-mediated cytoplasmic general 3′-to-5′ pathway, non-stop decay and nonsense-mediated 3′-to-5′ decay: Ski2p, Ski3p, Ski8p, Ski7p (Araki et al., 2001; Jacobs Anderson and Parker, 1998; Mitchell and Tollervey, 2003; Takahashi et al., 2003; van Hoof et al., 2002; van Hoof et al., 2000b). Ski2p, Ski3p, Ski8p and Ski7p are a putative RNA helicase, a tetratricopeptide-repeat protein, a protein containing WD motif, and a putative GTPase, respectively (Benard et al., 1999; Matsumoto et al., 1993; Rhee et al., 1989; Widner and Wickner, 1993). Ski2p, Ski3p, and Ski8p form a

stable heterotrimeric complex in the cytoplasm (Brown et al., 2000). Within the Ski complex, Ski3p serves as a scaffold protein with its C terminus interacting with Ski8p, and the sub-C terminus interacting with Ski2p, while no direct interaction between Ski2p and Ski8p was found (Wang et al., 2005). The Ski2p/Ski3p/Ski8p complex may act to recruit the exosome to the mRNA or remodel the mRNP to allow access by the exosome (Jacobs Anderson and Parker, 1998). The other SKI factor, Ski7p, interacts with the Ski2p/Ski3p/Ski8p complex via its interaction with Ski8p and Ski3p but not Ski2p (Wang et al., 2005). Ski7p is a GTP-binding protein containing a C-terminal domain that resembles the GTPase domains of translation elongation factor EF1α and termination factor eRF3 (Benard et al., 1999; van Hoof et al., 2000b). However, the N-terminal domain, but not the C-terminal GTPase domain, of Ski7p is necessary and sufficient for exosome-mediated general 3′-to-5′ mRNA decay by interacting with the exosome and the Ski2p/Ski3p/Ski8p complex (Araki et al., 2001). Instead, the C-terminal domain of Ski7p is required for nonstop decay by binding to the empty A site on ribosomes stalled at the end of a transcript lacking a stop codon (van Hoof et al., 2002). Furthermore, Ski7p interacts with Upf1p and is required in the nonsense-mediated 3′-to-5′ mRNA decay in yeast (Takahashi et al., 2003).

5. Autoregulation of *YRA1*

Yra1p is an essential RNA-binding protein encoded by the spliced *YRA1* transcript. It is a small RNA-binding protein first identified on the basis of its potent RNA annealing

activity *in vitro* (Portman et al., 1997). It was subsequently shown to be essential for mRNA export (Strasser and Hurt, 2000) and to be recruited to mRNA co-transcriptionally (Abruzzi et al., 2004; Lei et al., 2001; Lei and Silver, 2002; Strasser and Hurt, 2000). As an adaptor, Yra1p recruits Mex67p, the mRNA export receptor, to form an export-competent mRNA. Current views about the role of Yra1p in RNA export are reviewed in the "Export of mRNA" section of this chapter. In addition, over-expressing Yra1p has a dominant–negative effect, arresting cell growth (Espinet et al., 1995). Recent data show that Yra1p is required for S phase entry and plays a role in DNA replication by recruiting Dia2p, a protein of the ubiquitin ligase family involved in DNA replication and genome stability (Swaminathan et al., 2007).

The *YRA1* gene is one of the 5% of budding yeast genes that undergo splicing. It contains a 766-nt intron, the second largest intron in the yeast genome; its intron is located far downstream of the translation initiation codon; and its branch-point region diverges from the consensus motif common to the vast majority of yeast intron-containing pre-mRNAs (Portman et al., 1997; Strasser and Hurt, 2000). Previous studies suggested that *YRA1* expression is controlled through an autoregulated negative feedback loop (Preker and Guthrie, 2006; Preker et al., 2002; Rodriguez-Navarro et al., 2002). The *YRA1* intron is required for this autogenous regulation of Yra1p expression, which causes a dominant negative growth phenotype when elevated. Replacement of the intron with the intron sequences from other genes can partially recover the autoregulation of *YRA1* (Rodriguez-Navarro et al., 2002). Guthrie and colleagues proposed that inefficient co-transcriptional splicing is required for maintaining the appropriate levels of Yra1p (Preker

and Guthrie, 2006). However, the underlying mechanism of *YRA1* autoregulation remains unknown.

6. Rationale of this work

The detection of physical interactions between Edc3p and the Dcp1p/Dcp2p decapping enzyme (Gavin et al., 2002; Ho et al., 2002; Ito et al., 2001; Uetz et al., 2000) strongly suggests that Edc3p plays a role in mRNA decay. To assess the role of Edc3p in mRNA decay, we analyzed the effect of *EDC3* deletion on global RNA accumulation using high-density genome-wide microarrays in the yeast model system. Our analysis identified only two transcripts, the *RPS28B* mRNA and the *YRA1* pre-mRNA, that are up-regulated when *EDC3* is deleted. The *RPS28B* mRNA codes for a 40S ribosomal protein (Lecompte et al., 2002) and the spliced product of *YRA1* pre-mRNA codes for Yra1p which has an essential function in an early stage of mRNA export, as reviewed above. Interestingly, in a similar screen, the *RPS28B* transcript was shown by the Jacquier group to be the sole differentially expressed mRNA they could detect in *edc3Δ* cells (Badis et al., 2004). Their experiments showed that Edc3p directly controls *RPS28B* mRNA degradation through a deadenylation-independent decapping mechanism that requires the Dcp1p/Dcp2p decapping enzyme and a *cis*-regulatory element located in the 3′ UTR of *RPS28B* mRNA. In addition, they found that: 1) the expression of the *RPS28B* gene is autoregulated through a negative feedback loop, 2) *RPS28B* autoregulation is dependent on the Edc3p-mediated degradation of its mRNA, and 3) the *RPS28B* protein *in vivo* binds to the *cis*-

regulatory element in the 3′ UTR of *RPS28B* mRNA. Both *YRA1* and *RPS28B* are autoregulated. Moreover, both have sequence anomalies: *YRA1* has a long, unusual intron and *RPS28B* has a long, unusual 3′ UTR. Finally, both are regulated by Edc3p.These observations raised our interest and led us to ask how Edc3p regulates the expression of *YRA1*.In the following studies, we focused on the effect of Edc3p on *YRA1* pre-mRNA and tested two hypotheses: that Edc3p is engaged in the autoregulation of *YRA1* by regulating the stability of the *YRA1* pre-mRNA and that regulation of Edc3p-mediated *YRA1* pre-mRNA degradation is achieved by the combined effects of *cis*-acting elements and *trans*-regulatory factors.

CHAPTER II

YRA1 Autoregulation Requires Nuclear Export and Cytoplasmic Edc3p-mediated Degradation of its Pre-mRNA

Running title: Transcript-specific degradation mediated by Edc3p

Attribution: Dr. Daniel Zenklusen carried out the fluorescent *in situ* hybridization (FISH) experiment.

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* exon1 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 post-transcriptional 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/Dcp2 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 deadenlylated 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 (non-stop 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 were carried out with *EDC3* and *edc3Δ* strains and differentially expressed transcripts were initially identified based on three stringent criteria. First, the hybridization signal values of a specific transcript in the wild-type and the *edc3Δ* strains had to have a relative change of at least 2-fold and an absolute change of at least 200 units. Second, these changes had to be reproducible in at least 80% of the independent replicate experiments. Third, these changes had to demonstrate statistically significant P values ≤ 0.05. To our surprise, this data analysis revealed that, among the 7839 potential transcripts analyzed, only a single transcript met these criteria. In *edc3Δ* cells, the *EDC3* mRNA itself was decreased more than 10-fold relative to its level in wild-type cells (Table 2.4), consistent with the fact that the *edc3Δ* strain harbors a complete *EDC3* deletion. Although this observation validated the overall experiment, we reasoned that our stringent criteria may have overlooked at least two classes of transcripts. For example, highly expressed transcripts are prone to signal saturation and intron-containing transcripts might be missed since the oligonucleotide probes on our arrays do not differentiate intron-containing pre-mRNA signals from mRNA signals. We, therefore, lowered the analysis stringency by eliminating the minimum 2-fold change requirement and reanalyzed our data. This new analysis identified four additional differentially

expressed transcripts in the *edc3Δ* strain: two transcripts showed increased expression and two others showed decreased expression (Table 2.4). One of the up-regulated transcripts is encoded by the *RPS28B* gene and codes for a 40S ribosomal protein (Lecompte et al., 2002). The other up-regulated transcript is encoded by the *YRA1* gene and codes for an hnRNP-like protein (Yra1p) involved in an early stage of mRNA export (Portman et al., 1997; Strasser and Hurt, 2000). The two down-regulated transcripts, encoded by the *URA1* and *URA4* genes, may well reflect our use of the *URA3* gene as a selectable marker for replacement of the *EDC3* coding region. *URA1*, *URA3*, and *URA4* all code for enzymes involved in uracil biosynthesis (Denis-Duphil, 1989) and the decreased expression of *URA1* and *URA4* transcripts in the *edc3Δ* strain is presumably related to the expression of *URA3*, not to the deletion of *EDC3*.

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 2.1A, deletion of *EDC3* had no effect on the level of *YRA1* mRNA, but resulted in a five-fold increase in *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 >60 min in *edc3Δ* cells and ~15 min in *EDC3* cells (Figure 2.1B). 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 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 2.2A). 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 2.2A, 2.2B,

and 2.2C). 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-labelled oligonucleotide probes to respectively detect *YRA1* intron and exon sequences. In wild-type cells, the exon signal was detected in the nucleus and cytoplasm, whereas the intron signal was mainly detected in the nucleus, co-localizing with the exon signal and likely reflecting nascent *YRA1* transcripts (Figure 2.3A, panels a,b,c). Compared to the *YRA1* exon and intron signals in wild-type cells, *edc3Δ*, *xrn1Δ, dcp1Δ, and xrn1Δedc3Δ* cells all showed significant increase in both the exon and intron signals in the cytoplasm. In addition, the cytoplasmic intron signals in these cells co-localized largely with the exon signals (Figure 2.3A, panels e-t). Interestingly, *dcp1Δ c*ells 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 (Figure 2.3A, panels q-t). 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 2.3B and 2.3C). 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 2.2D, *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.

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 observation 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 2.4A, introduction of extra copies of the intron-containing *YRA1* 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* exon1, intron, and exon2 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* exon1 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. First, replacement of *YRA1* exon1 with the *CYH2* or *MER2* exon1, or the *HIS3* coding sequence differentially affected pre-mRNA splicing efficiency. Substitution of *YRA1* exon1 with the shorter *CYH2* exon1 (285 nt vs. 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 2.4B). In contrast, substitution of *YRA1* exon1 by the comparably sized *MER2* exon1 (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 (Figures 2.4B and 2.5A). 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 (Figures 2.4C and 2.5B). Third, replacement of *YRA1* exon2 with the *CYH2*, *MER2* or *RPS51A* exon2 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 (Figures 2.4D and 2.5C). 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 2.4D). This is most likely due to increased stability of these chimeric mRNAs. These results indicate that sequences in exon1 and the intron of the *YRA1* pre-mRNA are required for Yra1p autoregulation and that these sequences have distinct functions. Sequences in exon1 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: a) a 252-nt internal deletion in exon1 promoted more efficient splicing of the *YRA1* pre-mRNA; b) 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 c) 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 2.4E).

YRA1 Exon1 Sequences Inhibit its Pre-mRNA Splicing in a Size-dependent But Sequence-independent Manner

Since *YRA1* exon1 appeared to regulate its pre-mRNA splicing through a size-dependent but sequence-independent mechanism (see above) we analyzed the effects of shortening *YRA1* exon1 or replacing exon1 coding sequences with their complementary sequences. This analysis revealed that incremental deletions of exon1 resulted in incremental increases in *YRA1* mRNA levels in *EDC3* and *edc3Δ* strains (Figure 2.6A). 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 2.6A). These data show that *YRA1* exon1 functions to inhibit pre-mRNA splicing in *cis*.

Figure 2.6A 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 exon1, suggesting that the inhibitory function of exon1 on *YRA1* pre-mRNA splicing is dictated by its length. Replacing the *YRA1* exon1 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 2.6B). Since the complementary sequences of exon1 function as well as the coding sequences the ability of *YRA1* exon1 to inhibit its pre-mRNA splicing must be dictated by its size, but not its primary sequence.

Our analysis of exon1 deletion mutants in the *edc3Δ* strain also revealed an inverse correlation between the levels of *YRA1* pre-mRNA and mRNA (Figure 2.6A). 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 exon1 functions primarily to inhibit pre-mRNA splicing in *cis* and thus results in nuclear export of the pre-mRNA. Alternatively, *YRA1* exon1 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* exon1. We used *yra1-N84,* a complete loss-of-regulation allele that contains a 252-nt deletion in the coding region of exon1 and encodes a pre-mRNA that is efficiently spliced, as evidenced by high levels mRNA but almost no pre-mRNA in wild-type, *upf1Δ*, *edc3Δ*, and *upf1Δedc3Δ* strains (Figure 2.6C). 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 2.6C). 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 2.6C). 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 2.6C). 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 exon2 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* 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* exon1. These results indicate that: a) the primary function of *YRA1* exon1 is to inhibit *YRA1* pre-mRNA splicing, not to promote *YRA1* pre-mRNA nuclear export and b) *YRA1* exon1 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 examined the effects of mutations in the *YRA1* gene on levels of *YRA1* pre-mRNA and mRNA in an *edc3Δ* background. We reasoned that some loss of function mutations may also fail to autoregulate. We first analyzed *yra1-1*, a ts allele whose encoded protein contains multiple amino acid substitutions and is defective in mRNA export even when cells are grown at room temperature (Strasser and Hurt, 2000). As shown in Figure 2.6D, when cells were grown at 25°C (t=0) or were shifted to 37°C for 30 min, *edc3Δyra1-1* cells accumulated significantly lower levels of *YRA1* pre-mRNA than *edc3ΔYRA1* cells. In contrast, under these two growth conditions, *edc3Δyra1-1* cells accumulated significantly higher levels of *YRA1* mRNA than *edc3ΔYRA1* cells. The lower *YRA1* pre-mRNA to mRNA ratios in *edc3Δyra1-1* cells indicate that the *yra1-1* allele is defective in

autoregulation and suggest that Yra1p regulates its own 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 2.5E). These data show that introduction of wild-type *YRA1* into *yra1Δ* strains recapitulated *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 2.6E). 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 2.6E). 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 branch-point 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 2.6E). 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 2.7A, compare t_0 samples). At 37°C, the same strains exhibited dramatically different *YRA1* expression patterns (Figure 2.7A), including: a) *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; b) *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 c) 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 with 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 2.7B), we conclud 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 and 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 2.7C). 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 2.7A). 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 *mex67-5edc3Δ* cells in 24 min (Figure 2.7A). 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, *YRA1* pre-mRNA is predominantly cytoplasmic in *edc3Δ* cells (Figure 2.3A) and since, in a *MEX67edc3Δ* background, *YRA1* pre-mRNA has a half-life of ≥60 min (Figure 2.1B), 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 2.7D shows that deletion of *UPF1* restored *YRA1* pre-mRNA levels at early time points and 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* resulted 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 2.7 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 2.8A). Since the human Crm1p homolog is implicated in the nuclear export of unspliced or incompletely spliced HIV RNAs in mammalian cells (Cullen, 2003a), 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 (Figure 2.8A and 2.8B). The increased accumulation of *YRA1* mRNA and the appearance of *YRA1* mRNA with 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 2.8C, 2.8D and 2.8E).

Leptomycin treatment also resulted in the accumulation of longer *YRA1* pre-mRNA transcripts in both *EDC3* and *edc3Δ* cells (Figure 2.8E). These longer pre-mRNA species hybridize with an oligonuclotide probe downstream of the mapped canonical poly(A) site (Figure 2.8B), 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 (Figures 2.3C and 2.8E-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 2.8E). 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 2.8A). 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 2.8C and 2.1B), 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 where such export occurs these transcripts are often degraded by the translation-dependent NMD pathway (He et al., 2003; He et al., 1993; 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: a) the involvement of the Dcp1p/Dcp2p complex and Xrn1p, two cytoplasmic ribonucleases (Figure 2.2A), b) the *in situ* detection of *YRA1* pre-mRNA in the cytoplasm of *edc3Δ, dcp1Δ,* or *xrn1Δ* cells (Figure 2.3A), c) the resistance of *YRA1* pre-mRNA degradation to inactivation or depletion of the nuclear exonucleases, Rat1p or Rrp6p (Figures 2.3B and 2.3C), and d) the unchanged levels of *YRA1* mRNA in *edc3Δ* cells (Figure 2.1), 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), 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 2.4) 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: a) Edc3p appears to exist in a complex with Dcp1p and Dcp2p *in vivo* (Gavin et al., 2002; Ho et al., 2002), b) Edc3p and Dcp1p have comparable numbers of molecules per cell (Huh et al., 2003), and c) simultaneous inactivation of Edc3p and mutation of *DCP1* or *DCP2* promotes 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 multi-step 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 2.4) 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 (Figures 2.6D and 2.6E). 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 2.6E), 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 2.6E), these findings also suggest that Yra1p inhibits *YRA1* pre-mRNA splicing by committing the transcript to nuclear export. A simple interpretation 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 Exon1

We have identified two functionally distinct *cis*-regulatory elements that are involved in *YRA1* autoregulation: exon1 inhibits *YRA1* pre-mRNA splicing and the intron is required for Edc3p-mediated *YRA1* pre-mRNA degradation (Figures 2.4, 2.5, and 2.6). Unexpectedly, splicing autoregulation by exon1 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 exon1 (Figure 2.6). These findings indicate that: exon1 exerts its inhibitory function at or before the first step of the splicing reaction, the sole autoregulatory role of exon1 is to inhibit *YRA1* pre-mRNA splicing, and exon1 *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 exon1.

The primacy of size, not sequence, for the splicing inhibition function of *YRA1* exon1 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 exon1 can result in more than 8-fold increases in the level of *YRA1* mRNA (Figure 2.6A), but internal deletions in the intron had little (<2-fold) or no comparable effect (Figure 2.4E, 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 2.4B and 2.4C; (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 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, co-transcriptional 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. a) Mex67p shuttles between the nucleus and the cytoplasm (Segref et al., 1997), b) 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 c) 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, 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 commiting 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, 2003a). 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 PROCESURES

Yeast Strains

All strains used in this study are listed in Table 2.1. Strains containing deletions of *EDC3*, *UPF1*, *DCP1*, *XRN1*, *SKI2*, *SKI7*, *CCR4*, or *RRP6* were constructed by gene replacement (Guthrie and Fink, 1991), using DNA fragments harboring the corresponding null alleles. Each genomic DNA deletion was confirmed by PCR analysis. Strains containing deletions of *DHH1*, *PAT1*, *LSM1*, or *LSM7* were purchased from Open Biosystems. Strains harboring the temperature-sensitive *rpb1-1*, *prt1-1*, or *sup45-2* alleles were constructed by the pop-in and pop-out technique (Guthrie and Fink, 1991). Strains harboring the temperature-sensitive *yra1-1* allele were constructed by plasmid shuffling (Guthrie and Fink, 1991).

Plasmids

All plasmids used in this study are listed in Table 2.2. *YRA1* exon1, intron, or exon2 chimeric alleles were all constructed through *in vivo* recombination in yeast cells as described previously (He et al., 1996). *YRA1* alleles harboring deletions of exon1, intron, or exon2 sequences, or containing insertions of a stem-loop structure, were generated by PCR and molecular cloning. *YRA1* alleles harboring mutations in the *YRA1* translation initiation codon, the 5′ splice site, the branch-point region, or the 3′ splice site were generated using the QuikChange Site-DirectedMutagenesis Kit (Stratagene). All *YRA1* alleles were confirmed by DNA sequencing.

Oligonucleotides

The oligonucleotides used in this study were obtained from Operon, Inc., and are listed in Table 2.3.

Cell Growth Conditions

Cells were grown in YPD medium (Microarray analysis and Figures 2.1, 2.2, 2.3A, and 2.7), or in synthetic complete (SC) medium lacking tryptophan (Figures 2.4, 2.5, 2.6A, 2.6B, 2.6C, and 2.6E-right side), uracil (Figures 2.3B, 2.3C and 2.6D), histidine (Figure 2.8B and 2.8D), uracil and tryptophan (Figure 2.6E-left side), or histidine and tryptophan (Figure 2.8E and 2.8F) to select for plasmids. Cultures (15 ml) not involving drug treatment or temperature shifts were grown at 30°C to an OD_{600} of 0.7 and harvested by centrifugation. Cell pellets were frozen on dry ice and then stored at -80°C until RNA was isolated. Cultures involving drug treatment were grown at 30°C to an OD_{600} of 0.7 in a large volume and then concentrated five-fold in the same medium. Where appropriate, the following were added to concentrated cultures: cycloheximide (100 μg/ml), leptomicin (100 ng/ml), or thiolutin (15μg/ml). Drug-treated cells (2ml) were harvested at different time points. For temperature shifts, cells were first grown at 25°C and then treated as described previously (He and Jacobson, 1995).

Microarray Analysis

Five independent expression profiling experiments were carried out for isogenic wild-type (HFY114) and *edc3Δ* (CFY25) strains using Affymetrix Yeast Genome S98 Arrays.

Microarray procedures, including RNA isolation, cRNA preparation, microarray hybridization, and data analysis, were as previously described (He et al., 2003), with the following modifications: First, all microarrays were analyzed with Affymetrix Microarray Suite 5.0 software. Second, a transcript was considered to be differentially expressed if it met two of three previously defined criteria, i.e., it must have an absolute change threshold of 200 units and a change P value ≤0.05. The entire data set can be found at: http://jacobsonlab.umassmed.edu/cgi-bin/pubcontents.cgi?pubcontents=2006-Feng.

Yeast Two-hybrid Analysis

Two-hybrid interactions between full-length Crm1p fused to the *LexA* DNA- binding domain and full-length Edc3p fused to the Gal4p activation domain were assayed as described previously (He et al., 1997).

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).

Analysis of mRNA Decay Rate

mRNA decay rates were measured as previously described (Herrick et al., 1990). RNA decay curves were generated by plotting log RNA levels versus the relative time of transcription shut off. k, the slope of the RNA decay curve, is determined by liear regression equation $\ln A_t = \ln A_0 - kt$ (A_0 is the RNA concentration at time 0, A_t is the RNA concentration at time t after transcription shut off). $t_{1/2} = 0.693/k$.

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 using Cy3 and Cy5 fluorochrome-conjugated oligonucletides as described at http://www.singerlab.org/protocols/insitu_yeast.htm.

ACKNOWLEDGMENTS

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ACCESSION NUMBERS

Microarray data described in this study have been deposited into the Gene Expression Omnibus public database under the accession number GSE6647.

FIGURE LEGENDS

Figure 2.1. *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 panels A and B blots were hybridized with probes complementary to *YRA1* and *SCR1* transcripts, with the latter serving as a loading control.

Figure 2.2. *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-m^7G immunoprecipitation and northern blotting. T: total input RNA, S: supernatant (uncapped), P: pellet (capped).

Figure 2.3. Edc3p-mediated *YRA1* Pre-mRNA Degradation Occurs in the Cytoplasm

(A) Localization of *YRA1* pre-mRNA in wild-type, *edc3Δ*, *xrn1Δ, xrn1Δedc3Δ, 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.

(B) Effect of inactivation of Rat1p on levels of *YRA1* pre-mRNA and mRNA. *Rat1-1* or *rat1-1xrn1Δ* cells were grown in SC minus uracil medium at 25°C and then shifted to 37°C for 30, 60, and 120 min. The levels of *YRA1* pre-mRNA and mRNA were analyzed by northern blotting.

(C) Effect of depletion of Rrp6 on the levels of *YRA1* pre-mRNA and mRNA. Cells of the indicated genotypes were grown in YEPD medium and the levels of *YRA1* pre-mRNA and mRNA in these cells were analyzed by northern blotting. Note that *rrp6Δ* cells accumulated higher levels of *YRA1* mRNA than *RRP6* cells. The simplest explanation for this observation is that, in wild-type cells, a fraction of the *YRA1* pre-mRNA that is committed to the splicing pathway is degraded by the nuclear exosome.

Figure 2.4. *YRA1* Autoregulation Requires Edc3p and Two Distinct Regulatory Elements Localized in Exon1 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* exon1 (B), intron (C), and exon2 (D) with different sequences on *YRA1* pre-mRNA and mRNA expression. A three-letter code was used to denote the order of exon1, intron, and exon2 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* exon1, intron, or exon2 on *YRA1* pre-mRNA and mRNA expression.

In panels B, C, D, and 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 panel A.

Figure 2.5. *Cis*-acting Determinants of *YRA1* Expression. Alterations of *YRA1* pre-mRNA and mRNA expression mediated by: (A) replacing *YRA1* exon1 with *MER2* exon1, (B) replacing the *YRA1* intron with the *MER2* intron; and (C) replacing *YRA1* exon2 with *MER2* exon2 were analyzed. YCp low-copy plasmids harboring a chimeric

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. Blots A, B, and C were hybridized to *MER2* exon1, intron, or exon2-specific probes, respectively. The positions of chimeric pre-mRNAs and mRNAs are indicated. Y: *YRA1*, and M: *MER2*. Blots were hybridized to a *SCR1* probe to serve as a loading control.

Figure 2.6. *YRA1* Exon1 and Yra1p are *cis*- and *trans*-acting Negative Regulators of *YRA1* Pre-mRNA Splicing

(A-C) and (E) Effects on the levels of *YRA1* pre-mRNA and mRNA engendered by: (A) internal deletions in *YRA1* exon1; (B) substituting *YRA1* exon1 coding sequences with their complementary sequences; (C) mutations in the 5′ splice site, the 3′ splice site, or the branchpoint region of the *YRA1* intron; and (E) 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 panel B, the replaced complementary sequence does not contain in-frame premature stop codons.

In panels A, B, C, and E 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 (panel A) or denoted by numbers in panels B, C, and E: 1: *wild-type*, 2: *upf1Δ,* 3*: edc3Δ,* 4: *upf1Δ edc3Δ,* 5: *EDC3 yra1Δ yra2Δ*

(YEplac112-YRA2), and 6: *edc3Δ yra1Δ yra2Δ (YEplac112-YRA2)*. In panel 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.

(D) Cells of the indicated genotypes were grown in SC minus uracil medium at 25°C and then shifted to 37°C. The levels of *YRA1* pre-mRNA and mRNA were analyzed by northern blotting.

Figure 2.7. *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 in *edc3Δ* cells.

(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 panel A were analyzed with a *YRA1* oligonucleotide probe that spans exon1 and exon2 (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 requires ongoing transcription.

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

In panels 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 panel 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 panel A is marked by *. Blots were hybridized to a *SCR1* probe to serve as a loading control.

Figure 2.8. Crm1p Mediates *YRA1* Pre-mRNP Export to the Cytoplasm

(A) Crm1p Interacts With Edc3p in the Yeast Two-hybrid System. Yeast plasmids harboring LexA(DB)-*CRM1* and Gal4(AD)-*EDC3* fusions were co-transformed into the L40 tester strain. Transformants were selected and the β-galactosidase activity of individual transformants was assayed on plates containing X-Gal.

(B) 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

(C) 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 panel A were analyzed with the same set of oligonucleotide probes used in Figure 2.7B.

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

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

In panels B, D, E, and F, yeast strains of the indicated genotypes (all harboring the *CRM1-T539C* allele*)* were treated with leptomycin and the effect on *YRA1* pre-mRNA and mRNA were analyzed by northern blotting. In panel D, thiolutin and leptomycin were simultaneously added to *EDC3* and *edc3Δ* cells. In panel E, a YCp plasmid harboring the *YRA1* gene or its *yra1-m5SS* allele, as well as the empty vector, were introduced into *EDC3* and *edc3Δ* strains. In panel F, 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.

FIGURE 2.1

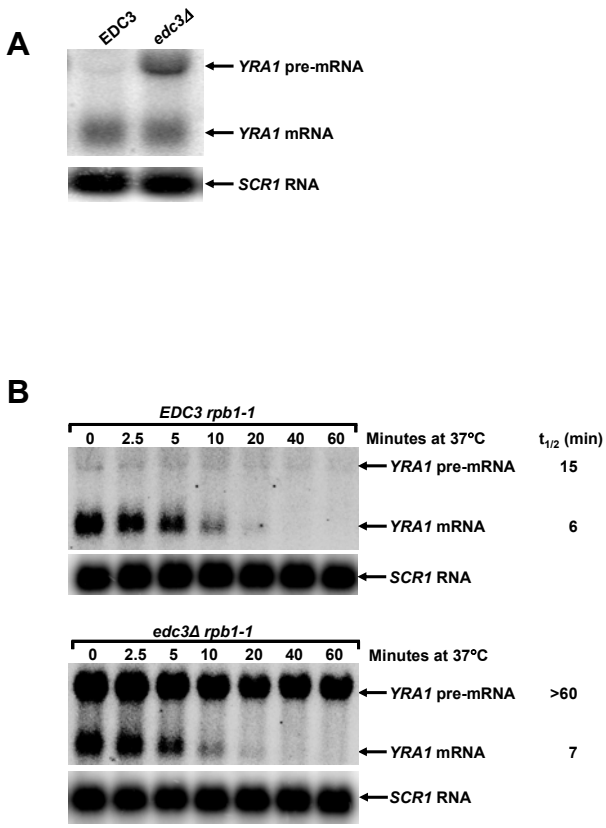

A
EDC3
edc3Δ
YRA1 pre-mRNA
YRA1 mRNA
SCR1 RNA
B
EDC3 rpb1-1
0 2.5 5 10 20 40 60
Minutes at 37°C
t1/2 (min)
YRA1 pre-mRNA
15
YRA1 mRNA
6
SCR1 RNA
edc3Δ rpb1-1
0 2.5 5 10 20 40 60
Minutes at 37°C
YRA1 pre-mRNA
>60
YRA1 mRNA
7
SCR1 RNA

FIGURE 2.2

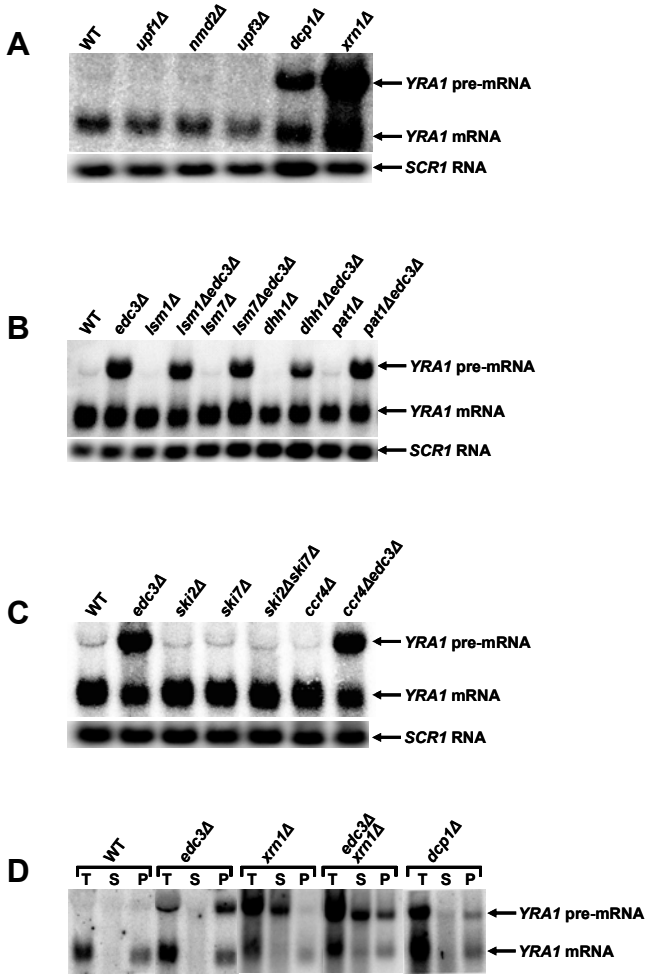

A
WT
upf1Δ
nmd2Δ
upf3Δ
dcp1Δ
xrn1Δ
YRA1 pre-mRNA
YRA1 mRNA
SCR1 RNA
B
WT
edc3Δ
lsm1Δ
lsm1Δedc3Δ
lsm7Δ
lsm7Δedc3Δ
dhh1Δ
dhh1Δedc3Δ
pat1Δ
pat1Δedc3Δ
YRA1 pre-mRNA
YRA1 mRNA
SCR1 RNA
C
WT
edc3Δ
ski2Δ
ski7Δ
ski2Δski7Δ
ccr4Δ
ccr4Δedc3Δ
YRA1 pre-mRNA
YRA1 mRNA
SCR1 RNA
D
WT
edc3Δ
xrn1Δ
edc3Δ xrn1Δ
dcp1Δ
T S P
YRA1 pre-mRNA
YRA1 mRNA

FIGURE 2.3

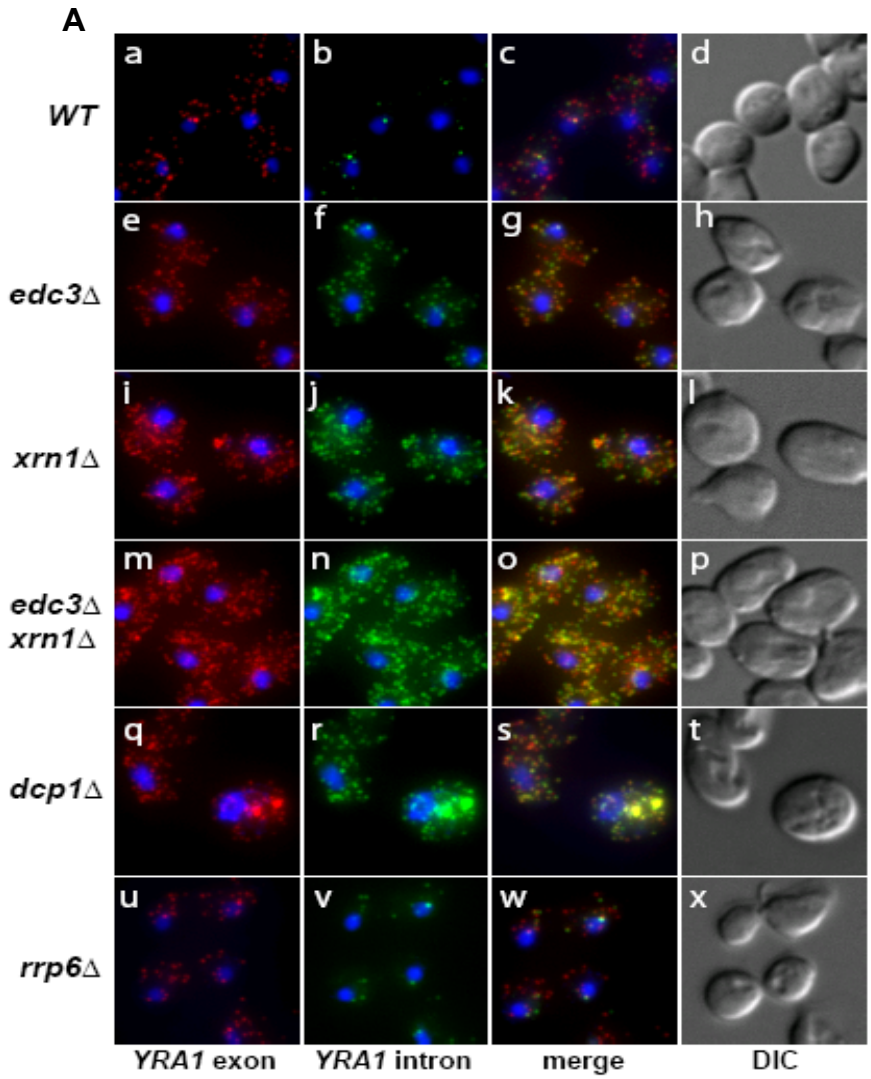

A
a
b
c
d
WT
e
f
g
h
edc3Δ
i
j
k
l
xrn1Δ
m
n
o
p
edc3Δ
xrn1Δ
q
r
s
t
dcp1Δ
u
v
w
x
rrp6Δ
YRA1 exon
YRA1 intron
merge
DIC
B
rat1-1
rat1-1 xrn1 Δ
0
30
60
120
0
30
60
120
Minutes at 37° C
YRA1 pre-mRNA
YRA1 mRNA
C
WT
edc3 Δ
rrp6 Δ
rrp6 Δ edc3 Δ
YRA1 pre-mRNA
YRA1 mRNA

FIGURE 2.4

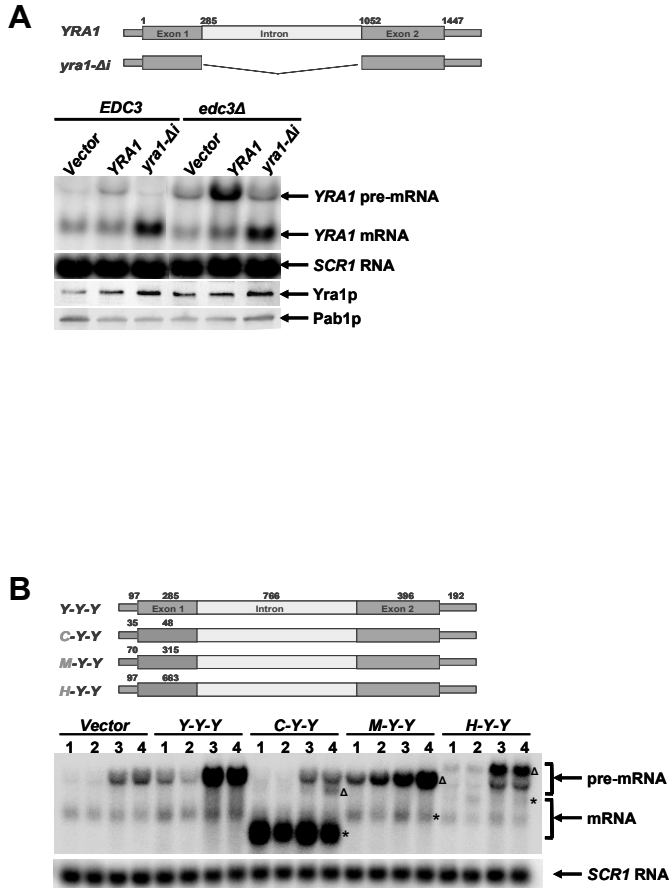

A
YRA1
yra1-Δi
1
285
1052
1447
Exon 1
Intron
Exon 2
EDC3
edc3Δ
Vector
YRA1
yra1-Δi
YRA1 pre-mRNA
YRA1 mRNA
SCR1 RNA
Yra1p
Pab1p
B
Y-Y-Y
C-Y-Y
M-Y-Y
H-Y-Y
97
285
766
396
192
35
48
70
315
663
Vector
pre-mRNA
mRNA
SCR1 RNA

FIGURE 2.4

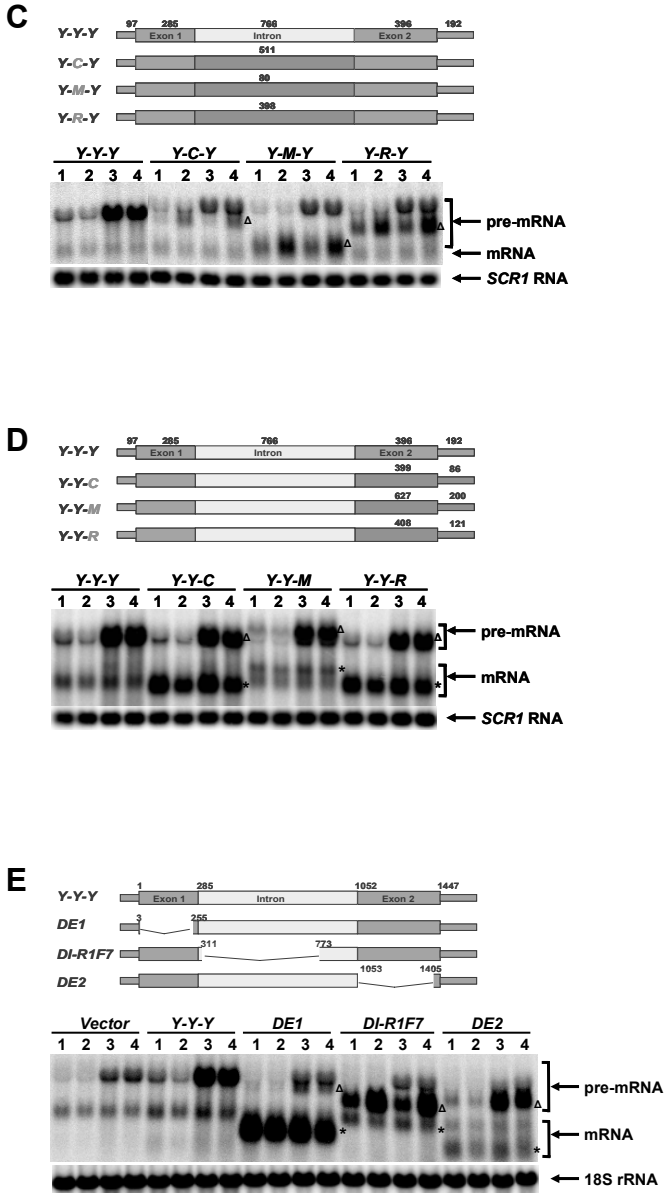

C
Y-Y-Y
Y-C-Y
Y-M-Y
Y-R-Y
97
285
766
396
192
Exon 1
Intron
Exon 2
611
80
398
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
pre-mRNA
mRNA
SCR1 RNA
D
Y-Y-C
Y-Y-M
Y-Y-R
399
86
627
200
408
121
E
DE1
DI-R1F7
DE2
1
285
1052
1447
3
255
311
773
1053
1405
Vector
18S rRNA

FIGURE 2.5

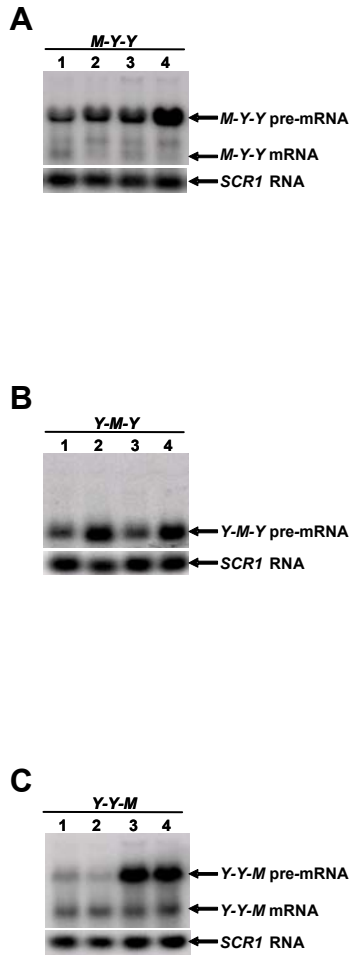

A
M-Y-Y
1 2 3 4
M-Y-Y pre-mRNA
M-Y-Y mRNA
SCR1 RNA
B
Y-M-Y
1 2 3 4
Y-M-Y pre-mRNA
SCR1 RNA
C
Y-Y-M
1 2 3 4
Y-Y-M pre-mRNA
Y-Y-M mRNA
SCR1 RNA

FIGURE 2.6

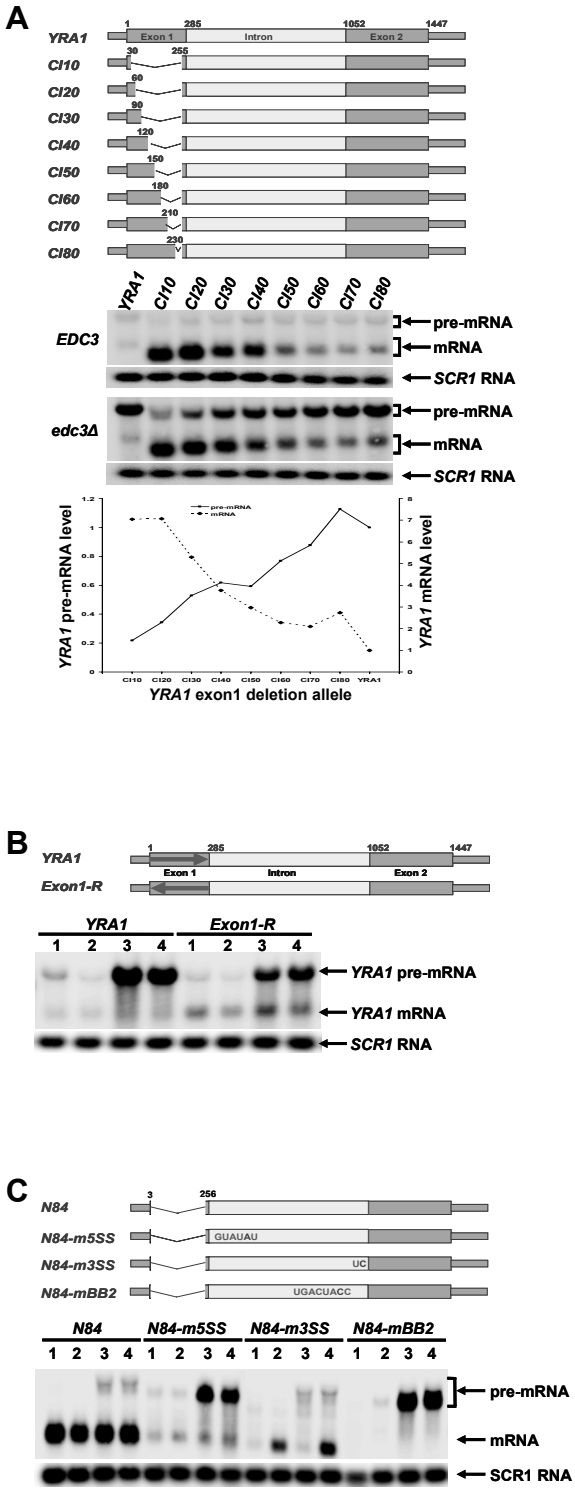

A
YRA1
CI10
CI20
CI30
CI40
CI50
CI60
CI70
CI80
Exon 1
Intron
Exon 2
EDC3
edc3Δ
pre-mRNA
mRNA
SCR1 RNA
YRA1 pre-mRNA level
YRA1 mRNA level
YRA1 exon1 deletion allele
B
Exon1-R
N84
N84-m5SS
N84-m3SS
N84-mBB2
GUAUAU
UC
UGACUACC
pre-mRNA
mRNA
SCR1 RNA

FIGURE 2.6

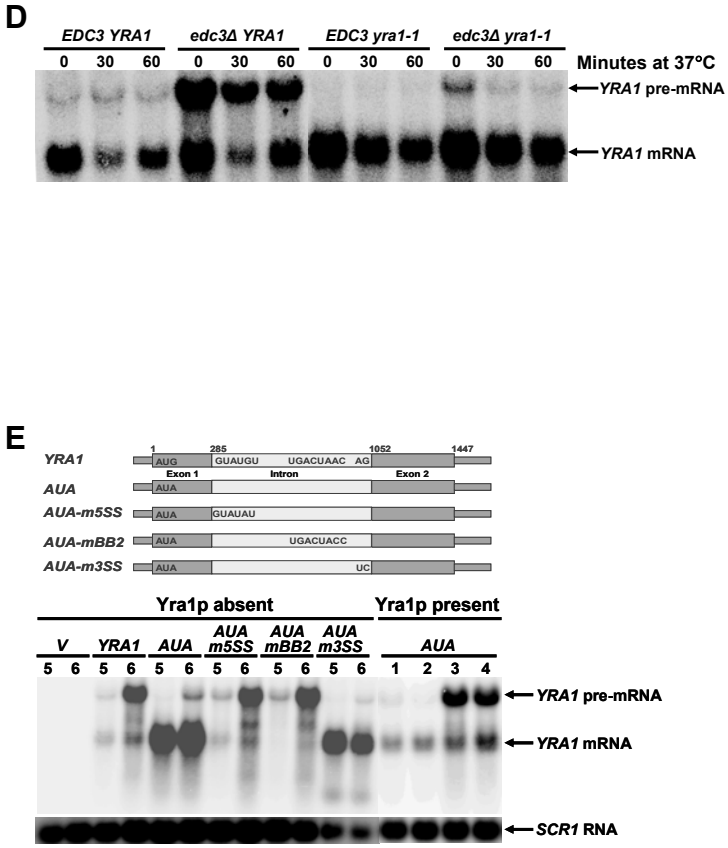

D
EDC3 YRA1
edc3Δ YRA1
EDC3 yra1-1
edc3Δ yra1-1
0 30 60 0 30 60 0 30 60 0 30 60
Minutes at 37°C
YRA1 pre-mRNA
YRA1 mRNA
E
YRA1
AUA
AUA-m5SS
AUA-mBB2
AUA-m3SS
1
285
1052
1447
AUG
GUAUGU
UGACUAAC
AG
Exon 1
Intron
Exon 2
AUA
GUAUAU
UGACUACC
UC
Yra1p absent
Yra1p present
V
YRA1
AUA
AUA m5SS
AUA mBB2
AUA m3SS
AUA
5 6 5 6 5 6 5 6 5 6 5 6 1 2 3 4
YRA1 pre-mRNA
YRA1 mRNA
SCR1 RNA

FIGURE 2.7

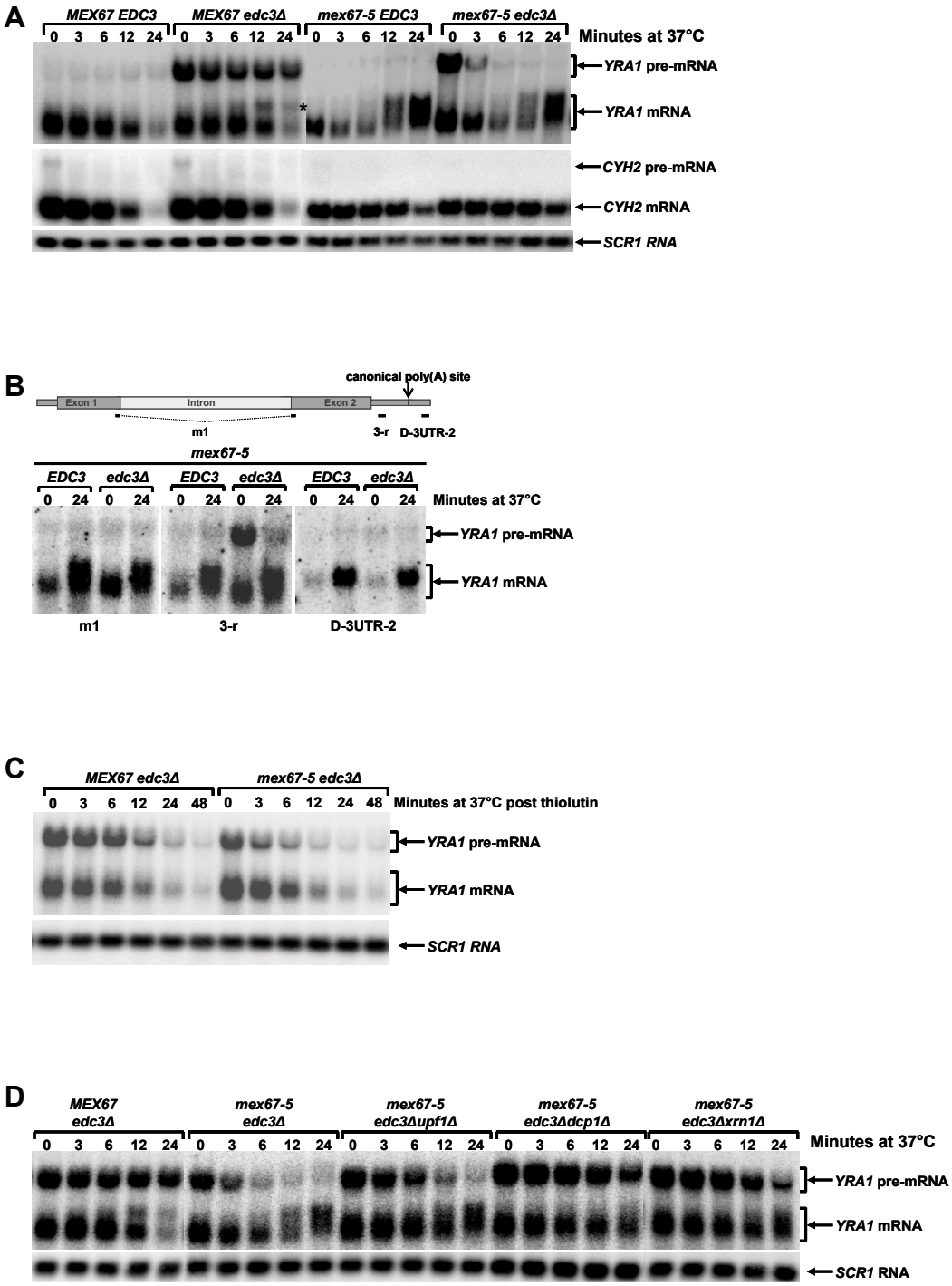

A
MEX67 EDC3
MEX67 edc3Δ
mex67-5 EDC3
mex67-5 edc3Δ
0 3 6 12 24
Minutes at 37°C
YRA1 pre-mRNA
YRA1 mRNA
CYH2 pre-mRNA
CYH2 mRNA
SCR1 RNA
B
canonical poly(A) site
Exon 1
Intron
Exon 2
m1
3-r
D-3UTR-2
mex67-5
EDC3
edc3Δ
0 24
Minutes at 37°C
YRA1 pre-mRNA
YRA1 mRNA
m1
3-r
D-3UTR-2
C
MEX67 edc3Δ
mex67-5 edc3Δ
0 3 6 12 24 48
Minutes at 37°C post thiolutin
YRA1 pre-mRNA
YRA1 mRNA
SCR1 RNA
D
MEX67 edc3Δ
mex67-5 edc3Δ
mex67-5 edc3Δupf1Δ
mex67-5 edc3Δdcp1Δ
mex67-5 edc3Δxrn1Δ
0 3 6 12 24
Minutes at 37°C
YRA1 pre-mRNA
YRA1 mRNA
SCR1 RNA

FIGURE 2.8

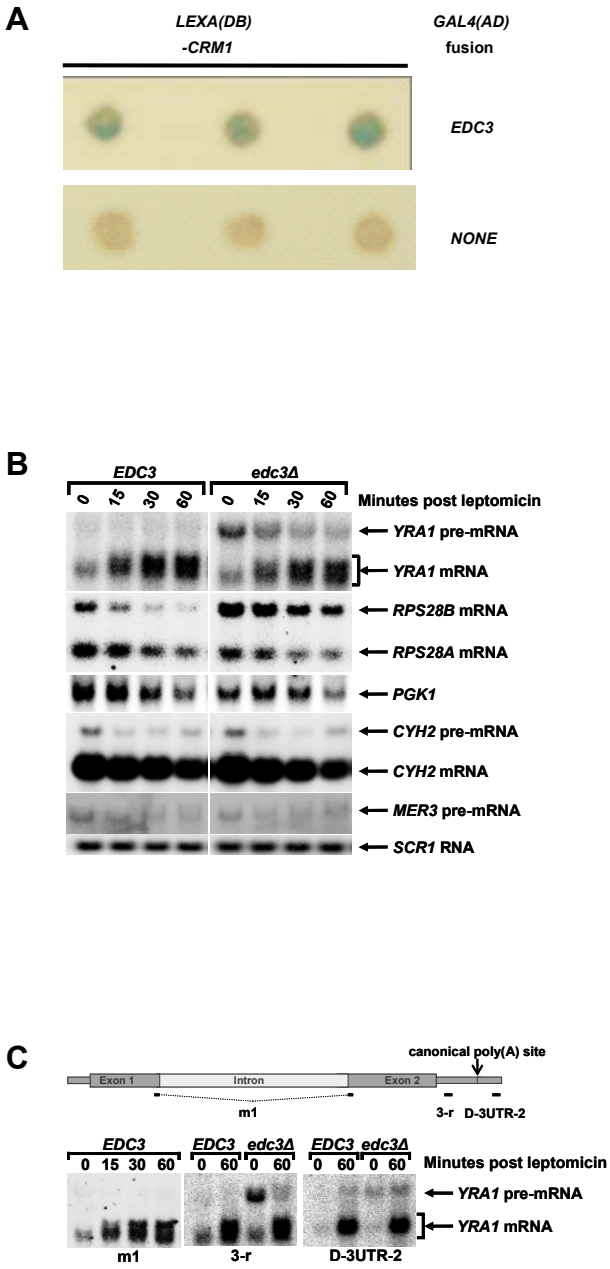
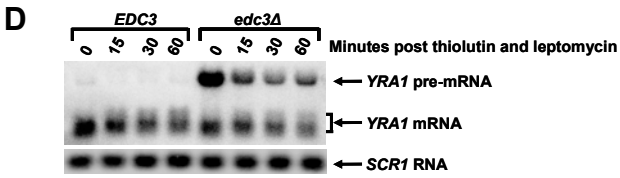
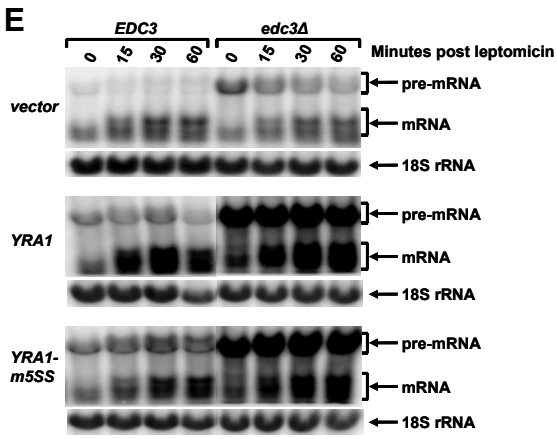

A
LEXA(DB)
-CRM1
GAL4(AD)
fusion
EDC3
NONE
B
EDC3
edc3Δ
0 15 30 60 0 15 30 60
Minutes post leptomicin
YRA1 pre-mRNA
YRA1 mRNA
RPS28B mRNA
RPS28A mRNA
PGK1
CYH2 pre-mRNA
CYH2 mRNA
MER3 pre-mRNA
SCR1 RNA
C
canonical poly(A) site
Exon 1
Intron
Exon 2
m1
3-r
D-3UTR-2
EDC3
EDC3 edc3Δ
EDC3 edc3Δ
0 15 30 60
0 60 0 60
0 60 0 60
Minutes post leptomicin
YRA1 pre-mRNA
YRA1 mRNA
m1
3-r
D-3UTR-2

FIGURE 2.8


D
EDC3
edc3Δ
0 15 30 60 0 15 30 60
Minutes post thiolutin and leptomycin
YRA1 pre-mRNA
YRA1 mRNA
SCR1 RNA


E
EDC3
edc3Δ
0 15 30 60 0 15 30 60
Minutes post leptomicin
vector
pre-mRNA
mRNA
18S rRNA
YRA1
pre-mRNA
mRNA
18S rRNA
YRA1-m5SS
pre-mRNA
mRNA
18S rRNA

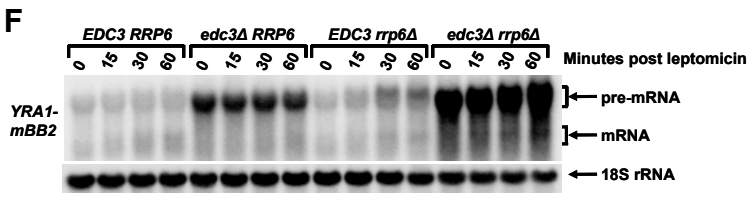

F
EDC3 RRP6
edc3Δ RRP6
EDC3 rrp6Δ
edc3Δ rrp6Δ
0 15 30 60 0 15 30 60 0 15 30 60 0 15 30 60
Minutes post leptomicin
YRA1-mBB2
pre-mRNA
mRNA
18S rRNA

TABLE 2.1. Yeast Strains Used in this Study

Strain	Genotype	References
HFY114	*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 UPF1 NMD2 UPF3*	(He et al., 2003)
HFY871	*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 upf1::HIS3 NMD2 UPF3*	(He et al., 2003)
HFY116	*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 UPF1 nmd2::HIS3 UPF3*	(He et al., 2003)
HFY881	*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 UPF1 NMD2 upf3::HIS3*	(He et al., 2003)
HFY1067	*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 UPF1 NMD2 UPF3 dcp1::URA3*	(He et al., 2003)
HFY1080	*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 UPF1 NMD2 UPF3 xrn1::ADE2*	(He et al., 2003)
CFY25	*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 UPF1 NMD2 UPF3 edc3::URA3*	This study
SYY158	*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 UPF1 NMD2 UPF3 edc3::URA3 upf1::HIS3*	This study
SYY9	*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 UPF1 NMD2 UPF3 rpb1-1*	This study
SYY43	*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 UPF1 NMD2 UPF3 edc3::URA3 rpb1-1*	This study
HFY1170	*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 UPF1 NMD2 UPF3 ski2::URA3*	This study
SYY17	*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 UPF1 NMD2 UPF3 ski7::URA3*	This study
SYY21	*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 UPF1 NMD2 UPF3 ski2::URA3 ski7::ADE2*	This study
CFY13	*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 UPF1 NMD2 UPF3 prt1-1*	This study
HFY1218	*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 UPF1 NMD2 UPF3 sup45-2*	This study
SYY60	*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 UPF1 NMD2 UPF3 edc3::URA3 xrn1::ADE2*	This study
SYY110	*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 UPF1 NMD2 UPF3 ccr4::LEU2*	This study
SYY114	*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 UPF1 NMD2 UPF3 edc3::URA3 ccr4::LEU2*	This study
DAt1-1	*MATa ura3-52 trp1-D63 leu2-D1 rat1-1*	(He and Jacobson, 2001)
HFY1102	*MATa ura3-52 trp1-D63 leu2-D1 rat1-1 xrn1::URA3*	(He and Jacobson, 2001)
SYY573	*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 UPF1 NMD2 UPF3 rrp6::LEU2*	This study
SYY577	*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 UPF1 NMD2 UPF3 edc3::URA3 rrp6::LEU2*	This study
BY4741	*MATa hisΔ1 leu2Δ0 met15Δ0 ura3Δ0*	open biosystems
SYY160	*MATa hisΔ1 leu2Δ0 met15Δ0 ura3Δ0 edc3::URA3*	This study
BY1301	*MATa hisΔ1 leu2Δ0 met15Δ0 ura3Δ0 lsm1::kanMX*	open biosystems
SYY176	*MATa hisΔ1 leu2Δ0 met15Δ0 ura3Δ0 lsm1::kanMX edc3::URA3*	This study
BY7383	*MATa hisΔ1 leu2Δ0 met15Δ0 ura3Δ0 lsm7::kanMX*	open biosystems
SYY172	*MATa hisΔ1 leu2Δ0 met15Δ0 ura3Δ0 lsm7::kanMX edc3::URA3*	This study
BY5797	*MATa hisΔ1 leu2Δ0 met15Δ0 ura3Δ0 pat1::kanMX*	open biosystems
SYY164	*MATa hisΔ1 leu2Δ0 met15Δ0 ura3Δ0 pat1::kanMX edc3::URA3*	This study
BY3858	*MATa hisΔ1 leu2Δ0 met15Δ0 ura3Δ0 dhh1::kanMX*	open biosystems
SYY214	*MATa hisΔ1 leu2Δ0 met15Δ0 ura3Δ0 dhh1::kanMX edc3::URA3*	This study
yra1 shuffle	*MATa ade2 his3 leu2 trp1 ura3 yra1::HIS3 pRS316-YRA1*	(Strasser and Hurt, 2000)
SYY88	*MATa ade2 his3 leu2 trp1 ura3 yra1::HIS3 pRS314-yra1-1*	This study
SYY131	*MATa ade2 his3 leu2 trp1 ura3 yra1::HIS3 pRS314-yra1-1 edc3::URA3*	This study
SYY529	*MATa ade2 his3 leu2 trp1 ura3 yra1::HIS3 pRS316-YRA1 edc3::LEU2*	This study
FSY1135	*MATa ade2 his3 leu2 trp1 ura3 yra1::HIS3 yra2::kan pRS316-YRA1*	(Zenklusen et al., 2001)
SYY606	*MATa ade2 his3 leu2 trp1 ura3 yra1::HIS3 yra2::kan yeplac112-YRA2*	This study
SYY621	*MATa ade2 his3 leu2 trp1 ura3 yra1::HIS3 yra2::kan yeplac112-YRA2 edc3::LEU2*	This study
MNY12	*MATa his3 leu2 trp1 ura3 crm1::kanR pRS313-GFP-crm1(T539C)*	(Neville and Rosbash, 1999)
SYY614	*MATa his3 leu2 trp1 ura3 crm1::kanR pRS313-GFP-crm1(T539C) edc3::URA3*	This study
SYY834	*MATa his3 leu2 trp1 ura3 crm1::kanR pRS313-GFP-crm1(T539C) rrp6::LEU2*	This study
SYY838	*MATa his3 leu2 trp1 ura3 crm1::kanR pRS313-GFP-crm1(T539C) edc3::URA3 rrp6::LEU2*	This study
SYY571	*MATa* or α *ade2 his3 15 leu2 trp1 ura3 mex67::HIS3 pRS316-URA3-MEX67*	From Dr. Rosbash
SYY572	*MATa ade2 his3 15 leu2 trp1 ura3 mex67::HIS3 pRS314-mex67-5*	From Dr. Rosbash
SYY1902	*MATa* or α *ade2 his3 15 leu2 trp1 ura3 mex67::HIS3 edc3::LEU2 pRS316-URA3-MEX67*	This study
SYY605	*MATa ade2 his3 15 leu2 trp1 ura3 mex67::HIS3 edc3::LEU2 pRS314-mex67-5*	This study
SYY749	*MATa ade2 his3 15 leu2 trp1 ura3 mex67::HIS3 edc3::LEU2 upf1::URA3 pRS314-mex67-5*	This study
SYY1913	*MATa ade2 his3 15 leu2 trp1 ura3 mex67::HIS3 edc3::LEU2 dcp1::ADE2 pRS314-mex67-5*	This study
SYY1920	*MATa ade2 his3 15 leu2 trp1 ura3 mex67::HIS3 edc3::LEU2 xrn1::ADE2 pRS314-mex67-5*	This study
L40	*MATa ade2 his3D200 leu2-3,112 trp1-901 LYS2::(lexAop)4-HIS3 URA3::(lexAop)8lacZ gal4 gal80*	Stanley Hollenberg

TABLE 2.2. Plasmids Used in this Study

Name	Allele	Description
SYE22	pRS406*-rpb1-1(-XbaI)	Contains the ts rpb1-1allele as an 4.0 kb EcoRI-HindIII fragment
SYE26	Bs-ski7::URA3	Contains the ski7::URA3 null allele as a NotI-SalI fragment
SYE28	Bs-ski7::ADE2	Contains the ski7::ADE2 null allele as a NotI-SalI fragment
SYE200	Bs-edc3::LEU2	Contains the edc3::LEU2 null allele as a NotI-SalI fragment
CFE7	Bs-edc3::URA3	Contains the edc3::URA3 null allele as a NotI-SalI fragment
HFE1397	Bs-upf1-1::URA3	Contains the upf1::URA3 null allele as a NotI-SalI fragment
	Bs-rrp6::LEU2	Contains the rrp6::LEU2 null allele as a HindIII-BamHI fragment
SYE44 (PIA278-1)	pRS314-YRA1	Contains wild-type YRA1 allele as a 2.0 kb BamHI-XhoI fragment
SYE46(PIA286-1)	pRS314-YRA1-ΔIVS	Same as SYE44 but lacks the *YRA1* intron
SYE58	pRS316-YRA1	Contains wild-type YRA1 allele as a 3.0 kb HindIII-SacI fragment
SYE60	pRS314-yra1-1	Contains the yra1-1 allele as a 3.0 kb HindIII-SacI fragment
SYE181(pFS2261)	YepLac112-Myc-YRA2	Contains Myc-tagged *YRA2* allele as a 1.7 kb BamHI-BamHI fragment
SYE70	pRS316-YRA1 (HindIII-SacI)	Contains wild-typ YRA1 allele as a 2.2 Kb HindIII-SacI fragment
SYE74	pRS314-YRA1(XhoI-SacI)	Contains wild-typ YRA1 allele as a 2.2 Kb XhoI-SacI fragment
SYE80	pRS314-YRA1-C-Y-Y	Same as in SYE74 but the 382-nt YRA1exon 1 was replaced by the 83-nt CYH2 exon1
SYE82	pRS314-YRA1-M-Y-Y	Same as in SYE74 but the 382-nt YRA1exon 1was replaced by the 385-nt MER2 exon1
SYE507	pRS314-YRA1-H-Y-Y	Same as in SYE74 but the 285-nt coding region of YRA1exon 1was replaced by the 663-nt HIS3 coding re
SYE474	pRS314-YRA1-exon1-reverse	Same as in SYE74 but the last 286 nts of YRA1exon 1 was replaced by its complementay sequences
SYE84	pRS314-YRA1-Y-C-Y	Same as in SYE74 but the 766-nt YRA1 intron was replaced by the 511-nt CYH2 intron
SYE87	pRS314-YRA1-Y-M-Y	Same as in SYE74 but the 766-nt YRA1 intronwas replaced by the 80-nt MER2 intron
SYE90	pRS314-YRA1-Y-R-Y	Same as in SYE74 but the 766-nt YRA1 intronwas replaced by the 398-nt RPS51A intron
SYE92	pRS314-YRA1-Y-Y-C	Same as in SYE74 but the 588-nt YRA1 exon 2 was replaced by the 485-nt CYH2 exon2
SYE92	pRS314-YRA1-Y-Y-M	Same as in SYE74 but the 588-nt YRA1 exon2 was replaced by the 827-nt MER2 exon2
SYE93	pRS314-YRA1-Y-Y-R	Same as in SYE74 but the 588-nt YRA1 exon2 was replaced by the 529-nt RPS51A exon2
SYE73	pRS314-YRA1-N84	Same as in SYE74 but contains an internal deletion from A.A. 2 to 85 in exon1
SYE137	pRS314-YRA1-DE-CI10	Same as in SYE74 but contains an internal deletion from A.A. 11 to 85 in exon1
SYE139	pRS314-YRA1-DE-CI20	Same as in SYE74 but contains an internal deletion from A.A. 21 to 85 in exon1
SYE141	pRS314-YRA1-DE-CI30	Same as in SYE74 but contains an internal deletion from A.A. 31 to 85 in exon1
SYE143	pRS314-YRA1-DE-CI40	Same as in SYE74 but contains an internal deletion from A.A. 41 to 85 in exon1
SYE145	pRS314-YRA1-DE-CI50	Same as in SYE74 but contains an internal deletion from A.A. 51 to 85 in exon1
SYE147	pRS314-YRA1-DE-CI60	Same as in SYE74 but contains an internal deletion from A.A. 61 to 85 in exon1
SYE149	pRS314-YRA1-DE-CI70	Same as in SYE74 but contains an internal deletion from A.A. 71 to 85 in exon1
SYE151	pRS314-YRA1-DE-CI80	Same as in SYE74 but contains an internal deletion from A.A. 81 to 85 in exon1
SYE374	pRS314-YRA1-DI-R1-F7	Same as SYE74 but contains a 461-nt internal deletion from nts 27 to 487 of YRA1 intron
SYE78	pRS314-YRA1-DE2	Same as SYE74 but contains a deletion of first 351nts of YRA1exon2
SYE160	pRS314-YRA1-AUA	Same as in SYE74 but contains AUG to AUA mutation in the transaltion initiation codon
SYE161	pRS314-YRA1-AUC	Same as in SYE74 but contains AUG to AUC mutation in the transaltion initiation codon
SYE163	pRS314-YRA1-AUU	Same as in SYE74 but contains AUG to AUU mutation in the transaltion initiation codon
SYE240	pRS316-YRA1-AUA	Same as in SYE74 but contains AUG to AUA mutation in the transaltion initiation codon
SYE298	pRS314-YRA1-m5SS	Same as in SYE74 but contains GUAUGU to GUAUAU mutation in the 5' splicing site
SYE300	pRS314-YRA1-m3SS	Same as in SYE74 but contains AG to UC mutation in the 3' splicing site
SYE302	pRS314-YRA1-mBB2	Same as in SYE74 but contains UGACUAAC to UGACUACC mutation in the branchpoint region
SYE415	pRS314-YRA1-N84-m5SS	Same as in SYE73 but contains GUAUGU to GUAUAU mutation in the 5' splicing site
SYE417	pRS314-YRA1-N84-m3SS	Same as in SYE73 but contains AG to UC mutation in the 3' splicing site
SYE419	pRS314-YRA1-N84-mBB2	Same as in SYE73 but contains UGACUAAC to UGACUACC mutation in the branchpoint region
SYE515	pRS316-YRA1-AUA-m5SS	Same as in SYE240 but contains GUAUGU to GUAUAU mutation in the 5' splicing site
SYE517	pRS316-YRA1-AUA-m3SS	Same as in SYE240 but contains AG to UC mutation in the 3' splicing site
SYE519	pRS316-YRA1-AUA-mBB2	Same as in SYE240 but contains UGACUAAC to UGACUACC mutation in the branchpoint region
SYE234	pGAD-C2-EDC3-FL	Contains the entire EDC3 coding region as an EcoR-SalI fragment
SYE324	pBTM116-CRM1-FL	Contains the entire CRM1 coding region as an EcoR-SalI fragment

TABLE 2.3. Oligonucleotides Used in this Study

Name	Sequences
YRA1-1	AACCAAAGAGAGAAAAGCCTGCTAA
YRA1-2	GATCGAGCTCTGAGGACCATCAATTAGTAAG
YRA1-3	ACTTGAAGAACTATAAAAGGCCGC
YRA1-4	GTGTGCCATATCCTTCCTTACAAA 3'
YRA1-5	GTATGTTAATACGTGAAATGAGAGCT
YRA1-6	GAGTTGCCAAGCTCTTGGACACCACTA
YRA1-5-R	TTCGTCTAAGGATTTATCTAAGTTAGC
YRA1-7-R	GCAGATGTAGGTATTTTCTTAATATGG
Y-C-1A	TCGTGTGTTTAGTAGTAGGTTTTTTTGTTAGAAGAAGTTTATTAAATCCCAGAACAATCATCCAA
Y-C-1B	GTAACTAAACAAATAGCTCTCATTTCACGTATTAACATACTGAGACGTGACCTCTGTGCTTTCTA
Y-C-2A	CGAAGGTTTGCCAAGGGACATTAAGCAGGATGCTGTAAGAGTATGTAGTTCCATTTGGAAGAGGG
Y-C-2B	AAACTCTTTGAACACCACCTACTTGAGATGCAAAAAATTCCTGTACAAAAAAAATATTGTAATGA
Y-C-3A	TCATAGAGATATATGACTAACTTTTTTTTTTTTATTATAGGGTAAAGGTCGTATCGGTAAGCACA
Y-C-3B	GACATATTATGAGTCAAATATGCCGAATAAACTTTAAAAGGAAATAATACAGAAGTAGATGTTGA
Y-M-1A	TCGTGTGTTTAGTAGTAGGTTTTTTTGTTAGAAGAAGTTTTCAACAAGAACAGAAAGAAGGAAAA
Y-M-1B	GTAACTAAACAAATAGCTCTCATTTCACGTATTAACATACGTTCGTAGCAGCATCTTGTTCCAGT
Y-M-2A	CGAAGGTTTGCCAAGGGACATTAAGCAGGATGCTGTAAGAGTTCGTACCAACACAGTGCATACCC
Y-M-2B	AAACTCTTTGAACACCACCTACTTGAGATGCAAAAAATTCCTATACTACAGTTGTTAGTAAATGT
Y-M-3A	TCATAGAGATATATGACTAACTTTTTTTTTTTTATTATAGAACGTGAAAACCTTAATAAAGGATT
Y-M-3B	GACATATTATGAGTCAAATATGCCGAATAAACTTTAAAAGCAACTGGCGTGGTTTTTCATTGTAG
Y-R-2A	CGAAGGTTTGCCAAGGGACATTAAGCAGGATGCTGTAAGAGTATGTTAATATGGACTAAAGGAGG
Y-R-2B	AAACTCTTTGAACACCACCTACTTGAGATGCAAAAAATTCCTATTAAAATGACGAAAAGCAATAC
Y-R-3A	TCATAGAGATATATGACTAACTTTTTTTTTTTTATTATAGGGTAGAGTTAGAACCAAGACCGTCA
Y-R-3B	GACATATTATGAGTCAAATATGCCGAATAAACTTTAAAAGAATTAGTAAATATAATAATATTTTT
Y-H-F1	TTTAAGAAATCCATATTAAGAAAATACCTACATCTGCTAAATGACAGAGCAGAAAGCCCTAGTAA
Y-H-R1	GTAACTAAACAAATAGCTCTCATTTCACGTATTAACATACCTACATAAGAACACCTTTGGTGGAG
YRA1-Ex1R-F	CTTTAAGAAATCCATATTAAGAAAATACCTACATCTGCTATCTTACAGCATCCTGCTTAATGTCC
YRA1-Ex1R-R	GTAACTAAACAAATAGCTCTCATTTCACGTATTAACATACAATGTCTGCTAACTTAGATAAATCC
YRA1-DE1-5'	GATCCTCGAGGGTATATTAAAGCTATTTTACCACT
YRA1-DE1-3'	GATCCCTTGGCATTTAGCAGATGTAGGTATTTTCT
YRA1-CI10	GATCCCTTGGGTCTAAGGATTTATCTAAGTTAGCA
YRA1-CI20	GATCCCTTGGACTTCCTGCTTTGTTAGAGCCAATG
YRA1-CI30	GATCCCTTGGACCACGAGTACCACCGACACGGGCT
YRA1-CI40	GATCCCTTGGAACTTGCTTACCAACTCTTCTTGGA
YRA1-CI50	GATCCCTTGGTCTGTTTGGAAGGCTCCTACGTTGG
YRA1-CI60	GATCCCTTGGTGCCCTAGTATTTTTTCTGATAGGG
YRA1-CI70	GATCCCTTGGCTTGGCAACTCTAGCGACTGCGTTT
YRA1-CI80	GATCCCTTGGGACCTTGACCTCTCTAGTGGTGTCC
YRA1-DI-3'	AATCTTGATCTGCCTCCATCGATTG
YRA1-DI-F7	GATCGAATTCTAGCTATGGGAAACATGTCTTTCAT
YRA1-DI-R1	GATCGAATTCAGCTCTCATTTCACGTATTAACATA
YRA1-DE2-5'	TGATCAAGAATTCTCTTTTTAGAGA
YRA1-DE2-3'	GATCAGATCTTCCTATAATAAAAAAAAAAAGTTA
YRA1-mAUU-F	AATACCTACATCTGCTAAATTTCTGCTAACTTAGATAAATC

YRA1-mAUU-R	GATTTATCTAAGTTAGCAGAAATTTAGCAGATGTAGGTATT
YRA1-mAUC-F	AATACCTACATCTGCTAAATCTCTGCTAACTTAGATAAATC
YRA1-mAUC-R	GATTTATCTAAGTTAGCAGAGATTTAGCAGATGTAGGTATT
YRA1-mAUA-F	AATACCTACATCTGCTAAATATCTGCTAACTTAGATAAATC
YRA1-mAUA-R	GATTTATCTAAGTTAGCAGATATTTAGCAGATGTAGGTATT
YRA1-m5SS-F	GCAGGATGCTGTAAGAGTATATTAATACGTGAAATGAGAGC
YRA1-m5SS-R	GCTCTCATTTCACGTATTAATATACTCTTACAGCATCCTGC
YRA1-mBB2-F	ATCATAGAGATATATGACTACCTTTTTTTTTTTTATTATAG
YRA1-mBB2-R	CTATAATAAAAAAAAAAAAGGTAGTCATATATCTCTATGAT
YRA1-m3SS-F	AACTTTTTTTTTTTTATTATTCGAATTTTTTGCATCTCAAGT
YRA1-m3SS-R	ACTTGAGATGCAAAAAATTCGAATAATAAAAAAAAAAAAGTT
yra1-2-r	CGCCATTTCCTTGTCCAGATCTTC
YRA1-m1	GATGCAAAAAATTCTCTTACAGCATCC
YRA1-p1	AGCTCTCATTTCACGTATTAACATAC
YRA1-D-3UTR-1	CATCGCACTGTAAATAGTGACATATTATGA
YRA1-D-3UTR-2	ACGAGTAACACACGTTTAATCAACCTATCC
YRA1-1-r	TCGAGGCGACACAATACGCCAGCT
YRA1-IN-1	AAACTCGAGAGAGGTCAAGGTCAACGTCGAAGG
YRA1-IN-2	GGGAAGCTTGTTATAACTCAACAAAACTCTTTG
YRA1-DI-5'	GATCGAATTCCTCCTTGATTGTTTGTGTATTGTCC
YEL015W-1	AATTGCGGCCGCGGTCAACAGGTTGCTCGAAAAGAAGCA
YEL015W-2	AGAGGATCCTATGGTTTCTTTTACGAATTACTGTATTGT
EDC3-DS-2A	GATCGGATCCTCTAGATATGGTTTCTTTTACGAATTACTGTATTG
YEL015W-3	AGAGGATCCAACATCGATACCAAGAATTACTTTAGCCTAAACTGGATA
EDC3-DS-3A	GATCGGATCCCTCGAGACCAAGAATTACTTTAGCCTAAACTGGAT
YEL015W-4	AATTGTCGACGCTCAGTTCCTGTCTTCGTAGGATTGG
CRM1-TH-F1	GATCGAATTCATGGAAGGAATTTTGGATTTTTCTAACGAC
CRM1-TH-C1	GATCGTCGACCTAATCATCAAGTTCGGAAGGTTTTAATAA
CRM1-F-S1	AGTTGAGCCATTATTGAACGCTGT
CRM1-F-S2	AGGCAAATCGATGGTTCCGAATGG
CRM1-R-S1	GTCCAAAGTGCATTCAAATACACTC
DBP2-2-r	CTCTGTTACCCCAGCCACCATCTC
RPS28B	CGCAAACGACGAGCTTCACGTTCA
MER2-P	TGAGGGTATGCACTGTGTTGGTACGAAC
MER2-exon1-F	ATGGTCGCTAGAGGTAGAACAGACGAGA
MER2-exon1-R	CGTTCGTAGCAGCATCTTGTTCCAGTAG
MER2-exon2-F	GAAACGTGAAAACCTTAATAAAGGATTT
MER2-exon2-R	TCACAGCTCAGATTCCAGAGTGTCGGGT
18S	CATGGCTTAATCTTTGAGAC
RRP6-1	CCCGGAATTCCCAAAAATATGAGGGCATCGG
RRP6-2	CTAGTCTAGATTCGAGCGAAGTATAATCCTGC

TABLE 2.4. Transcripts Differentially Expressed in *edc3Δ* Cells

Probe set	ORF	Gene	A-WT Signal	A-edc3 Signal	B-WT Signal	B-edc3 Signal	C-WT Signal	C-edc3 Signal	E-WT Signal	E-edc3 Signal	G-WT Signal	G-edc3 Signal
6078_at	YDR381W	YRA1	622.5	944.9	801.4	1092	644.3	1038.9	568.4	1010.9	568.3	961.1
6079_at	YDR381W	YRA1	585.1	1158	561.2	944.7	645.4	1040.5	593.6	1165.5	578.5	1209.1
10069_f_at	YLR264W	RPS28B	2214	2722.5	2219.4	2966.9	2221.2	2744.2	2090.5	3027.8	2013.6	2799.1
10068_i_at	YLR264W	RPS28B	4649.6	4855.4	4040.4	4755.4	4869.1	5681.9	4692.4	6727.4	4337.1	5260.2
5732_at	YEL015W	EDC3	473.6	10	480.5	10	466.8	10	465.4	10	456.8	10
10796_at	YKL216W	URA1	1838.5	786.6	1963.9	1019	1805.3	831.3	1649.7	827.3	1620.7	857.9
9911_at	YLR420W	URA4	790.5	544.9	826.8	577.2	780.1	574.8	741.1	466.5	750.2	471.1

Five independent expression profiling experiments were carried out with wild-type (*EDC3*) and *edc3Δ* strains. Our data analysis indicates that only five transcripts (represented by seven probe sets) are differentially expressed in the *edc3Δ* strain. The raw signal values of each of these probe sets in different experiments (A, B, C, E, and G) are shown in the table.

CHAPTER III

Edc3p-mediated Cytoplasmic *YRA1* Pre-mRNA Degradation Requires Multiple Intronic Modular Elements, Translational Repression, and Mex67p

Running title: *cis* and *trans* regulation of Edc3p-mediated *YRA1* pre-mRNA decay

SUMMARY

mRNA decay pathways regulate gene expression and ensure transcript quality control, two principles illustrated by Edc3p-mediated cytoplasmic degradation of yeast *YRA1* pre-mRNA. Here, we have delineated the *cis*- and *trans*-regulators of this transcript-specific decay mechanism. Our experiments revealed five structurally distinct but functionally interdependent modular elements in the *YRA1* intron, of which two control Edc3p substrate specificity and three mediate translational repression of *YRA1* pre-mRNA. We find that translational repression requires Mex67p, but not Edc3p, and serves to enhance the Edc3p substrate specificity by preventing *YRA1* pre-mRNA from becoming a substrate for nonsense-mediated mRNA decay (NMD). Degradation of *YRA1* pre-mRNA, an integral part of *YRA1* autoregulation, thus appears to proceed through a series of ordered events, including translational repression, substrate recognition by Edc3p, recruitment of the Dcp1p/Dcp2p decapping enzyme, and activation of decapping.

INTRODUCTION

mRNA degradation controls the level of gene expression and ensures transcript quality control. In the yeast *Saccharomyces cerevisiae*, degradation of wild-type mRNAs generally proceeds from poly(A) shortening to either decapping and 5′ to 3′ exonucleolytic digestion or to exosome-mediated 3′ to 5′ decay (Anderson and Parker, 1998; Cao and Parker, 2001; Coller and Parker, 2004; Garneau et al., 2007; Mitchell et al., 1997). Yeast mRNAs containing premature translation termination codons are degraded by nonsense-mediated mRNA decay (NMD), a mechanism for deadenylation-independent entry into either the 5′ to 3′ or 3′ to 5′ decay pathways (Amrani et al., 2006a; Cao and Parker, 2003; Mitchell and Tollervey, 2003; Muhlrad and Parker, 1994). Intron-containing pre-mRNAs are normally retained and processed in the nucleus (Bousquet-Antonelli et al., 2000; Kufel et al., 2004; Moore, 2002), but are sometimes exported to the cytoplasm and degraded by the NMD pathway as a consequence of in-frame termination codons present in the introns (He et al., 1993; Vilardell et al., 2000). However, we have recently shown that the intron-containing *YRA1* pre-mRNA evades the NMD pathway in the cytoplasm and is degraded by a transcript-specific decay pathway mediated by the decapping activator, Edc3p (Dong et al., 2007). Genome-wide microarray analysis has indicated that this Edc3p-mediated decay pathway principally targets only two transcripts, *YRA1* pre-mRNA and *RPS28B* mRNA (Badis et al., 2004; Dong et al., 2007).

The substrate status of a transcript for the different decay pathways can be regulated by specific *cis*-acting elements and their respective *trans*-regulatory RNA-

binding factors. The former include the extensively characterized AU-rich and UG-rich elements present in the 3′-untranslated regions (3′-UTRs) of diverse eukaryotic mRNAs, sequences whose interacting proteins can accelerate transcript-specific decay by recruitment of the PARN and Ccr4p deadenylases, the exosome, or the Dcp1p/Dcp2p decapping enzyme (Anderson et al., 2006; Barreau et al., 2005; Goldstrohm et al., 2007; Mukherjee et al., 2002). For *RPS28B* mRNA, a stem-loop structure in its 3′-UTR was shown to bind Rps28bp, which, in turn, interacted with Edc3p to recruit the decapping enzyme (Badis et al., 2004).

Edc3p-mediated *YRA1* pre-mRNA degradation is dependent on the presence of the *YRA1* intron and appears to require the function of the general mRNA export factor Mex67p (Dong et al., 2007). Here, we have dissected the intronic decay-regulating element and the role of Mex67p in Edc3p-mediated *YRA1* pre-mRNA decay. Our experiments delineated five structurally distinct but functionally interdependent modules. Two modules dictate Edc3p substrate specificity and are designated as Edc3p responsive elements (EREs). The other three modules, designated as translational repression elements (TREs), inhibit the translation of *YRA1* pre-mRNA. This translational repression requires Mex67p, but not Edc3p, and prevents *YRA1* pre-mRNA from becoming a substrate for the NMD pathway.

RESULTS

YRA1 Pre-mRNA is Translationally Repressed by a Mechanism Independent of Edc3p Activity

YRA1 pre-mRNA contains multiple in-frame nonsense codons in its intron and, as such, could be considered to be a typical NMD substrate when present in the cytoplasm (He et al., 2003; He et al., 1993). Nevertheless, upon export from the nucleus, this pre-mRNA is degraded by the Edc3p-mediated decay pathway, not the NMD pathway. Since activation of NMD is dependent on translation, the NMD resistance of *YRA1* pre-mRNA could be attributable to translational repression of the transcript. Moreover, since *YRA1* pre-mRNA is still resistant to NMD in the absence of Edc3p (Dong et al., 2007), the hypothetical translational repression mechanism must not require Edc3p. To assess these possibilities, we utilized sucrose gradient fractionation to analyze polyribosome profiles of cytoplasmic extracts derived from wild-type and *edc3Δ* strains, and then determined the distribution of *YRA1* pre-mRNA in these gradients by northern blotting. As shown in Figure 3.1A, the majority of the *YRA1* pre-mRNA population in wild-type cells was present in the non-polysomal mRNP fractions (70%), with only modest representation in the polyribosome fractions (30%). In contrast, most of the *YRA1* mRNA (75%) was associated with the polyribosome fractions and only 25% was located in the mRNP fractions. These results suggest that *YRA1* pre-mRNA is indeed translationally repressed in wild-type cells whereas *YRA1* mRNA is not. Deletion of *EDC3* did not affect the overall polyribosome profile and only mildly altered the distribution of *YRA1* pre-mRNA

in the gradient (Figure 3.1B). In *edc3Δ* cells, the majority (64%) of pre-mRNA transcripts was still present in the mRNP fractions. Likewise, the distribution of *YRA1* mRNA was essentially unaffected by deletion of *EDC3*. In control experiments, deletion of *EDC3* did not alter the distribution of the *CYH2* mRNA. *CYH2* mRNA was predominantly localized to polyribosomal fractions in both wild-type and *edc3Δ* extracts (data not shown). These results indicate that, in spite of its cytoplasmic localization (Dong et al., 2007), *YRA1* pre-mRNA accumulating in wild-type or *edc3Δ* cells is translationally repressed, and Edc3p plays little, if any, role in establishing this repression.

Edc3p-mediated *YRA1* Pre-mRNA Degradation Does Not Require Translation

The observed translational repression of *YRA1* pre-mRNA suggests that Edc3p-mediated *YRA1* pre-mRNA degradation occurs independently of translation. To test this idea, we carried out two sets of experiments. First, we examined the effects of *trans* inhibition of translation initiation, elongation, or termination on *YRA1* pre-mRNA accumulation. In these experiments, initiation was inhibited by using the temperature-sensitive *prt1-1* allele to inactivate Prt1p, a component of the translation initiation factor eIF3 complex (Keierleber et al., 1986); elongation was inhibited by treating cultures with the drug cycloheximide (Zhang et al., 1997); and termination was inhibited by using the temperature-sensitive *sup45-2* allele to inactivate Sup45p, the yeast paralog of eukaryotic release factor 1 (eRF1) (Stansfield et al., 1997). We evaluated the effects of each of these three translation blocks in both *EDC3* and *edc3Δ* backgrounds and found that none of them affected the accumulation of *YRA1* pre-mRNA. *EDC3 s*trains all accumulated low

levels of *YRA1* pre-mRNA before or after translational inhibition and *edc3Δ* strains all accumulated high levels of *YRA1* pre-mRNA before or after any of the three translation blocks (Figure 3.2A). As controls, we found that each of the three translation blocks caused 2- to 5-fold increases in the expression of nonsense-containing *can1-100* and *ade2-1* mRNAs (data not shown). These results show that inhibition of any of the three basic steps in translation has no effect on the degradation of *YRA1* pre-mRNA and that, even when general translation is inhibited, *YRA1* pre-mRNA is still degraded in an Edc3p-dependent manner.

Second, we examined whether inclusion of a *cis*-acting inhibitor of translation initiation in the 5′-UTR of *YRA1* pre-mRNA has any effect on the decay of that transcript. In this experiment, we inserted a stem-loop structure known to inhibit translation initiation (Beelman and Parker, 1994) 31 nt upstream of the *YRA1* initiator AUG and analyzed the steady-state levels of the resulting *SL31-YRA1* pre-mRNA in wild-type, *upf1Δ*, *edc3Δ*, and *upf1Δedc3Δ* cells. Northern blotting analyses indicated that the *SL31-YRA1* pre-mRNA behaved like the wild-type pre-mRNA, i.e., it accumulated to low levels in wild-type or *upf1Δ* cells, but increased 5-fold in *edc3Δ* or *upf1Δedc3Δ* cells (Figure 3.2C). As a control, we also inserted the stem-loop structure into the 5′-UTR of the *yra1 C-775* allele. This allele contains a large 5′ deletion of the *YRA1* intron and codes for a pre-mRNA that is degraded by the NMD pathway but not by the Edc3p-mediated pathway (Figure 3.2C). As expected, *SL31-C-775* pre-mRNA containing this stem-loop structure was stabilized more than 3-fold in cells with wild-type NMD function (Figure 3.2C). These data show that inclusion of a *cis*-acting inhibitor of translation in the

5′-UTR of *YRA1* pre-mRNA does not affect its decay behavior. Collectively, the experiments utilizing *cis*- or *trans*-inhibition of protein synthesis indicate that Edc3p-mediated *YRA1* pre-mRNA degradation does not require concomitant translation of the transcript and suggest that translational repression may be an important component of Edc3p-mediated *YRA1* pre-mRNA decay.

Interestingly, *cis* and *trans* inhibition of translation differentially affected the degradation of *YRA1* mRNA. *Cis*-inhibition of initiation or *trans*-inhibition of elongation promoted stabilization of the *YRA1* mRNA, whereas *trans*-inhibition of initiation or termination appeared to destabilize the same transcript (Figures 3.2A and 3.2C). This result indicates that different modes of translational inhibition can have different consequences for mRNA stability, a conclusion borne out by genome-wide analyses of transcript levels in *prt1-1*, *sup45-2*, and cycloheximide-treated cells (FH, SD, C. Li, and AJ, manuscript in preparation).

Edc3p-mediated *YRA1* Pre-mRNA Degradation Requires Multiple Elements of the *YRA1* Intron

Replacing the *YRA1* intron with the intron of the *CYH2*, *MER2* or *RPS51A* genes yields, in each case, a pre-mRNA that is degraded by the NMD pathway (Dong et al., 2007). This intron dependence of Edc3p-mediated *YRA1* pre-mRNA degradation, as well as the observation that *YRA1* pre-mRNA is translationally repressed, suggests that the *YRA1* intron may have two cytoplasmic functions, i.e., repressing pre-mRNA translation and recruiting Edc3p to *YRA1* pre-mRNPs. To delineate the *cis*-acting elements involved in

these potential regulatory functions, we generated a set of *YRA1* pre-mRNA transcripts harboring 3′ or 5′ deletions of the *YRA1* intron and analyzed the effects of each of the intron deletions on the steady-state levels of *YRA1* pre-mRNA in wild-type, *upf1Δ*, *edc3Δ*, and *upf1Δedc3Δ* cells. Each of the mutant *YRA1* pre-mRNA transcripts contain in-frame nonsense codons and we, therefore, included *upf1Δ* strains in these analyses because some intron deletions that inactivate Edc3p-mediated decay may simultaneously render a transcript sensitive to NMD.

Deletions from the 3′ end of the intron yielded three distinct pre-mRNA decay phenotypes. First, deletions up to nt 744 (including constructs *N-942*, *N-852*, *N-771*, *N-753*, and *N-744*; Figure 3.3A) left the *YRA1* pre-mRNA decay phenotypes unchanged. Much like full-length wild-type *YRA1* pre-mRNA, the pre-mRNA transcripts encoded by these alleles showed increased accumulation (at least 5-fold) only in *edc3Δ* and *upf1Δedc3Δ* cells (Figure 3.3B), indicating that these transcripts are still degraded exclusively by the Edc3p-mediated pathway. Second, further deletions from nt 744 to nt 372 (including constructs *N-711*, *N-543*, *N-399*, and *N-372*; Figure 3.3A) resulted in partial activation of NMD without complete elimination of Edc3p-mediated decay. The pre-mRNA transcripts encoded by these alleles showed modest increases in *upf1Δ* or *edc3Δ* strains, but exhibited dramatic increases in the *upf1Δedc3Δ* strain (Figure 3.3C), indicating that these transcripts are degraded by either the Edc3p pathway or the NMD pathway. Since activation of NMD requires translation, these results suggest that sequences 5′ of nt 744 of the *YRA1* intron are not required for the Edc3p response *per se*, but are required for repressing *YRA1* pre-mRNA translation, thus inhibiting NMD.

Finally, further deletions from nt 372 (including constructs *N-342* and *N-312*, Figure 3.3A) resulted in full activation of NMD and the elimination of Edc3p-mediated decay. The pre-mRNA transcripts encoded by these alleles showed increased accumulation only in *upf1Δ* and *upf1Δedc3Δ* cells (Figure 3.3D), indicating that these transcripts are degraded exclusively by the NMD pathway. These results suggest that sequences 5′ of nt 372 of the *YRA1* intron are required for the Edc3p response. Since the smallest transcript capable of responding to Edc3p is that derived from construct *N-372*, which contains intron sequences from nt 286-372, this region must contain a ERE and we designate this segment as module A.

Deletions from the 5′ end of the intron, up to nt 559 (including constructs *C-397*, *C-544*, *C-549*, *C-553*, and *C-559*; Figure 3.4A), did not affect the *YRA1* pre-mRNA decay phenotype. All of the pre-mRNA transcripts encoded by these alleles accumulated to higher levels only in *edc3Δ* and *upf1Δedc3Δ* cells (Figure 3.4B), indicating that these transcripts are degraded exclusively by the Edc3p pathway. Further 5′ deletions, from nt 559 to nt 673 of the intron (including constructs *C-565*, *C-574*, *C-625*, *C-637*, *C-649*, *C-661*, and *C-673*; Figure 3.4A), eliminated the Edc3p response. Interestingly, and in sharp contrast to the 3′ deletions described above, these deletions did not result in the activation of NMD. The pre-mRNA transcripts encoded by these alleles accumulated to higher levels than the full-length *YRA1* pre-mRNA in wild-type cells, but exhibited similar levels in all four strains (Figure 3.4C). These results indicate that the region between nt 559-673 of the *YRA1* intron is required for triggering Edc3p-mediated decay. This segment must also contain an ERE and we designate this region as module B.

Further 5′ deletions, from nt 673 to 943 (including constructs *C-679*, *C-685*, *C-715*, *C-853*, and *C-943*; Figure 3.4A) resulted in the activation of NMD. The pre-mRNA transcripts encoded by these alleles maintained the same levels in wild-type and *edc3Δ* cells, but increased in abundance 2- to 5-fold in *upf1Δ* and *upf1Δedc3Δ* cells (Figure 3.4D). These results suggest that these transcripts are primarily degraded by NMD and indicate that sequences downstream of nt 673 might function in repressing *YRA1* pre-mRNA translation and thus inhibit the NMD pathway. Since the 3′ deletion analyses suggested that sequences upstream of nt 744 inhibit NMD, the region from nt 673-744 is most likely involved in repressing *YRA1* pre-mRNA translation. Accordingly, we consider it to function as a TRE and designate this region as module C.

Taken together, we identified three intronic regions that are involved in the decay of *YRA1* pre-mRNA. Modules A and B are required for the Edc3p response, and module C appears to function in repressing *YRA1* pre-mRNA translation, thus inhibiting the transcript's susceptibility to NMD.

Functional Interdependence of the Intron Modules Controlling the Degradation of *YRA1* Pre-mRNA

Our analysis of 5′ and 3′ deletions of the *YRA1* intron identified three critical regions ("modules") that are involved in Edc3p-mediated *YRA1* pre-mRNA decay (See Figures 3.3 and 3.4). Modules A and B are EREs and are required for the Edc3p response. Module C is a TRE and is required for inhibiting *YRA1* pre-mRNA degradation by NMD. The presence of two distinct EREs that lack synergistic activity suggests that the EREs

may have redundant functions. In addition, although module C is required for inhibiting NMD, it is unknown whether this TRE is sufficient for this inhibitory function. To address these issues, we generated *YRA1* pre-mRNAs harboring internal deletions or containing different internal fragments of the *YRA1* intron and analyzed the decay phenotypes of these transcripts in wild-type, *upf1Δ*, *edc3Δ*, and *upf1Δedc3Δ* cells. These experiments, described below, revealed two additional modules (D and E) involved in the translational repression of *YRA1* pre-mRNA and delineated the functional relationships of all five modules in Edc3p-mediated *YRA1* pre-mRNA decay.

Modules B and C lack independent activities in Edc3p-mediated YRA1 pre-mRNA decay

The module A ERE can function independently to trigger a partial Edc3p response (see Figure 3.3). To assess whether modules B and C also have independent activities, we generated *YRA1* alleles harboring different fragments of the module B/C region and analyzed the decay phenotypes of *YRA1* transcripts encoded by these alleles. As shown in Figure 3.5, *YRA1* pre-mRNAs harboring an intact module B (alleles *I-R4-NR5* and *I-R4-NR2*) or C (alleles *I-F1B-R6A* and *I-F13-R6A*) or both modules B and C (alleles *I-R4-NR6A* and *I-F1-R6A*) are all sensitive to *UPF1* deletion but insensitive to *EDC3* deletion, indicating that these transcripts are all degraded by the NMD pathway. These results indicate that modules B and C each lack an independent activity in Edc3p-mediated *YRA1* pre-mRNA decay.

ERE modules A and B have redundant activities in Edc3p-mediated YRA1 pre-mRNA decay

To test whether the two EREs have redundant functions, we generated a set of complete loss-of-function deletions of either module A or module B and analyzed the effects of these deletions on *YRA1* pre-mRNA decay. In contrast to complete deletion of both modules A and B (Figure 3.4, allele *C-673*), complete deletion of either module A (Figure 3.6, alleles *R1* and *R2*) or module B (Figure 3.6, alleles *F13*, *F14*, *F15* and *F2*) had no effect on *YRA1* pre-mRNA decay. *YRA1* pre-mRNAs harboring each of these deletions behaved like the wild-type *YRA1* pre-mRNA. All these pre-mRNAs are sensitive to *EDC3* deletion but are insensitive to *UPF1* deletion, indicating that these transcripts are all degraded by the Edc3p-mediated decay pathway. These results argue that ERE modules A and B function redundantly in Edc3p-mediated *YRA1* pre-mRNA.

ERE modules A and B exhibit distinct functional interactions with TRE modules C and D

To further assess the potential functional interactions and redundancy of *YRA1* intron modules A, B and C, we generated *YRA1* pre-mRNAs containing combinations of different modules and analyzed the steady-state levels of the transcripts encoded by these alleles in wild-type, *upf1Δ*, *edc3Δ*, and *upf1Δedc3Δ* cells. As already shown in Figure 3.3C, *N-711* pre-mRNA, a transcript containing modules A and B, behaved like *N-372* pre-mRNA, a transcript containing only module A. These two transcripts had slightly increased levels in *upf1Δ* or *edc3Δ* cells, but showed dramatic increases in *upf1Δedc3Δ* cells (compared to wild-type cells), suggesting that they are susceptible to either NMD or

Edc3p-mediated decay. However, transcripts containing modules A and C (allele *AR3-F2-NR6C* in Figure 3.7A, and alleles *A-F2-NR7* and *A-F14B-NR6C* in Figure 3.8A,) exhibited the same specificity for the Edc3p-mediated pathway as did full-length *YRA1* pre-mRNA, with its level increased 5-fold in *edc3Δ* and *upf1Δedc3Δ* cells compared to that observed in wild-type and *upf1Δ* cells (Figures 3.7B and 3.8B). However, when module A was combined with each of the fragments that lack an intact module C (Figure 3.8, alleles *F5*, *F6*, and *F8*), the transcripts behaved like the *YRA1* pre-mRNA that contains module A alone (Figure 3.3, allele *N372*). These transcripts were degraded by either NMD or the Edc3p pathway. These results indicate that ERE module A collaborates with TRE module C to promote Edc3p-mediated *YRA1* pre-mRNA decay.

In contrast to what we observed for module A, transcripts containing modules B and C (allele *I-F1A-R6A* in Figure 3.7A, and alleles *I-R4-NR6A* and *I-F1-R6A* in Figure 3.5A) exhibited specificity for NMD but not for the Edc3p-mediated pathway, with its level increased in *upf1Δ* and *upf1Δedc3Δ* cells compared to that observed in wild-type and *edc3Δ* cells (Figures 3.7B and 3.5B). Notably, transcripts containing modules B and C plus downstream sequences up to nt 942, (allele *I-F1-F16N* in Figure 3.7A and allele *I-R4-F16N* in Figure 3.9A), exhibited specificity for the Edc3p-mediated pathway comparable to that manifested by full-length *YRA1* pre-mRNA, with its level increased 5-fold in *edc3Δ* and *upf1Δedc3Δ* cells compared to that observed in wild-type and *upf1Δ* cells (Figures 3.7B and 3.9B). This result suggests that the segment downstream of module C, from nt 745 to nt 942, is also involved in Edc3p-mediated *YRA1* pre-mRNA degradation and we, therefore, designated this region as module D. Significantly, when

module B was combined with a fragment that contains the region except module A and C (Figure 3.9, allele dA-C), the transcript behaved like an NMD substrate. These data show that ERE module B collaborates with TRE modules C and D to promote Edc3p-mediated *YRA1* pre-mRNA decay.

TRE Modules C, D, and E Inhibit YRA1 Pre-mRNA Degradation by NMD

Interestingly, transcripts containing modules C and D (allele *I-F15B-F16N* in Figure 3.7A, and alleles *I-F14-F16N* and *I-F14A-F16N* in Figure 3.9A), still exhibited specificity for NMD but not for the Edc3p pathway, with its level increased in *upf1Δ* and *upf1Δedc3Δ* cells compared to that observed in wild-type and *edc3Δ* cells (Figures 3.4B and 3.9B). However, transcripts containing modules C and D plus downstream sequences up to nt 1052 (allele *C-673* in Figure 3.4A and allele *F15B-NR15* in Figure 3.10A) are refractory to the NMD and the Edc3p pathways (Figures 3.4C and 3.10B). This result suggests that the segment downstream of module D, from nt 942-1052, also plays a role in *YRA1* pre-mRNA degradation. We designated this region as module E. As described below, modules C, D, and E can function together to repress *YRA1* pre-mRNA translation and they were, therefore, all designated as TREs.

Taken together, these experiments indicate that *YRA1* pre-mRNA degradation likely involves five intronic sequence elements (Figure 3.7A). These five modules appear to encompass two distinct functions and to exhibit partially redundant activities (Figure 3.6). Modules A and B are required for the Edc3p response. Modules C, D, and E are not

required for the Edc3p response *per se*, but are most likely involved in repressing *YRA1* pre-mRNA translation since these modules together can inhibit NMD. Importantly, a combination of modules A and C, or B, C, and D, is sufficient to trigger a robust Edc3p response. These results indicate that the respective ERE and TRE activities appear to function synergistically in *YRA1* pre-mRNA decay.

YRA1 Intron Modules C, D, and E Mediate Translational Repression of *YRA1* Pre-mRNA

The deletion analyses described above implicate *YRA1* intron modules C, D, and E in the repression of *YRA1* pre-mRNA translation because deletion of these three modules activates degradation of the respective *YRA1* pre-mRNA transcripts by NMD, and *YRA1* pre-mRNA transcripts containing modules C, D, and E but lacking the two EREs are refractory to NMD. To further assess the role of modules C, D, and E in the translational repression of *YRA1* pre-mRNA, we examined the translation status of pre-mRNAs that contain or lack these modules. In this experiment, polysome profiles of two different *YRA1* pre-mRNA transcripts were analyzed by northern blotting subsequent to sucrose gradient fractionation of cytoplasmic extracts of wild-type or *upf1Δ* cells. The first transcript, *C-565* pre-mRNA (Figure 3.4A), contains intact modules C, D, and E, but lacks the two EREs, and is refractory to both Edc3p-mediated decay and NMD (Figure 3.4C). The second transcript, *C-775* pre-mRNA (Figure 3.2B), is almost identical to *C-565* pre-mRNA except that it lacks module C and part of module D. This transcript is susceptible to NMD but not to Edc3p-mediated decay (Figure 3.2C). We analyzed the

first transcript in wild-type cells but the second transcript in *upf1Δ* cells, because the second transcript is susceptible to NMD and has low abundance in wild-type cells. As shown in Figure 3.11A, the majority (62%) of the *C-565* transcript in wild-type cells was present in the non-polysomal fractions, with only modest representation in the polyribosome and 80S fractions (38%). In contrast, the majority (80%) of the *C-775* transcript in *upf1Δ* cells was present in the polyribosome and 80S fractions, with only modest representation (20%) in the non-polysomal fractions (Figure 3.11B). These data show that the *C-565* transcript, which contains intact modules C, D, and E, is largely translationally repressed. In contrast, the *C-775* transcript, which contains only a part of module D and the entirety of module E, is actively engaged in translation. Consistent with these observations, we found that inclusion of the initiation-inhibiting stem-loop structure in the 5′-UTR of *C-755* pre-mRNA caused a significant shift of the transcript from the polyribosome and 80S fractions to the non-polyribosomal fractions, with 42% of the transcript now located in the former and 58% in the latter fractions (Figure 3.11C). These experiments provide direct evidence that *YRA1* intron modules C, D, and E function in repressing *YRA1* pre-mRNA translation and suggest that this translation repressing activity requires the combined actions of all three modules.

Our observation that intron modules C, D, and E play a role in repressing *YRA1* pre-mRNA translation further indicates that translational repression is an important component of Edc3p-mediated *YRA1* pre-mRNA decay. However, the complexity of the *YRA1* intron elements and their functional interaction patterns raises the question of whether modules C, D, and E are only involved in translational repression or have

additional regulatory functions (e.g., Edc3p substrate specificity). To address this issue, we tested whether *cis*-inhibition of translation initiation can suppress the defect caused by deletion of modules C, D, and E. In this experiment, we used the *yra1 F7* and *N-399* alleles. The *F7* allele contains module A, a part of module D, and the entire module E and the *N-399* allele contains only module A (Figures 3.11D and 3.3A). We inserted a stem-loop structure 31 nt upstream of the initiator AUG codon of the *F7* and *N-399* alleles to generate the *SL31-F7* and *SL31-N-399* alleles (Figure 3.11D), respectively, and analyzed the decay phenotypes of the transcripts encoded by these alleles in wild-type, *upf1Δ*, *edc3Δ*, and *upf1Δedc3Δ* cells. Unlike the *F7* and *N-399* transcripts, which are partial Edc3p substrates (Figures 3.11E and 3.3C), the *SL31-F7* and *SL31-N-399* pre-mRNAs behaved like *bona fide* Edc3p substrates, i.e., they accumulated to the same levels in wild-type and *upf1Δ* cells, but increased at least 3-fold in *edc3Δ* and *upf1Δedc3Δ* cells (Figure 3.11E, see bands marked with an arrowhead). These data show that *cis*-inhibition of translation initiation suppresses the defect caused by partial or complete deletion of modules C, D, and E and restores the Edc3p response of a module A-containing partial Edc3p substrate. These results indicate that the principal function of *YRA1* intron modules C, D, and E is the repression of *YRA1* pre-mRNA translation.

Consistent with previous observations (Figure 3.2C and (Beelman and Parker, 1994), the spliced product of the *SL31-F7* pre-mRNA accumulated to elevated levels in all four strains (Figure 3.11B, see bands marked by asterisks), presumably reflecting stabilization of the mRNA caused by the inhibition of its translation.

Mex67p is a Component of the Cyoplasmic *YRA1* pre-mRNP and Functions in Repressing *YRA1* pre-mRNA Translation to Enhance Edc3p-mediated Decay

As described above, our data indicate that Edc3p-mediated decay of *YRA1* pre-mRNA depends on a translational repression mechanism that requires specific intron segments but not Edc3p. These results implicate at least one other *trans*-acting factor in this process. Since inactivation of Mex67p triggers rapid degradation of *YRA1* pre-mRNA by NMD in *edc3Δ* cells (Dong et al., 2007), and NMD requires translation while Edc3p-mediated decay does not, we considered the possibility that Mex67p is a critical determinant of *YRA1* pre-mRNA translational repression.

To assess the role of Mex67p in translational repression, we tested whether tethering of this protein can inhibit the translation-dependent process of NMD. In this experiment, we initially used the intron-containing *yra1 F7* and *R1-F7* alleles. The *R1-F7* allele is almost identical to the *F7* allele except that it lacks a functional module A (Figure 3.12). Since the pre-mRNA transcript encoded by the *F7* allele is partially sensitive to deletion of either *UPF1* or *EDC3* (Figure 3.11E) it must be a substrate for NMD and Edc3p. In contrast, the pre-mRNA transcript encoded by the *R1-F7* allele must be a *bona fide* NMD substrate since this transcript is sensitive only to elimination of Upf1p but not to elimination of Edc3p (Figure 3.12). Two tandem MS2 coat protein binding sites were inserted into the intronic regions immediately upstream of the module D sequences of the *F7* and *R1-F7* alleles, generating alleles *F7-MS2* and *R1-F7-MS2* (Figure 3.13). The plasmids respectively harboring the *F7-MS2* or *R1-F7-MS2* alleles were then co-transformed with a plasmid encoding an MS2 coat protein/Mex67p fusion

protein (MS2p-Mex67p) into wild-type, *upf1Δ*, *edc3Δ*, and *upf1Δedc3Δ* strains and the steady-state levels of the *F7-MS2* and *R1-F7-MS2* transcripts in the resulting strains were determined.

As shown in Figure 3.13B, when MS2p-Mex67p was not co-expressed, the *F7-MS2* transcript behaved the same as the *F7* transcript lacking the MS2 binding sites (compare with Figure 3.11E). The *F7-MS2* transcript was a substrate for both NMD and Edc3p since its levels increased modestly in *upf1Δ* and *edc3Δ* cells, but increased substantially in *upf1Δedc3Δ* cells when compared to its level in wild-type cells (Figure 3.13B, lanes a-d). However, when MS2p-Mex67p was co-expressed, the *F7-MS2* transcript behaved like a *bona fide* Edc3p substrate, i.e., its level increased in *edc3Δ* cells, but was essentially unchanged in *upf1Δ* cells (Figure 3.13B, lanes m-p). These results show that tethering of Mex67p inhibits degradation of the transcript by NMD yet promotes its degradation by the Edc3p-mediated pathway. In contrast to the *F7-MS2* transcript, co-expression of MS2p-Mex67p did not change the decay phenotype of the *R1-F7-MS2* transcript. The latter pre-mRNA maintained its status as an NMD substrate, with its level increased significantly in *upf1Δ* and *upf1Δedc3Δ* cells but essentially unchanged in *edc3Δ* cells (Figure 3.13C, lanes m-p). These results indicate that the ability of tethered Mex67p to inhibit NMD depends on a functional ERE in the same pre-mRNA. As controls, we also analyzed the effects of tethering other proteins to the *F7-MS2* or *R4-F7-MS2* transcripts in wild-type, *upf1Δ*, *edc3Δ*, and *upf1Δedc3Δ* cells. The results of these experiments indicated that tethered Edc3p, Crm1p, Sub2p, and Yra1p did not

significantly affect the decay phenotypes of the *F7-MS2* or *R4-F7-MS2* transcripts (Figures 3.13B and 3.13C).

To determine whether the NMD-inhibitory effect of tethering Mex67p requires additional *cis* elements besides the module A ERE, we analyzed two additional intron-containing *yra1* alleles, *F12-MS2*, and *N-399-MS2*. These two alleles are identical to the *F7-MS2* allele except that *F12-MS2* lacks module D and *N-399-MS2* lacks both modules D and E. Much like the *F7-MS2* transcripts, the transcripts encoded by the *F12-MS2* or *N-399-MS2* alleles are also substrates for both NMD and Edc3p. However, our results indicated that tethering Mex67p neither inhibited the degradation by NMD nor promoted the degradation by Edc3p for either of these transcripts (Figure 3.13D).

Taken together, our data show that tethering Mex67p to *YRA1* pre-mRNA can inhibit its translation and this inhibitory effect requires at least intron modules A and D. Mex67p is thus likely to be a component of the cytoplasmic *YRA1* pre-mRNP that functions in repressing *YRA1* pre-mRNA translation.

The *YRA1* Intron Elements Are Conserved among *Saccharomyces* Species

To assess the biological significance of *YRA1* intron elements, we first identified putative *YRA1* orthologs from other yeast species. We performed BLASTN searches of the fungi database using the *S. cerevisiae YRA1* coding sequence as a query. We then aligned the genomic DNA sequences of *YRA1* orthologs of the yeast species. The results showed that the sizes of *YRA1* exons are similar and their sequences display high conservation. On the contrary, the size of *YRA1* intron varies significantly, ranging from

418 nts in *Candida lusitaniae* to 1297 nts in *Candida glabrata*. Interestingly, the size of *YRA1* intron in *Saccharomyces* species is similar. We then performed ClustalW to identify the conserved regions of the *YRA1* DNA sequences in 4 *Saccharomyces* species including *S. cerevisae*, *S. paradoxus*, *S. bayanus*, *S. kudriavzevi*. As shown in Figure 3.14 and Table 3.1, the coding sequences of *YRA1* are highly conserved. Exon1 sequences and exon2 sequences exhibit 81% and 64% identities. The entire intron sequences only show 49% identities. Significantly, modules A and C display 78% and 76% identities, respectively. Modules B, D, and E display 56%, 43%, and 65% identities, respectively. In contrast, the non-element region, sequences between modules A and B, display only 20% identity. We also used Mfold software (Zuker, 2003) to analyze the potential secondary structure of modules A and C. We found that the highly conserved regions of modules A and C from the 4 *Saccharomyces* species form the almost identical potential secondary structures. For example, the regions highlighted in Figure 3.15A are 100% conserved in the 4 *Saccharomyces* species. These computational analyses suggest that the *cis*-elements required for *YRA1* pre-mRNA degradation are evolutionally conserved in the yeast *Sacchromyces* species. Modules A and C display high identity in both primary sequences and secondary structures, suggesting that the functions of these two modules are also highly conserved. Modules B, D, and E display divergences in both primary sequences and secondary structures (data not shown), suggesting that the functions of these modules are divergent during the course of evolution.

DISCUSSION

Cytoplasmic Edc3p-mediated *YRA1* Pre-mRNA Degradation is Independent of Translation

Edc3p-mediated *YRA1* pre-mRNA decay occurs in the cytoplasm (Dong et al., 2007). In contrast to other cytoplasmic decay pathways, such as the NMD and the general 5′ to 3′ decay pathways (Amrani et al., 2006a; Coller and Parker, 2004; Jacobson and Peltz, 2000), our data indicate that Edc3p-mediated *YRA1* pre-mRNA decay is independent of translation. This conclusion is supported by several observations, notably: 1) *YRA1* pre-mRNA is in a translationally repressed state in wild-type and *edc3Δ* cells (Figures 3.1A and 3.1B), 2) *trans*-inhibition of general translation initiation, elongation, or termination has no significant effect on the steady-state levels of *YRA1* pre-mRNA in both *EDC3* and *edc3Δ* backgrounds (Figure 3.2A), and 3) inclusion of a *cis*-inhibitor of translation initiation in the 5′-UTR of *YRA1* pre-mRNA also has no effect on its decay (Figure 3.2C).

Our finding that Edc3p-mediated *YRA1* pre-mRNA decay does not involve translation explains why this transcript is resistant to NMD despite the fact that it resembles a typical NMD substrate (He et al., 2003). The translation independence of *YRA1* pre-mRNA decay also raises the possibility that Edc3p-mediated decay initiates immediately after nuclear export of the transcript or that decay may even be physically linked to export. The latter notion is further supported by the observation that Edc3p interacts with Crm1p, one of the *YRA1* pre-mRNA export factors (Dong et al., 2007).

Edc3p-mediated *YRA1* Pre-mRNA Degradation is Controlled by Multiple Intronic Elements with Both Independent and Interdependent Functions

Our analyses of the consequences of deleting specific segments of the *YRA1* intron identified five distinct modules critical for Edc3p-mediated *YRA1* pre-mRNA decay that appear to have both independent and interdependent functions. Modules A and B, spanning nt 286-372 and 543-672, respectively, are required for triggering an Edc3p response and are thus *bona fide* EREs (Figures 3.3C and 3.4C). Modules C, D and E, spanning nt 673-744, 745-942, and 943-1052, respectively, are required for inhibiting translation and translation-dependent NMD and, therefore, function as TREs (Figures 3.4C, 3.4D, 3.11A, and 3.10).

Although the deletion analyses revealed discrete roles for modules A, B, C D, and E, our observation that each of these modules except module A lacks an independent activity indicates that these intron modules function interdependently to ensure Edc3p-mediated *YRA1* pre-mRNA degradation. This conclusion is supported by the synergistic effects observed when different types of modules (i.e., EREs and TREs) are combined to promote efficient Edc3-mediated decay (module C is combined with module A or modules C and D are combined with module B) (Figures 3.7B, 3.8 and 3.9), or when the same type of modules (e.g., TREs C, D, and E) are combined to promote translational repression of *YRA1* pre-mRNA (Figures 3.4C and 3.10). The lack of synergistic effects when the two ERE modules (A and B) are combined (allele *N-711*, Figure 3.3), and the fact that these two ERE modules have different functional requirements for TRE modules C and D (Figures 3.7B, 3.8 and 3.9), suggest that the two ERE modules likely perform at

least partially redundant functions in Edc3p-mediated *YRA1* pre-mRNA decay. The underlying molecular mechanisms for the observed synergistic effects of *YRA1* intron modules are currently not clear, but may be indicative of cooperative binding to these intron modules by different factors or different modules by the same factor.

The observation that the *YRA1* intron contains two different functional elements, and that ERE module A, or the combination of TRE modules C, D, and E, exhibit independent activity indicates that the *YRA1* intron modules perform at least two essential yet physically separable functions in Edc3p-mediated *YRA1* pre-mRNA decay. The EREs appear to dictate Edc3p substrate specificity and most likely function to recruit Edc3p to the *YRA1* pre-mRNP whereas the TREs repress *YRA1* pre-mRNA translation, thus preventing degradation of the pre-mRNA by translation-dependent NMD and promoting degradation by the Edc3p pathway. Since elimination of the two EREs does not have any significant effects on TRE-mediated translational repression of *YRA1* pre-mRNA (Figure 3.11A), and in contrast, the elimination of the three TREs causes partial loss but does not eradicate the ERE-mediated Edc3p response (Figure 3.3C), the function of the TREs is independent of that of the EREs. However, the function of the EREs is partially dependent on that of TREs. These observations suggest that TRE-mediated translational repression is epistatic to ERE-mediated recruitment of Edc3p. When combined with the fact that Edc3p interacts with the Dcp1/Dcp2 decapping enzyme (Decker et al., 2007; Fromont-Racine et al., 2000; Gavin et al., 2002; Ho et al., 2002; Ito et al., 2001; Kshirsagar and Parker, 2004; Tritschler et al., 2007; Uetz and Hughes, 2000), these functional data indicate that Edc3p-mediated *YRA1* pre-mRNA degradation is a highly

regulated process that is most likely carried out through a series of ordered events including translational repression, recognition by Edc3p, recruitment of the decapping enzyme, and finally activation of decapping.

A key conundrum of *EDC3* regulation is why the positioning, sequence, and function of the *YRA1* ERE and TRE regulatory elements manifest so little similarity to the *cis*-element regulating decay of the only other known Edc3p substrate, namely *RPS28B* mRNA (Badis et al., 2004). The *cis*-element dictating Edc3p-specificy of *RPS28B* mRNA decay is localized in the 3′-UTR of the transcript where it appears to form a stem-loop structure that binds Rps28bp and recruits Edc3p to activate decapping and subsequent translation-dependent 5′ to 3′ decay (Badis et al., 2004). The combined activities of the *YRA1* and *RPS28B* regulatory elements strongly suggest that, although Edc3p association with a transcript appears to promote decapping and 5′ to 3′ decay, this association can be indirect, i.e., mediated by an additional factor, and does not require translational repression. Moreover, interaction of Edc3p with different mRNPs may involve distinct domains of this modular protein. Localization of the *YRA1* elements within the intron limits their activity to the appropriate substrate, namely the pre-mRNA. Hence, translational repression of the pre-mRNA may be essential to prevent intron-associated Edc3p-targeting factors from being displaced by translocating ribosomes. However, a more complex issue related to the *YRA1* pre-mRNA decay requirement for translational repression pertains to the concomitant avoidance of NMD, i.e., why is this transcript not simply a substrate for NMD? One possibility is that there may be physiological conditions where NMD is inactive and decay of the *YRA1* pre-mRNA

needs to be maintained, or vice versa, i.e., conditions where NMD is active, but *YRA1* pre-mRNA decay needs to be reduced.

Complex *cis*-regulatory elements control other aspects of mRNA function. In *Drosophila melanogaster*, for example, localized translation of *nanos* mRNA is controlled by multiple, partially independent sequences localized in the 3′-UTR of the transcript. The translational repression element and the localization signal overlap partially, and function independently, but act synergistically to ensure that *nanos* mRNA is translated in the proper location (Crucs et al., 2000; Forrest et al., 2004). In *Xenopus* oocytes, the *Vg1* mRNA also contains 3′-UTR sequences controlling localization and translation of the transcript. When *Vg1* mRNA is localized properly, a *Vg* mRNA-binding protein, VgBP71, binds to the localization element and stimulates cleavage at a polyadenylation signal to remove the downstream translational repression element and, therefore, activate translation (Kolev and Huber, 2003). As shown here, Edc3p-mediated *YRA1* pre-mRNA degradation requires two types of functional elements (EREs and TREs) that exhibit both independent and synergistic activities. These results suggest that mRNA decay may share common regulatory mechanisms with mRNA localization.

Mex67p Functions in the Repression of *YRA1* Pre-mRNA Translation to Promote Edc3p-mediated Decay

YRA1 pre-mRNA is translationally repressed in both wild-type and *edc3Δ* cells and the elimination of TRE function results in partial susceptibility of the transcript to the translation-dependent NMD pathway. Hence, translational repression is a critical

component of Edc3p-mediated *YRA1* pre-mRNA decay. Three lines of experimentation indicate that the general mRNA exporter Mex67p plays a specific role in this repression of *YRA1* pre-mRNA translation. First, we previously observed that inactivation of Mex67p in *edc3Δ* cells renders the *YRA1* pre-mRNA transcript susceptible to translation-dependent NMD (Dong et al., 2007). Second, tethering Mex67p to an NMD-susceptible partial Edc3p substrate inhibits the transcript's degradation by NMD but promotes its degradation by the Edc3p-mediated pathway. Finally, the NMD-inhibitory effect of tethering Mex67p requires the presence of specific *YRA1* intronic *cis*-regulatory elements.

Mex67p-mediated translational repression of *YRA1* pre-mRNA is most likely mediated through the intronic TREs since both the elimination of the TREs and inactivation of Mex67p have similar consequences on *YRA1* pre-mRNA decay, i.e., increased susceptibility to NMD and diminished susceptibility to the Edc3p pathway. One interesting possibility is that Mex67p binds directly to at least one of the TREs. In support of this idea, we have shown that the NMD-inhibitory effect of tethering Mex67p requires at least part of TRE module D (Figures 3.13B and 3.13D). Importantly, the human homolog of Mex67p, Tap, is a sequence-specific RNA-binding protein that binds directly to the constitutive transport elements (CTE) of both viral and cellular intron-containing mRNAs (Gruter et al., 1998; Li et al., 2006; Pasquinelli et al., 1997). The molecular mechanism by which Mex67p represses *YRA1* pre-mRNA translation is currently unknown, but our observation that *YRA1* pre-mRNA is mainly localized in the free mRNP fractions in both wild-type and *edc3Δ* cells suggest that Mex67p-mediated translational repression of *YRA1* pre-mRNA occurs at the level of translation initiation.

The requirement of a general mRNA export factor (i.e., Mex67p) for the translational repression of a specific mRNA is intriguing and raises the possibility that this factor may also have a role in the translational control of additional mRNAs.

EXPERIMENTAL PROCEDURES

General procedures

Most strains, protocols, and materials used in this study have been described previously (Dong et al., 2007). Additional procedures used herein are summarized below.

Yeast strains

All strains used in this study are listed in Table 3.2. Strains containing deletions of *EDC3* or *UPF1* were constructed by gene replacement (Guthrie and Fink, 1991), using DNA fragments harboring the corresponding null alleles. Each genomic DNA deletion was confirmed by PCR analysis. Strains harboring the temperature-sensitive *prt1-1* or *sup45-2* alleles were constructed by the pop-in and pop-out technique (Guthrie and Fink, 1991).

Plasmids

All plasmids used in this study are listed in Table 3.3. *YRA1* alleles harboring deletions of intron sequences, or containing insertions of a stem-loop structure, were generated by PCR and molecular cloning. *YRA1-MS2* constructs were prepared by annealing two oligonucleotides containing two tandem MS2-coat protein recognition sites and inserting the resulting DNA fragment into the BamHI and EcoRI sites in the intronic region of the *F7*, *R1-F7*, *F12*, and *N-399 YRA1* alleles. All *YRA1* alleles were confirmed by DNA sequencing. Plasmids expressing the MS2-coat protein fusions with Crm1p, Edc3p, Mex67p, Sub2p, and Yra1p were generated by PCR and molecular cloning. In each case,

a DNA fragment harboring the coding and 3′-UTR sequences of *CRM1*, *EDC3*, *MEX67*, *SUB2*, or *YRA1* was amplified using a pair of oligonucleotides that contain the NheI (5′ primer) and SalI (3′ primer) sites. The resulting DNA fragment was then inserted between the NheI and SalI sites of a plasmid that contains the *ADH1* promoter and the MS2 coding sequence. Each of these fusion proteins was expressed *in vivo* under the control of the *ADH1* promoter.

Oligonucleotides

The oligonucleotides used in this study were obtained from Operon, Inc., and are listed in Table 3.4.

RNA analysis

Cells were grown in either YEPD (Figures 3.1 and 3.2A), or synthetic complete (SC) medium lacking tryptophan (Figures 3.2C, 3.3, 3.4, 3.7, and 3.11), or leucine and tryptophan (Figure 3.13). For normal cell cultures, cells (15 ml) were grown at 30 °C to an OD_{600} of 0.7 and harvested by centrifugation. For cultures involving cycloheximide treatment, cells (100 ml) were grown at 30°C to an OD_{600} of 0.7, harvested by centrifugation, and resuspended in 20 ml of the same medium. Cycloheximide was added to cell cultures at a final concentration of 100µg/ml and 2ml of cell cultures were harvested at different time points. For cultures involving temperature-shifts, cells were grown at 25°C and treated as described previously (He and Jacobson, 1995). In each case, cell pellets were frozen on dry ice and then stored at -80°C until RNA isolation. The

procedures for RNA isolation and northern blotting were as previously described (He and Jacobson, 1995). Transcript-specific signals were determined with a FUJI film analyzer.

Polysome analysis

Cells were grown at 30°C in either YEPD medium or SC medium lacking tryptophan to an OD_{600} of 0.7. Cell extracts were prepared and fractionated on 7-47% sucrose gradients as previously described (Mangus and Jacobson, 1999).

Computational analysis

Putative homologs of *S. cerevisiae* Yra1p were identified by BLASTn serches of public databases (including http://www.ncbi.nlm.nih.gov/sutils/genome_table.cgi?organism=fungi; http://www.broad.mit.edu/cgi-bin/annotation/fgi/blast_page.cgi). The multiple sequence alignment was performed through http://www.ebi.ac.uk/Tools/clustalw2/index.html. The Mfold analysis was done through http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/rna-form1.cgi. Default search parameters were used.

ACKNOWLEDGMENTS

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FIGURE LEGENDS

Figure 3.1. *YRA1* pre-mRNA Is Translationally Repressed and Edc3p Plays a Minor Role in Repressing *YRA1* Pre-mRNA Translation.

The polyribosomal association of *YRA1* pre-mRNA and mRNA was analyzed by sucrose gradient fractionation and northern blotting. Upper panels: OD_{260} tracings; lower panels: northern blots of individual gradient fractions. Blots were hybridized with a probe complementary to *YRA1* transcripts. (A) wild-type cells and (B) *edc3Δ* cells.

Figure 3.2. *Trans*- and *Cis*-inhibition of Translation Have no Effect on Edc3p-Mediated *YRA1* Pre-mRNA Degradation.

(A) Effects of *trans*-inhibition of translation on the steady-state levels of *YRA1* pre-mRNA and mRNA. Initiation was inhibited by inactivation of Prt1p, termination was inhibited by inactivation of Sup45p, and elongation was inhibited by treating cells with cycloheximide. At the indicated times post-inhibition, RNA was isolated from culture aliquots and subjected to northern analysis. Blots were hybridized with probes complementary to the *YRA1* or *SCR1* transcripts, with the latter serving as a loading control.

(B) Schematic diagram of full-length *YRA1* pre-mRNA and the related transcripts derived from the *SL31-YRA1*, *C-775,* and *SL31-C-775* alleles. Smaller rectangles denote the 5′- and 3′-UTRs and larger rectangles denote the exons and the intron. The relative position of the 5′-UTR stem-loop structure is indicated, as are the nucleotides comprising the A of

the initiator AUG (1), the 5′ (285) and 3′ (1052) boundaries of the intron, and the terminal nucleotide of the termination codon (1447).

(C) The effects of *cis*-inhibition of translation initiation. Stem-loop structures were inserted into the 5′-UTRs of the *YRA1* gene or its *C-775* allele and the relative steady-state levels of the respective pre-mRNA and mRNA transcripts were determined by northern blotting as in (A). (1) wild-type cells, (2) *upf1Δ* cells, (3) *edc3Δ* cells, and (4) *upf1Δedc3Δ* cells.

Figure 3.3. Effects of 3′ Deletions of *YRA1* Intron on Edc3p-mediated *YRA1* Pre-mRNA Decay.

(A) Schematic diagram of full-length *YRA1* pre-mRNA and related transcripts derived from *yra1* alleles containing 3′ deletions of the *YRA1* intron. The relative position of each deletion is indicated. The transcripts are divided into three groups by broken lines based on their distinct decay phenotypes manifested in the northern blots of panels B, C, and D.

(B-D) Northern blotting analyses of the steady-state levels of *YRA1* pre-mRNAs harboring 3′ deletions of the *YRA1* intron up to nt 744 (B), or further deletions up to nt 372 (C), or nt 312 (D) in wild-type (1), *upf1Δ* (2), *edc3Δ* (3), and *upf1Δedc3Δ* (4) cells. Blots were hybridized with probes complementary to the *YRA1* or *SCR1* transcripts, with the latter serving as a loading control.

Figure 3.4. Effects of 5′ Deletions of the *YRA1* Intron on Edc3p-mediated *YRA1* Pre-mRNA Decay.

(A) Schematic diagram of full-length *YRA1* pre-mRNA and related transcripts derived from *yra1* alleles containing 5′ deletions of *YRA1* intron. The relative position of each deletion is indicated. The transcripts are divided into three groups by broken lines based on their distinct decay phenotypes manifested in the northern blots of panels B, C, and D.

(B-D) Northern blotting analyses of the steady-state levels of *YRA1* pre-mRNAs harboring 5′ deletions of the *YRA1* intron up to nt 559 (B), nt 673 (C) or nt 943 (D) in wild-type (1), *upf1Δ* (2), *edc3Δ* (3), and *upf1Δedc3Δ* (4) cells. Blots were hybridized with probes complementary to the *YRA1* or *SCR1* transcripts, with the latter serving as a loading control.

Figure 3.5. Intronic Modules B and C Lack Independent Activity in Edc3p-mediate *YRA1* Pre-mRNA Decay.

(A) Schematic diagram of the *YRA1* pre-mRNAs derived from *yra1* alleles containing different internal fragments of the module B and C region of the *YRA1* intron. The relative position of each deletion is indicated.

(B) Northern blotting analysis of the steady-state levels of *YRA1* pre-mRNAs encoded by each of the above depicted *yra1* alleles in wild-type (1), *upf1Δ* (2), *edc3Δ* (3), and *upf1Δedc3Δ* (4) cells. The blot was hybridized with a probe complementary to the *YRA1* transcripts. *18S* rRNA serves as a loading control.

Figure 3.6. ERE Modules A and B Function Redundantly in Edc3p-mediated *YRA1* Pre-mRNA Decay.

(A) Schematic diagram of the *YRA1* pre-mRNAs derived from *yra1* alleles harboring complete loss-of-function deletions of either module A or B. The relative position of each deletion is indicated.

(B) Northern blotting analysis of the steady-state levels of the *YRA1* pre-mRNAs encoded by each of the above depicted *yra1* alleles in wild-type (1), *upf1Δ* (2), *edc3Δ* (3), and *upf1Δedc3Δ* (4) cells. The blot was hybridized with a probe complementary to the *YRA1* transcripts. *18S* rRNA serves as a loading control.

Figure 3.7. *YRA1* Intron Modules Exhibit Synergistic and Partially Redundant Activities.

(A) Schematic diagram of full-length *YRA1* pre-mRNA and related transcripts derived from *yra1* alleles containing different combinations of the *YRA1* intron modules. The relative positions and the implicated functions of modules A, B, C, D, and E are indicated.

(B) Northern blotting analyses of the steady-state levels of *YRA1* pre-mRNAs containing different combinations of *YRA1* intron modules in wild-type (1), *upf1Δ* (2), *edc3Δ* (3), and *upf1Δedc3Δ* (4) cells. Blots were hybridized with probes complementary to the *YRA1* or *SCR1* transcripts, with the latter serving as a loading control.

Figure 3.8. The ERE Module A Collaborates with TRE Module C to Promote Edc3p-mediated *YRA1* Pre-mRNA Decay.

(A) Schematic diagram of the *YRA1* pre-mRNAs derived from the *yra1* alleles containing ERE module A and different fragments of the putative TRE region of the *YRA1* intron. The relative position of each deletion is indicated.

(B) Northern blotting analysis of the steady-state levels of the *YRA1* pre-mRNAs encoded by each of the above depicted *yra1* alleles in wild-type (1), *upf1Δ* (2), *edc3Δ* (3), and *upf1Δedc3Δ* (4) cells. The blot was hybridized with a probe complementary to the *YRA1* transcripts. *18S* rRNA serves as a loading control.

Figure 3.9. ERE Module B Collaborates with TRE Modules C and D to Promote Edc3p-mediated *YRA1* Pre-mRNA Decay.

(A) Schematic diagram of the *YRA1* pre-mRNAs derived from the *yra1* alleles containing ERE module B and different fragments of the putative TRE region of the*YRA1* intron. The relative position of each deletion is indicated.

(B) Northern blotting analysis of the steady-state levels of the *YRA1* pre-mRNAs encoded by each of the above depicted *yra1* alleles in wild-type (1), *upf1Δ* (2), *edc3Δ* (3), and *upf1Δedc3Δ* (4) cells. The blot was hybridized with a probe complementary to the *YRA1* transcripts. *18S* rRNA serves as a loading control.

Figure 3.10. TRE Modules C, D, and E Together Inhibit *YRA1* Pre-mRNA Degradation by NMD.

(A) Schematic diagram of the *YRA1* pre-mRNAs derived from the *yra1* alleles containing a complete or a 3′ truncated TRE region of the *YRA1* intron. The relative position of each

deletion is indicated. The bar on the F15B-NR15 allele represents an introduced NcoI restriction site.

(B) Northern blotting analysis of the steady-state levels of the *YRA1* pre-mRNAs encoded by each of the above depicted *yra1* alleles in wild-type (1), *upf1Δ* (2), *edc3Δ* (3), and *upf1Δedc3Δ* (4) cells. The blot was hybridized with a probe complementary to the *YRA1* transcripts. *18S* rRNA serves as a loading control.

Figure 3.11. Intron Modules C, D and E Mediate Translational Repression of *YRA1* Pre-mRNA.

(A-C) Analysis of the polyribosomal association of the *YRA1* transcripts encoded by the *C-565* allele in wild-type cells (A), or the *C-775* and *SL31-C-775* alleles in *upf1Δ* cells (B and C) by sucrose gradient fractionation and northern blotting. Upper panels: OD_{260} tracings; lower panels: northern blots of individual gradient fractions. Blots were hybridized with a probe complementary to *YRA1* transcripts.

(D) Schematic diagram of the *YRA1* pre-mRNA transcripts derived from the *F7*, *SL31-F7*, and *SL31-N-399* alleles. The relative positions of the 5′-UTR stem-loop structure, the intron modules, and the intron deletions are indicated.

(E) Northern blotting analyses of the steady-state levels of *YRA1* pre-mRNAs encoded by the *F7*, *SL31-F7*, and *SL31-N-399* alleles in wild-type (1), *upf1Δ* (2), *edc3Δ* (3), and *upf1Δedc3Δ* (4) cells. Blots were hybridized with probes complementary to the *YRA1* or *SCR1* transcripts, with the latter serving as a loading control. The positions of exogenous

YRA1 pre-mRNA and mRNA are marked by arrowheads (◄) and asterisks (*), respectively.

Figure 3.12. *YRA1* Pre-mRNA Lacking Modules A, B, and C Is a Substrate for NMD.

(A) Schematic diagram of the *YRA1* pre-mRNA derived from the *R1-F7* allele. The relative position of the intron deletion is indicated.

(B) Northern blotting analysis of the steady-state levels of the *YRA1* pre-mRNA encoded by *R1-F7* allele in wild-type (1), *upf1Δ* (2), *edc3Δ* (3), and *upf1Δedc3Δ* (4) cells. The blot was hybridized with a probe complementary to the *YRA1* or *SCR1* transcripts, with the latter serving as a loading control.

Figure 3.13. Tethering Mex67p Can Inhibit Translation-dependent NMD and Enhance Edc3p-mediated Decay.

(A) Schematic diagram of the *YRA1* pre-mRNAs derived from the *F7-MS2*, *R1-F7-MS2*, *F12-MS2*, and *N-399-MS2* alleles. The relative positions of the intron deletions and the inserted MS2 coat protein binding sites are indicated.

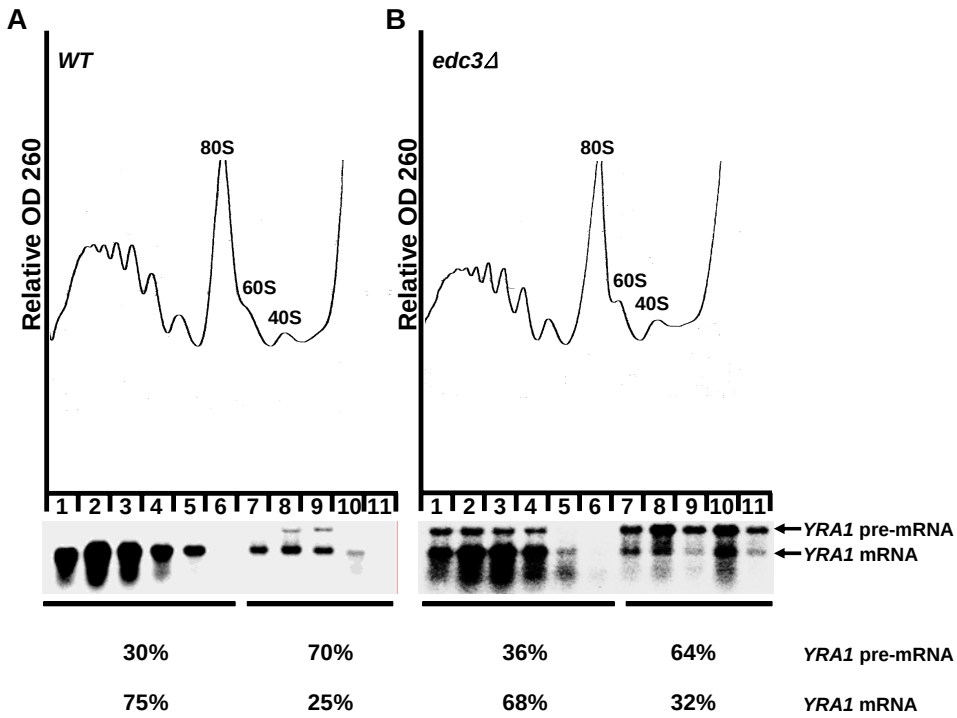
(B-C) Northern blotting analyses of the steady-state levels of the *YRA1* pre-mRNA transcripts encoded by the *F7-MS2* (B) and *R1-F7-MS2* (C) alleles in wild-type (1), *upf1Δ* (2), *edc3Δ* (3), and *upf1Δedc3Δ* (4) cells that express or do not express the Edc3p-, Crm1p-, Mex67p-, Sub2p-, or Yra1p-MS2p fusion proteins.

(D) Northern blotting analyses of the steady-state levels of the *YRA1* pre-mRNA transcripts encoded by the *F12-MS2* and *N-399-MS2* alleles in wild-type (1), *upf1Δ* (2), *edc3Δ* (3), and *upf1Δedc3Δ* (4) cells that express the Mex67p-MS2p fusion protein. Blots were hybridized with probes complementary to the *YRA1* (B), *MS2* (C and D), or *SCR1* transcripts, with the latter serving as a loading control.

Figure 3.14. The Sequences of *YRA1* Exons and Intron Elements Are Conserved among *Saccharomyces*. Alignment of *YRA1* DNA sequences in 4 *Saccharomyces* species including *S. cerevisae*, *S. paradoxus*, *S. bayanus*, *S. kudriavzevi*. The numbers indicate the position of the nucleotide start from translation initiator. The stars (*) indicate the nucleotide identical in the 4 *Saccharomyces* species. The translation initiator, 5´SS, branch point, 3´SS, translation terminator are highlighted. The different intron modules are underlined.

Figure 3.15. The Second Structures of Modules A and C Are Conserved among *Saccharomyces*. Mfold analyses for secondary structure prediction of modules A and C. The stars (*) indicate the nucleotide identical in the 4 *Saccharomyces* species. The highlighted areas indicate the regions which are 100% conserved in the 4 *Saccharomyces* species.

FIGURE 3.1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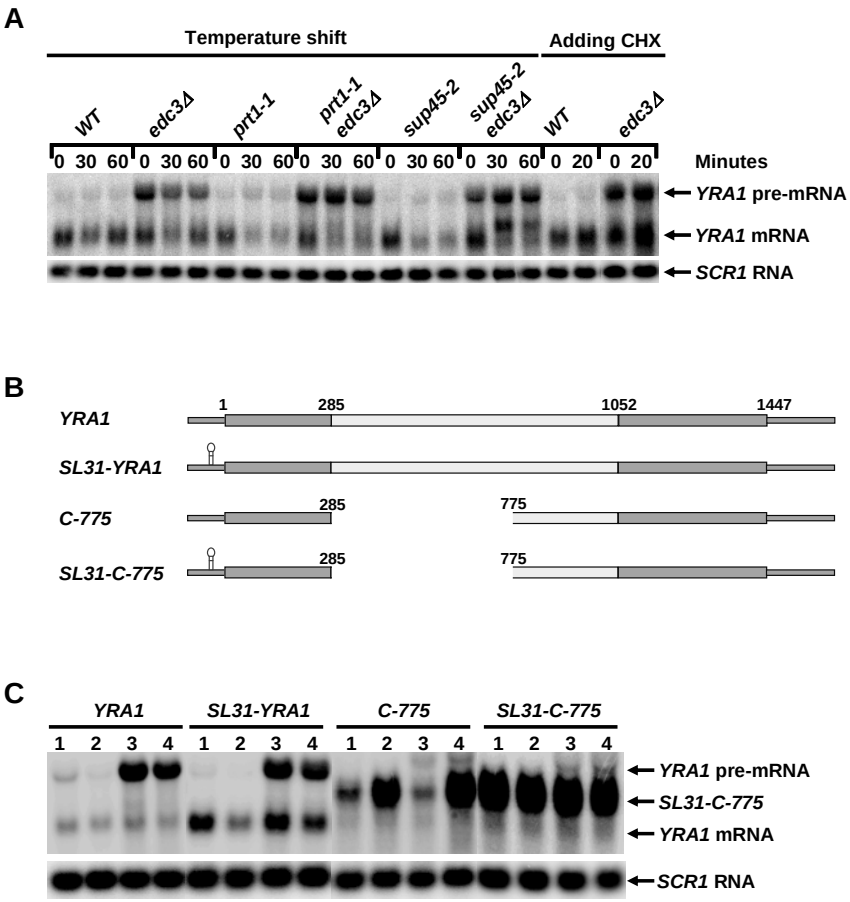
WT
Relative OD 260
80S
60S
40S
1 2 3 4 5 6 7 8 9 10 11
B
edc3Δ
Relative OD 260
80S
60S
40S
1 2 3 4 5 6 7 8 9 10 11
YRA1 pre-mRNA
YRA1 mRNA
30% 70% 36% 64% YRA1 pre-mRNA
75% 25% 68% 32% YRA1 mRNA

FIGURE 3.2


A
Temperature shift
Adding CHX
WT
edc3Δ
prt1-1
prt1-1 edc3Δ
sup45-2
sup45-2 edc3Δ
WT
edc3Δ
0 30 60 0 30 60 0 30 60 0 30 60 0 30 60 0 30 60 0 20 0 20
Minutes
YRA1 pre-mRNA
YRA1 mRNA
SCR1 RNA
B
YRA1
SL31-YRA1
C-775
SL31-C-775
1
285
1052
1447
775
C
YRA1
SL31-YRA1
C-775
SL31-C-775
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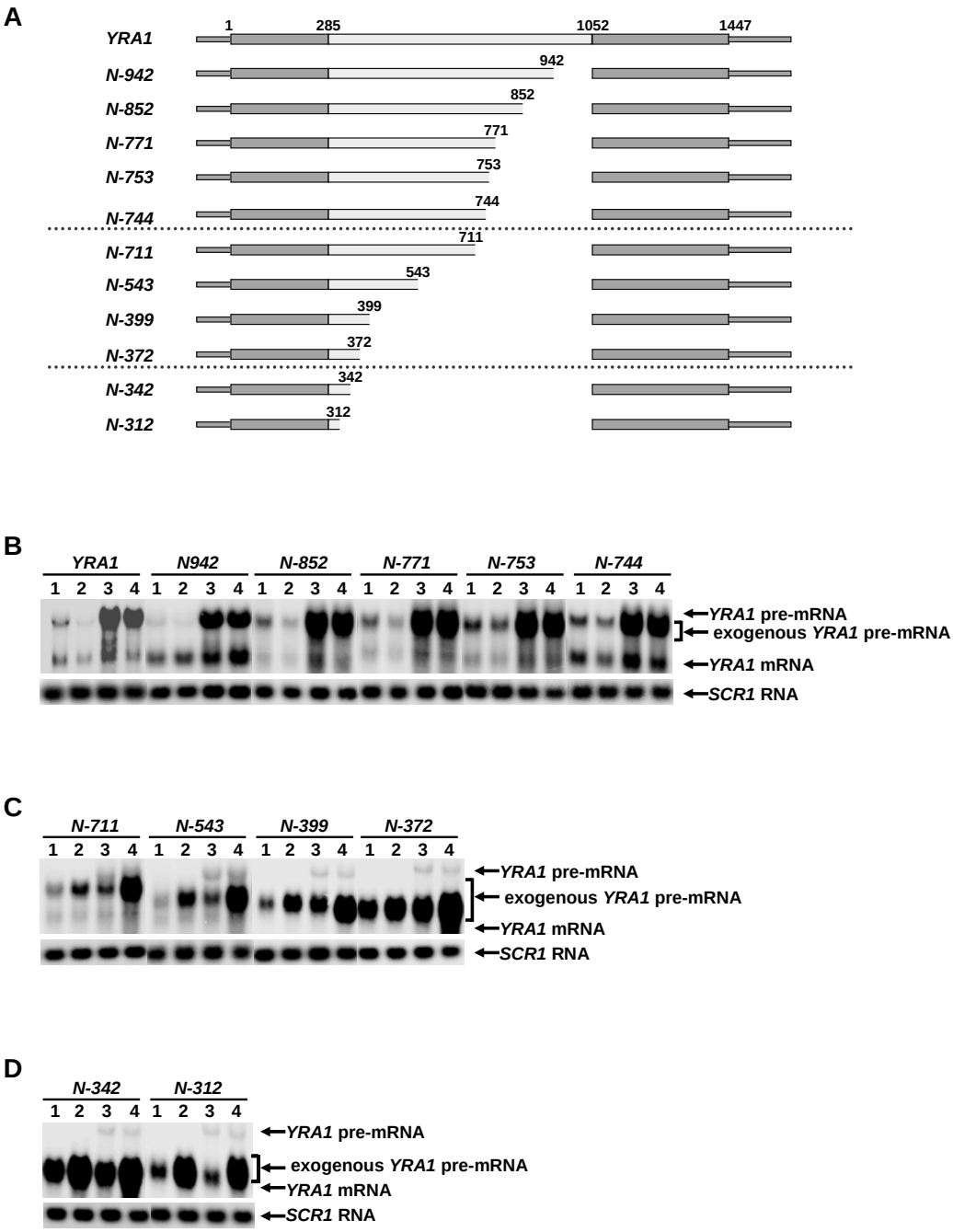
YRA1 pre-mRNA
SL31-C-775
YRA1 mRNA
SCR1 RNA

FIGURE 3.3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YRA1
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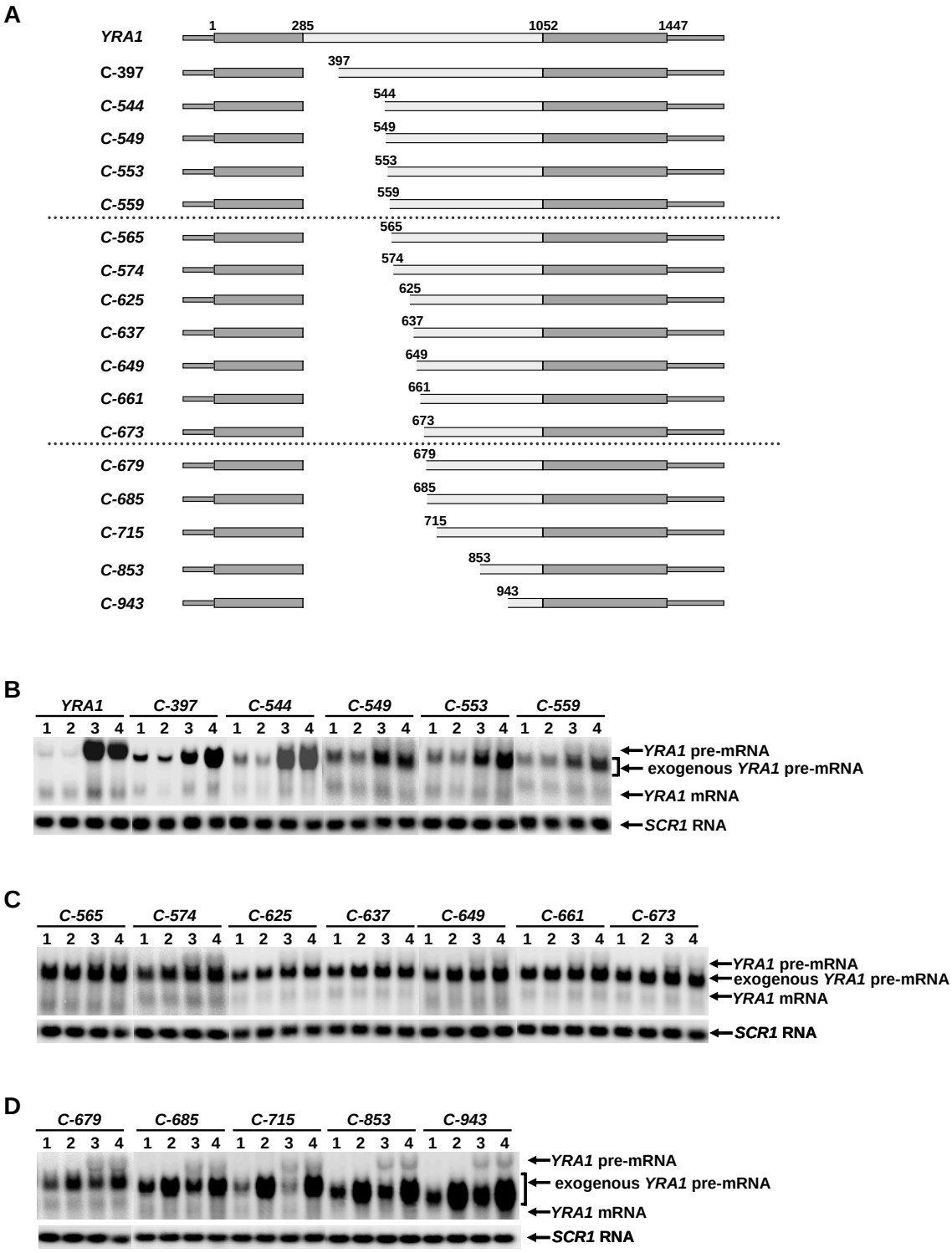
N-852
N-771
N-753
N-744
N-711
N-543
N-399
N-372
N-342
N-312
1
285
1052
1447
942
852
771
753
744
711
543
399
372
342
312
B
YRA1
N942
N-852
N-771
N-753
N-744
1 2 3 4
YRA1 pre-mRNA
exogenous YRA1 pre-mRNA
YRA1 mRNA
SCR1 RNA
C
N-711
N-543
N-399
N-372
1 2 3 4
YRA1 pre-mRNA
exogenous YRA1 pre-mRNA
YRA1 mRNA
SCR1 RNA
D
N-342
N-312
1 2 3 4
YRA1 pre-mRNA
exogenous YRA1 pre-mRNA
YRA1 mRNA
SCR1 RNA

FIGURE 3.4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YRA1
1
285
1052
1447
C-397
397
C-544
544
C-549
549
C-553
553
C-559
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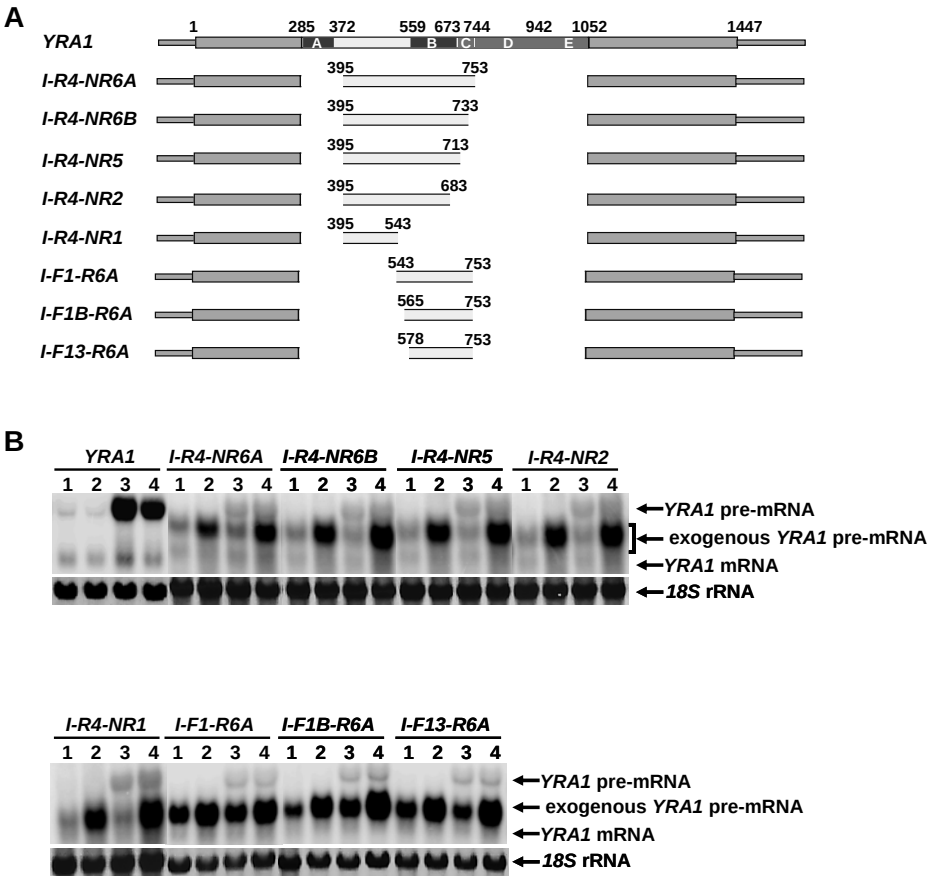
C-565
565
C-574
574
C-625
625
C-637
637
C-649
649
C-661
661
C-673
673
C-679
679
C-685
685
C-715
715
C-853
853
C-943
943
B
YRA1
C-397
C-544
C-549
C-553
C-559
1 2 3 4
YRA1 pre-mRNA
exogenous YRA1 pre-mRNA
YRA1 mRNA
SCR1 RNA
C
C-565
C-574
C-625
C-637
C-649
C-661
C-673
1 2 3 4
YRA1 pre-mRNA
exogenous YRA1 pre-mRNA
YRA1 mRNA
SCR1 RNA
D
C-679
C-685
C-715
C-853
C-943
1 2 3 4
YRA1 pre-mRNA
exogenous YRA1 pre-mRNA
YRA1 mRNA
SCR1 RNA

FIGURE 3.5


A
YRA1
1
285
372
559
673
744
942
1052
1447
A
B
C
D
E
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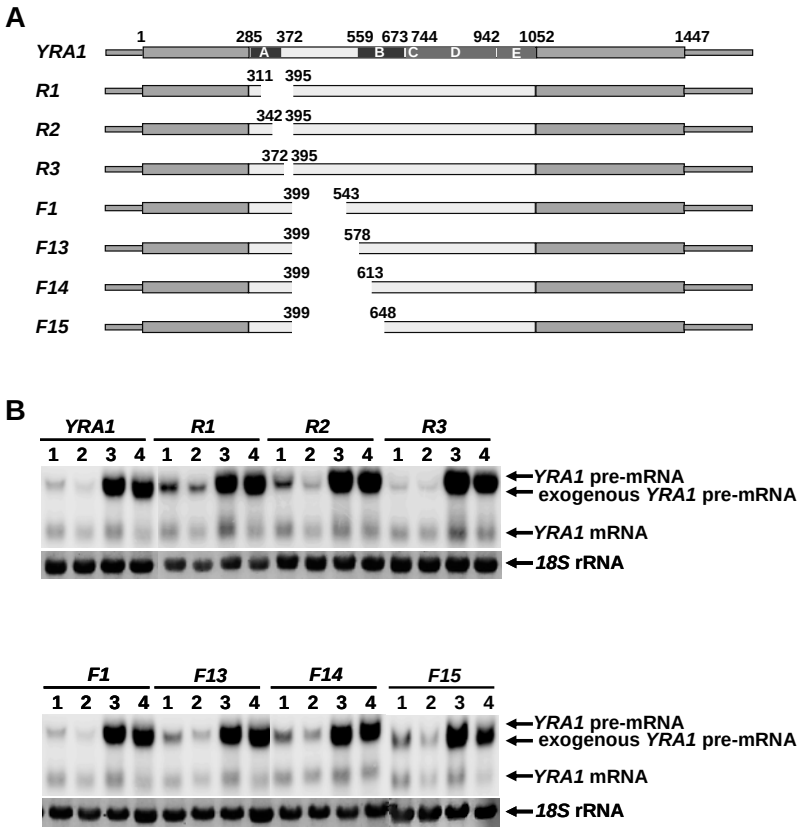
395
753
I-R4-NR6B
395
733
I-R4-NR5
395
713
I-R4-NR2
395
683
I-R4-NR1
395
543
I-F1-R6A
543
753
I-F1B-R6A
565
753
I-F13-R6A
578
753
B
YRA1
I-R4-NR6A
I-R4-NR6B
I-R4-NR5
I-R4-NR2
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
YRA1 pre-mRNA
exogenous YRA1 pre-mRNA
YRA1 mRNA
18S rRNA
I-R4-NR1
I-F1-R6A
I-F1B-R6A
I-F13-R6A
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
YRA1 pre-mRNA
exogenous YRA1 pre-mRNA
YRA1 mRNA
18S rRNA

FIGURE 3.6


A
YRA1
1
285
372
559
673
744
942
1052
1447
A
B
C
D
E
R1
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395
R2
342
395
R3
372
395
F1
399
543
F13
399
578
F14
399
613
F15
399
648
B
YRA1
R1
R2
R3
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
YRA1 pre-mRNA
exogenous YRA1 pre-mRNA
YRA1 mRNA
18S rRNA
F1
F13
F14
F15
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
YRA1 pre-mRNA
exogenous YRA1 pre-mRNA
YRA1 mRNA
18S rRNA

FIGURE 3.7

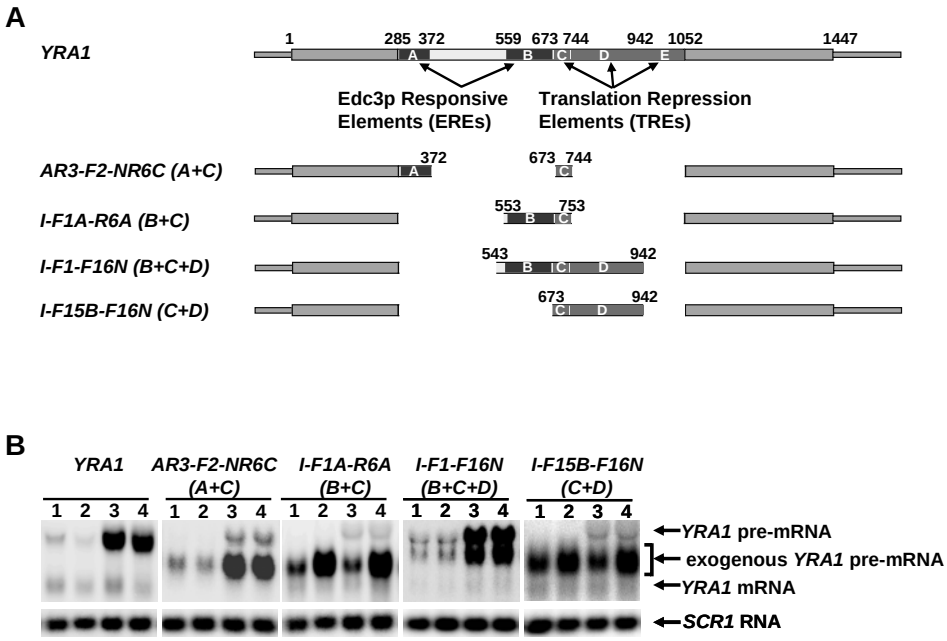
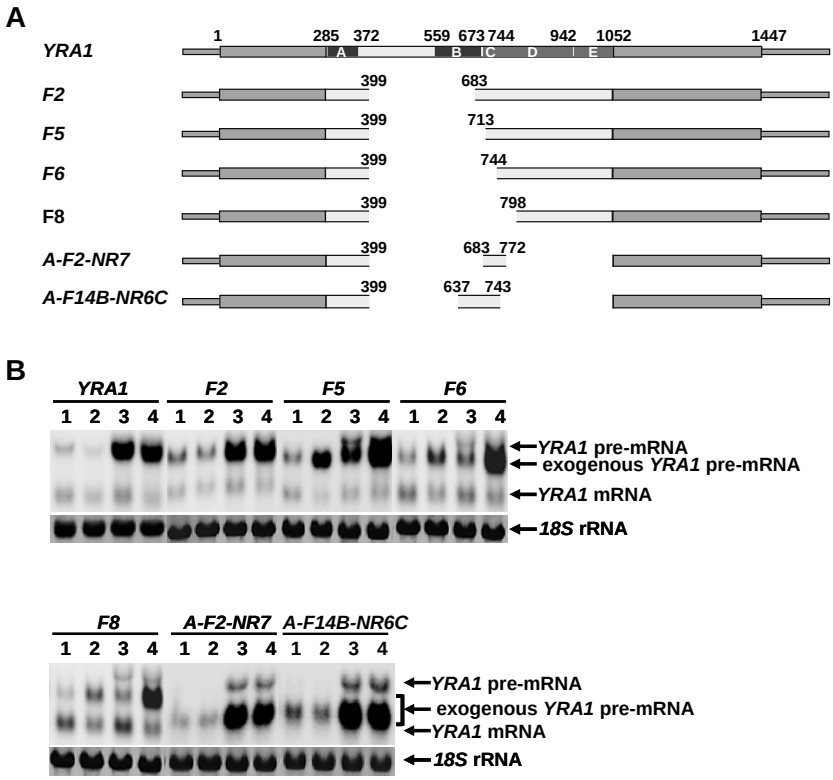

A
YRA1
1
285 372
559 673 744
942 1052
1447
A
B
C
D
E
Edc3p Responsive Elements (EREs)
Translation Repression Elements (TREs)
AR3-F2-NR6C (A+C)
372
673 744
I-F1A-R6A (B+C)
553 753
I-F1-F16N (B+C+D)
543 942
I-F15B-F16N (C+D)
673 942
B
YRA1
AR3-F2-NR6C (A+C)
I-F1A-R6A (B+C)
I-F1-F16N (B+C+D)
I-F15B-F16N (C+D)
1 2 3 4
YRA1 pre-mRNA
exogenous YRA1 pre-mRNA
YRA1 mRNA
SCR1 RNA

FIGURE 3.8


A
YRA1
1
285
372
559
673
744
942
1052
1447
A
B
IC
D
E
F2
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683
F5
399
713
F6
399
744
F8
399
798
A-F2-NR7
399
683
772
A-F14B-NR6C
399
637
743
B
YRA1
F2
F5
F6
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
YRA1 pre-mRNA
exogenous YRA1 pre-mRNA
YRA1 mRNA
18S rRNA
F8
A-F2-NR7
A-F14B-NR6C
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YRA1 pre-mRNA
exogenous YRA1 pre-mRNA
YRA1 mRNA
18S rRNA

FIGURE 3.9

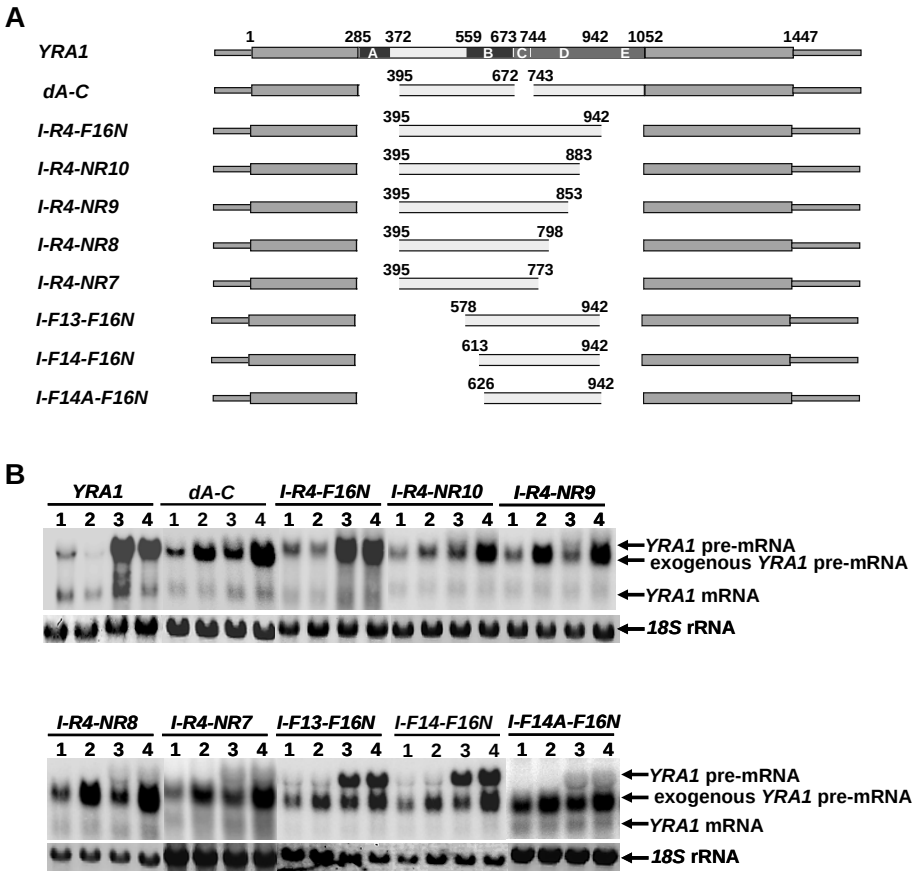

A
YRA1
1
285
372
559
673
744
942
1052
1447
A
B
C
D
E
dA-C
395
672
743
I-R4-F16N
395
942
I-R4-NR10
395
883
I-R4-NR9
395
853
I-R4-NR8
395
798
I-R4-NR7
395
773
I-F13-F16N
578
942
I-F14-F16N
613
942
I-F14A-F16N
626
942
B
YRA1
dA-C
I-R4-F16N
I-R4-NR10
I-R4-NR9
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
YRA1 pre-mRNA
exogenous YRA1 pre-mRNA
YRA1 mRNA
18S rRNA
I-R4-NR8
I-R4-NR7
I-F13-F16N
I-F14-F16N
I-F14A-F16N
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
YRA1 pre-mRNA
exogenous YRA1 pre-mRNA
YRA1 mRNA
18S rRNA

FIGURE 3.10

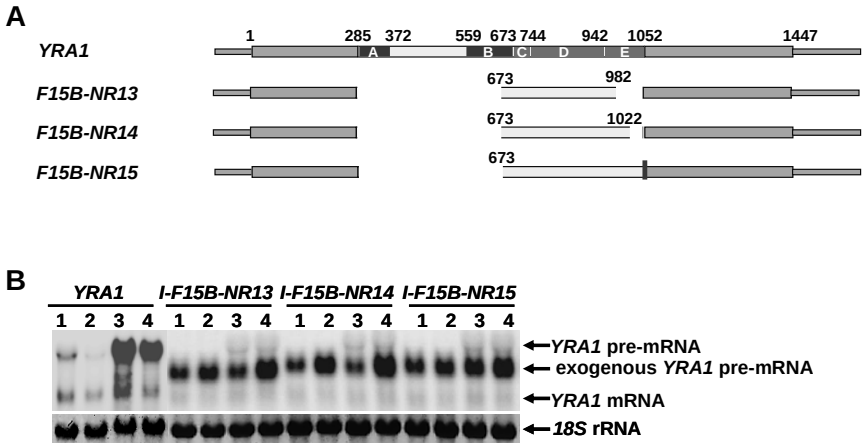

A
YRA1
F15B-NR13
F15B-NR14
F15B-NR15
1
285
372
559
673
744
942
1052
1447
A
B
C
D
E
673
982
673
1022
673
B
YRA1
I-F15B-NR13
I-F15B-NR14
I-F15B-NR15
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
YRA1 pre-mRNA
exogenous YRA1 pre-mRNA
YRA1 mRNA
18S rRNA

FIGURE 3.11

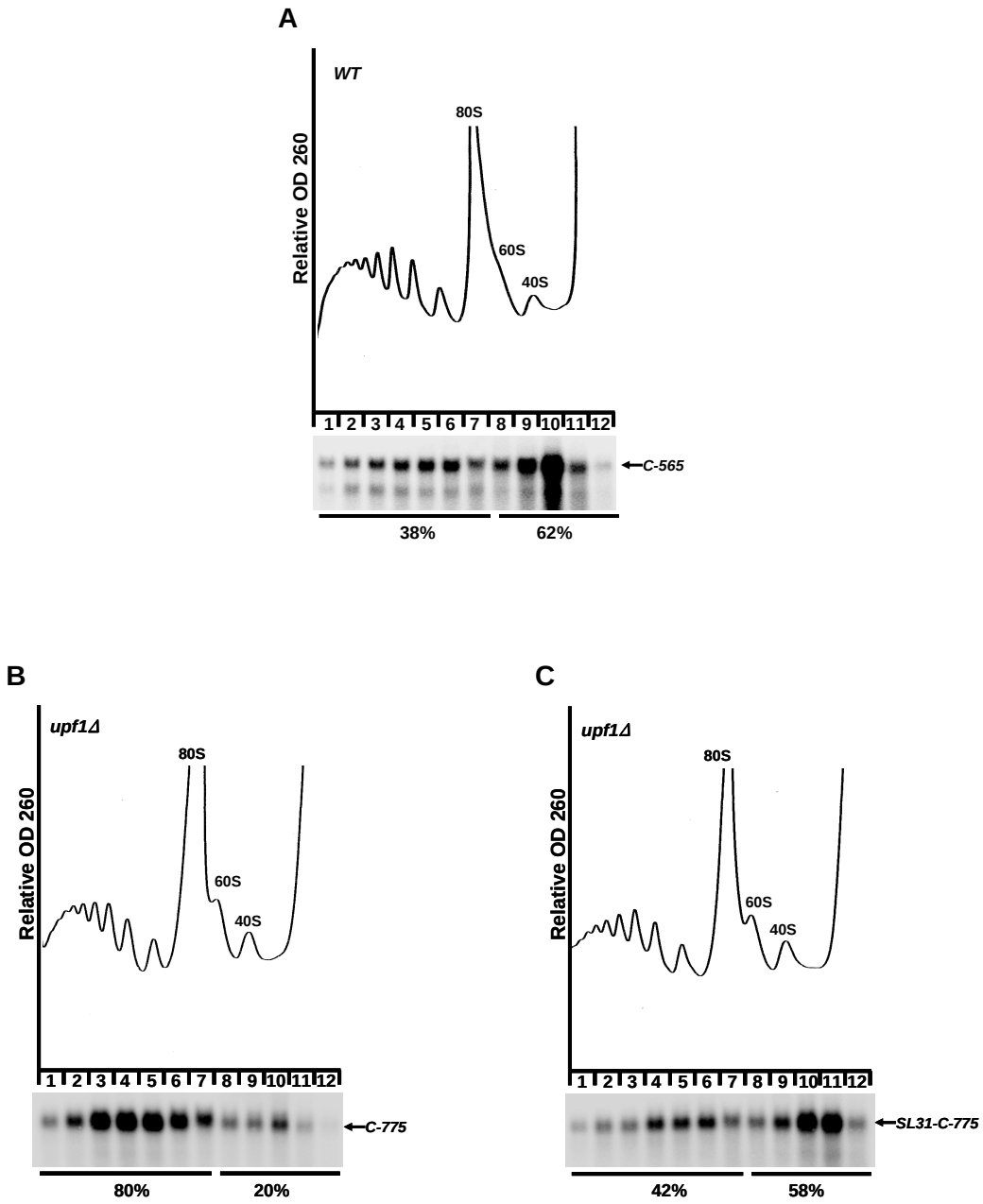

A
WT
80S
60S
40S
Relative OD 260
1 2 3 4 5 6 7 8 9 10 11 12
C-565
38%
62%
B
upf1Δ
80S
60S
40S
Relative OD 260
1 2 3 4 5 6 7 8 9 10 11 12
C-775
80%
20%
C
upf1Δ
80S
60S
40S
Relative OD 260
1 2 3 4 5 6 7 8 9 10 11 12
SL31-C-775
42%
58%

FIGURE 3.11

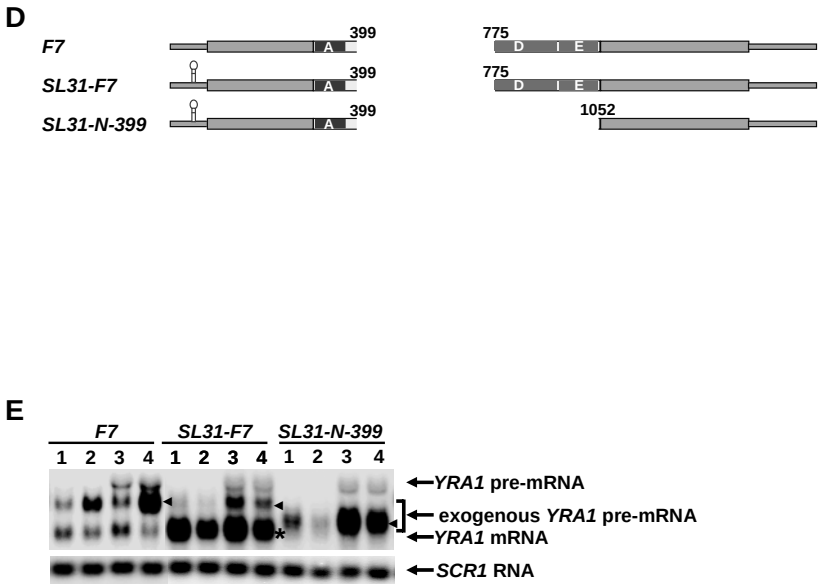

D
F7
SL31-F7
SL31-N-399
399
399
399
A
A
A
775
775
1052
D
I
E
D
I
E
E
F7
SL31-F7
SL31-N-399
1 2 3 4 1 2 3 4 1 2 3 4
YRA1 pre-mRNA
exogenous YRA1 pre-mRNA
YRA1 mRNA
SCR1 RNA
*

FIGURE 3.12

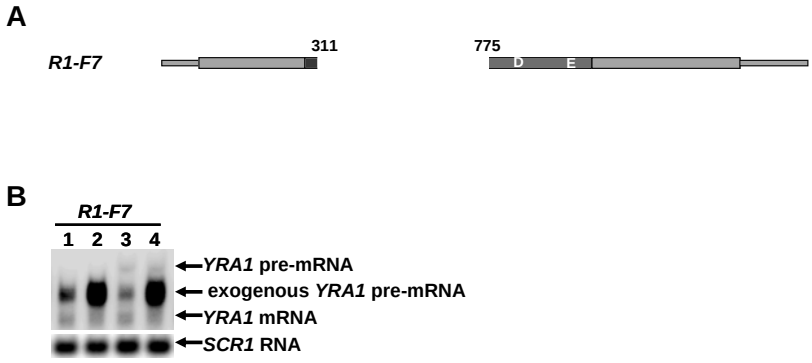

A
R1-F7
311
775
D
E
B
R1-F7
1 2 3 4
YRA1 pre-mRNA
exogenous YRA1 pre-mRNA
YRA1 mRNA
SCR1 RNA

FIGURE 3.13

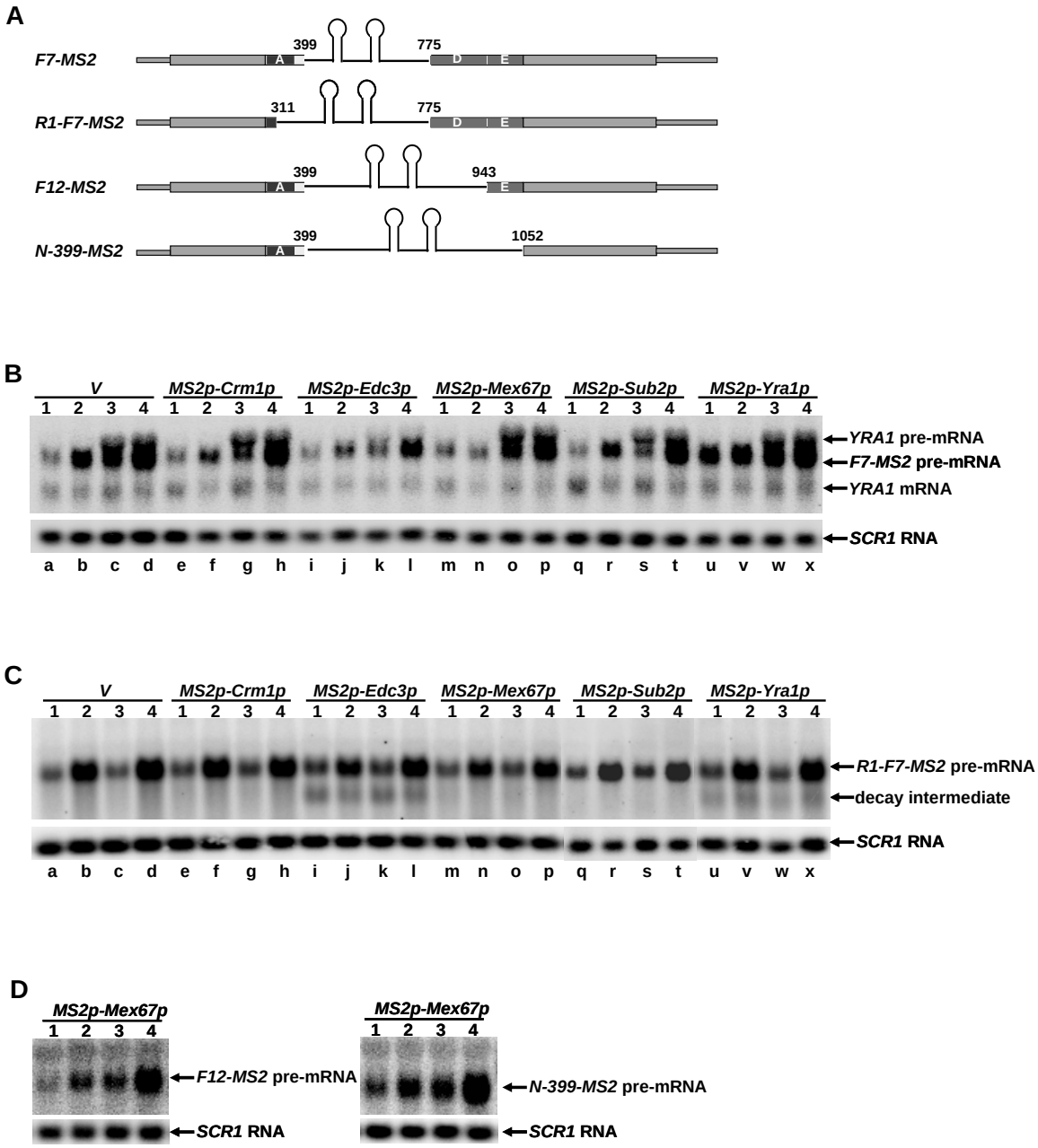
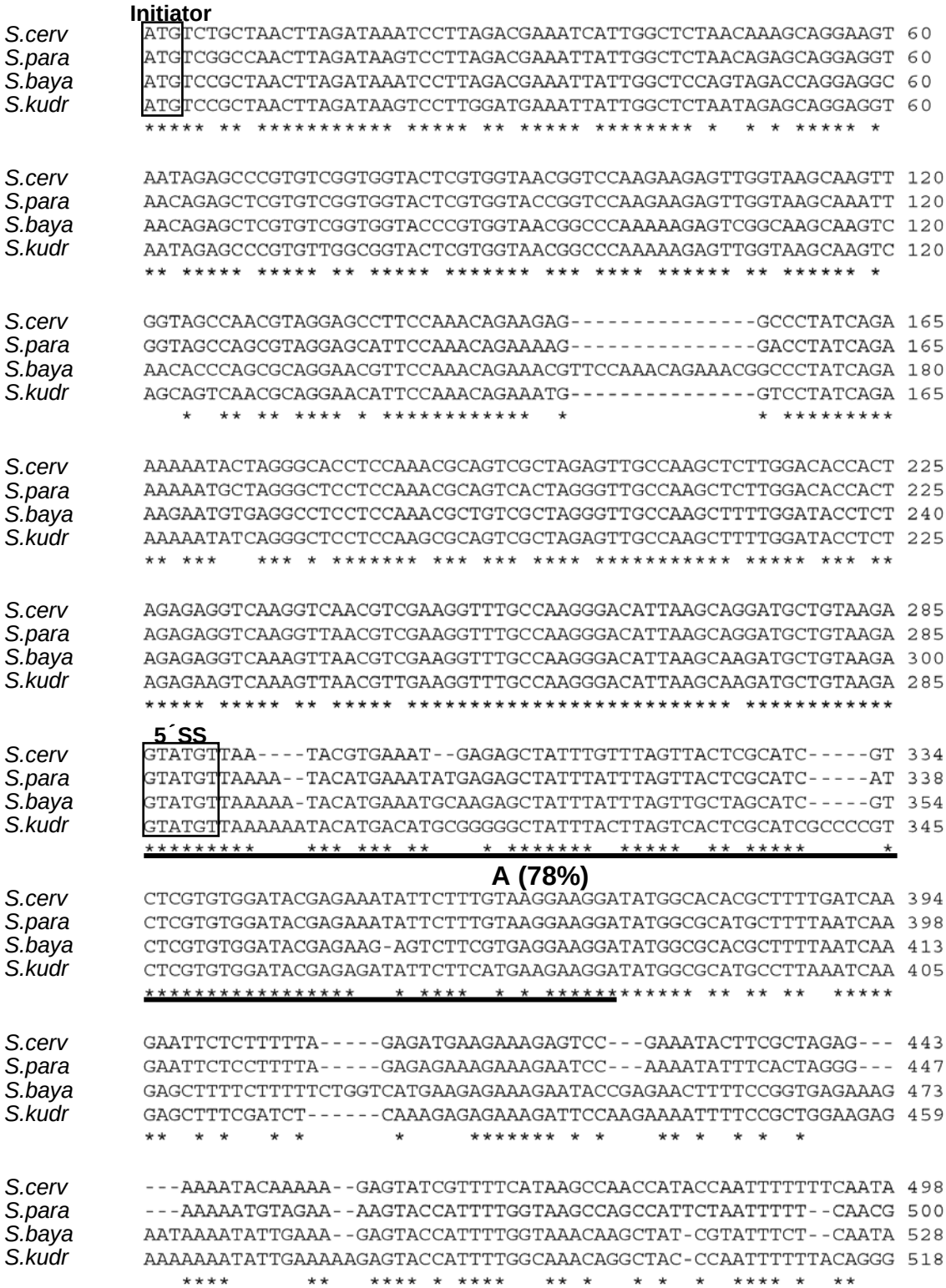
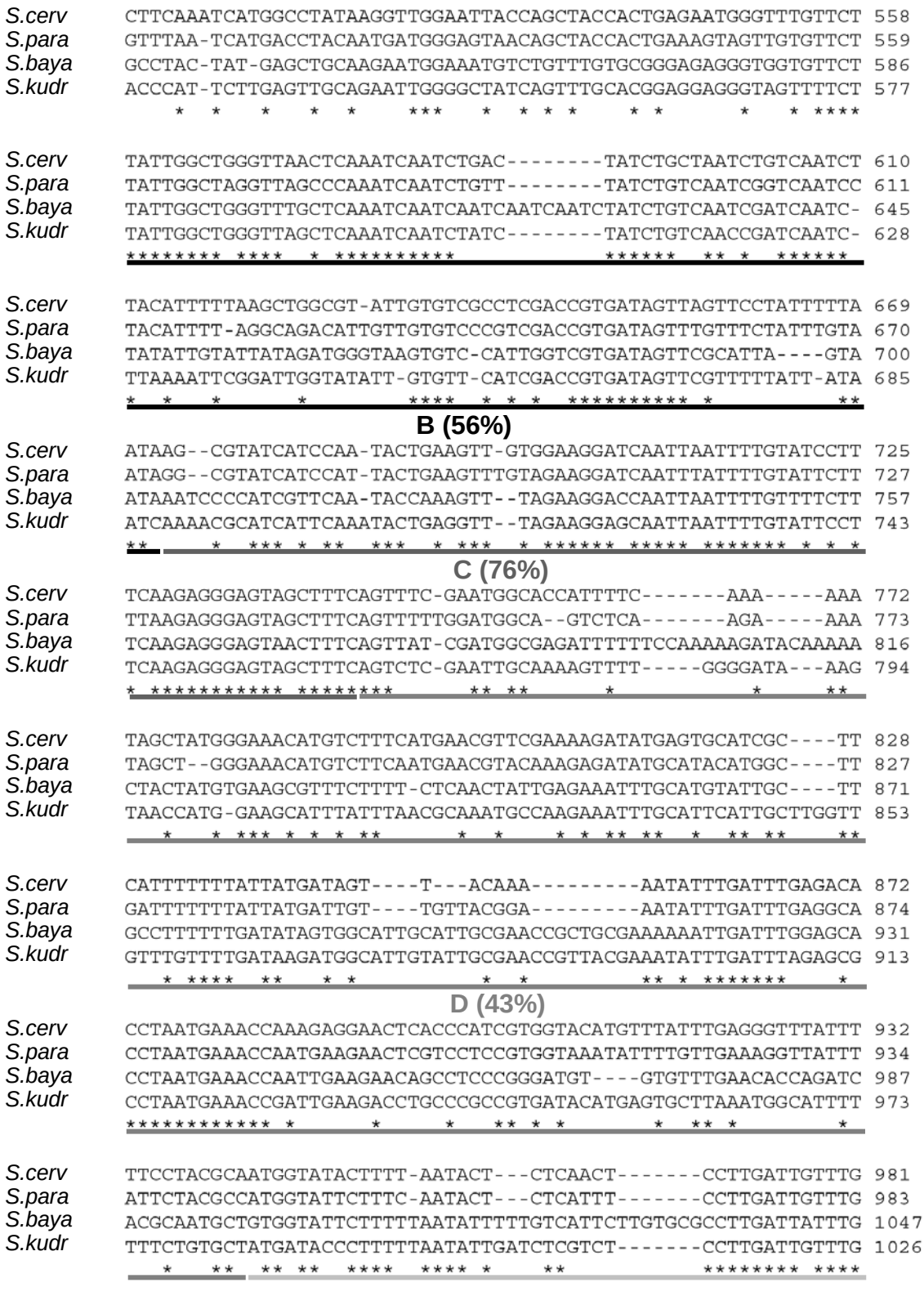

A
F7-MS2
399
775
A
D
E
R1-F7-MS2
311
775
F12-MS2
399
943
N-399-MS2
399
1052
B
V
MS2p-Crm1p
MS2p-Edc3p
MS2p-Mex67p
MS2p-Sub2p
MS2p-Yra1p
1 2 3 4
YRA1 pre-mRNA
F7-MS2 pre-mRNA
YRA1 mRNA
SCR1 RNA
a b c d e f g h i j k l m n o p q r s t u v w x
C
R1-F7-MS2 pre-mRNA
decay intermediate
SCR1 RNA
D
F12-MS2 pre-mRNA
N-399-MS2 pre-mRNA

FIGURE 3.14


Initiator
S.cerv ATGTCTGCTAACTTAGATAAATCCTTAGACGAAATCATTGGCTCTAACAAAGCAGGAAGT 60
S.para ATGTCGGCCAACTTAGATAAGTCCTTAGACGAAATTATTGGCTCTAACAGAGCAGGAGGT 60
S.baya ATGTCCGCTAACTTAGATAAATCCTTAGACGAAATTATTGGCTCCAGTAGACCAGGAGGC 60
S.kudr ATGTCCGCTAACTTAGATAAGTCCTTGGATGAAATTATTGGCTCTAATAGAGCAGGAGGT 60
S.cerv AATAGAGCCCGTGTCGGTGGTACTCGTGGTAACGGTCCAAGAAGAGTTGGTAAGCAAGTT 120
S.para AACAGAGCTCGTGTCGGTGGTACTCGTGGTACCGGTCCAAGAAGAGTTGGTAAGCAAATT 120
S.baya AACAGAGCTCGTGTCGGTGGTACCCGTGGTAACGGCCCAAAAAGAGTCGGCAAGCAAGTC 120
S.kudr AATAGAGCCCGTGTTGGCGGTACTCGTGGTAACGGCCCAAAAAGAGTTGGTAAGCAAGTC 120
S.cerv GGTAGCCAACGTAGGAGCCTTCCAAACAGAAGAG----------GCCCTATCAGA 165
S.para GGTAGCCAGCGTAGGAGCATTCCAAACAGAAAAG----------GACCTATCAGA 165
S.baya AACACCCAGCGCAGGAACGTTCCAAACAGAAACGTTCCAAACAGAAACGGCCCTATCAGA 180
S.kudr AGCAGTCAACGCAGGAACATTCCAAACAGAAATG----------GTCCTATCAGA 165
S.cerv AAAAATACTAGGGCACCTCCAAACGCAGTCGCTAGAGTTGCCAAGCTCTTGGACACCACT 225
S.para AAAAATGCTAGGGCTCCTCCAAACGCAGTCACTAGGGTTGCCAAGCTCTTGGACACCACT 225
S.baya AAGAATGTGAGGCCTCCTCCAAACGCTGTCGCTAGGGTTGCCAAGCTTTTGGATACCTCT 240
S.kudr AAAAATATCAGGGCTCCTCCAAGCGCAGTCGCTAGAGTTGCCAAGCTTTTGGATACCTCT 225
5´SS
S.cerv GTATGTTAA----TACGTGAAAT--GAGAGCTATTTGTTTAGTTACTCGCATC-----GT 334
S.para GTATGTTAAAA--TACATGAAATATGAGAGCTATTTATTTAGTTACTCGCATC-----AT 338
S.baya GTATGTTAAAAA-TACATGAAATGCAAGAGCTATTTATTTAGTTGCTAGCATC-----GT 354
S.kudr GTATGTTAAAAAATACATGACATGCGGGGGCTATTTACTTAGTCACTCGCATCGCCCCGT 345
A (78%)
S.cerv CTCGTGTGGATACGAGAAATATTCTTTGTAAGGAAGGATATGGCACACGCTTTTGATCAA 394
S.para CTCGTGTGGATACGAGAAATATTCTTTGTAAGGAAGGATATGGCGCATGCTTTTAATCAA 398
S.baya CTCGTGTGGATACGAGAAG-AGTCTTCGTGAGGAAGGATATGGCGCACGCTTTTAATCAA 413
S.kudr CTCGTGTGGATACGAGAGATATTCTTCATGAAGAAGGATATGGCGCATGCCTTAAATCAA 405
S.cerv GAATTCTCTTTTTA-----GAGATGAAGAAAGAGTCC---GAAATACTTCGCTAGAG--- 443
S.para GAATTCTCCTTTTA-----GAGAGAAAGAAAGAATCC---AAAATATTTCACTAGGG--- 447
S.baya GAGCTTTTCTTTTTCTGGTCATGAAGAGAAAGAATACCGAGAACTTTTCCGGTGAGAAAG 473
S.kudr GAGCTTTCGATCT------CAAAGAGAGAAAGATTCCAAGAAAATTTTCCGCTGGAAGAG 459
S.cerv ---AAAATACAAAAA--GAGTATCGTTTTCATAAGCCAACCATACCAATTTTTTTCAATA 498
S.para ---AAAAATGTAGAA--AAGTACCATTTTGGTAAGCCAGCCATTCTAATTTTT--CAACG 500
S.baya AATAAAATATTGAAA--GAGTACCATTTTGGTAAACAAGCTAT-CGTATTTCT--CAATA 528
S.kudr AAAAAAATATTGAAAAAGAGTACCATTTTGGCAAACAGGCTAC-CCAATTTTTTACAGGG 518


S.cerv CTTCAAATCATGGCCTATAAGGTTGGAATTACCAGCTACCACTGAGAATGGGTTTGTTCT 558
S.para GTTTAA-TCATGACCTACAATGATGGGAGTAACAGCTACCACTGAAAGTAGTTGTGTTCT 559
S.baya GCCTAC-TAT-GAGCTGCAAGAATGGAAATGTCTGTTTGTGCGGGAGAGGGTGGTGTTCT 586
S.kudr ACCCAT-TCTTGAGTTGCAGAATTGGGGCTATCAGTTTGCACGGAGGAGGGTAGTTTTCT 577
S.cerv TATTGGCTGGGTTAACTCAAATCAATCTGAC--------TATCTGCTAATCTGTCAATCT 610
S.para TATTGGCTAGGTTAGCCCAAATCAATCTGTT--------TATCTGTCAATCGGTCAATCC 611
S.baya TATTGGCTGGGTTTGCTCAAATCAATCAATCAATCAATCTATCTGTCAATCGATCAATC- 645
S.kudr TATTGGCTGGGTTAGCTCAAATCAATCTATC--------TATCTGTCAACCGATCAATC- 628
S.cerv TACATTTTTAAGCTGGCGT-ATTGTGTCGCCTCGACCGTGATAGTTAGTTCCTATTTTTA 669
S.para TACATTTT-AGGCAGACATTGTTGTGTCCCGTCGACCGTGATAGTTTGTTTCTATTTGTA 670
S.baya TATATTGTATTATAGATGGGTAAGTGTC-CATTGGTCGTGATAGTTCGCATTA----GTA 700
S.kudr TTAAAATTCGGATTGGTATATT-GTGTT-CATCGACCGTGATAGTTCGTTTTTATT-ATA 685
B (56%)
S.cerv ATAAG--CGTATCATCCAA-TACTGAAGTT-GTGGAAGGATCAATTAATTTTGTATCCTT 725
S.para ATAGG--CGTATCATCCAT-TACTGAAGTTTGTAGAAGGATCAATTTATTTTGTATTCTT 727
S.baya ATAAATCCCCATCGTTCAA-TACCAAAGTT--TAGAAGGACCAATTAATTTTGTTTTCTT 757
S.kudr ATCAAAACGCATCATTCAAATACTGAGGTT--TAGAAGGAGCAATTAATTTTGTATTCCT 743
C (76%)
S.cerv TCAAGAGGGAGTAGCTTTCAGTTTC-GAATGGCACCATTTTC-------AAA-----AAA 772
S.para TTAAGAGGGAGTAGCTTTCAGTTTTTGGATGGCA--GTCTCA-------AGA-----AAA 773
S.baya TCAAGAGGGAGTAACTTTCAGTTAT-CGATGGCGAGATTTTTTCCAAAAAGATACAAAAA 816
S.kudr TCAAGAGGGAGTAGCTTTCAGTCTC-GAATTGCAAAAGTTTT-----GGGGATA---AAG 794
S.cerv TAGCTATGGGAAACATGTCTTTCATGAACGTTCGAAAAGATATGAGTGCATCGC----TT 828
S.para TAGCT--GGGAAACATGTCTTCAATGAACGTACAAAGAGATATGCATACATGGC----TT 827
S.baya CTACTATGTGAAGCGTTTCTTTT-CTCAACTATTGAGAAATTTGCATGTATTGC----TT 871
S.kudr TAACCATG-GAAGCATTTATTTAACGCAAATGCCAAGAAATTTGCATTCATTGCTTGGTT 853
S.cerv CATTTTTTTATTATGATAGT----T---ACAAA---------AATATTTGATTTGAGACA 872
S.para GATTTTTTTATTATGATTGT----TGTTACGGA---------AATATTTGATTTGAGGCA 874
S.baya GCCTTTTTTGATATAGTGGCATTGCATTGCGAACCGCTGCGAAAAAATTGATTTGGAGCA 931
S.kudr GTTTGTTTTGATAAGATGGCATTGTATTGCGAACCGTTACGAAATATTTGATTTAGAGCG 913
D (43%)
S.cerv CCTAATGAAACCAAAGAGGAACTCACCCATCGTGGTACATGTTTATTTGAGGGTTTATTT 932
S.para CCTAATGAAACCAATGAAGAACTCGTCCTCCGTGGTAAATATTTTGTTGAAAGGTTATTT 934
S.baya CCTAATGAAACCAATTGAAGAACAGCCTCCCGGGATGT----GTGTTTGAACACCAGATC 987
S.kudr CCTAATGAAACCGATTGAAGACCTGCCCGCCGTGATACATGAGTGCTTAAATGGCATTTT 973
S.cerv TTCCTACGCAATGGTATACTTTT-AATACT---CTCAACT-------CCTTGATTGTTTG 981
S.para ATTCTACGCCATGGTATTCTTTC-AATACT---CTCATTT-------CCTTGATTGTTTG 983
S.baya ACGCAATGCTGTGGTATTCTTTTTAATATTTTTGTCATTCTTGTGCGCCTTGATTATTTG 1047
S.kudr TTTCTGTGCTATGATACCCTTTTTAATATTGATCTCGTCT-------CCTTGATTGTTTG 1026

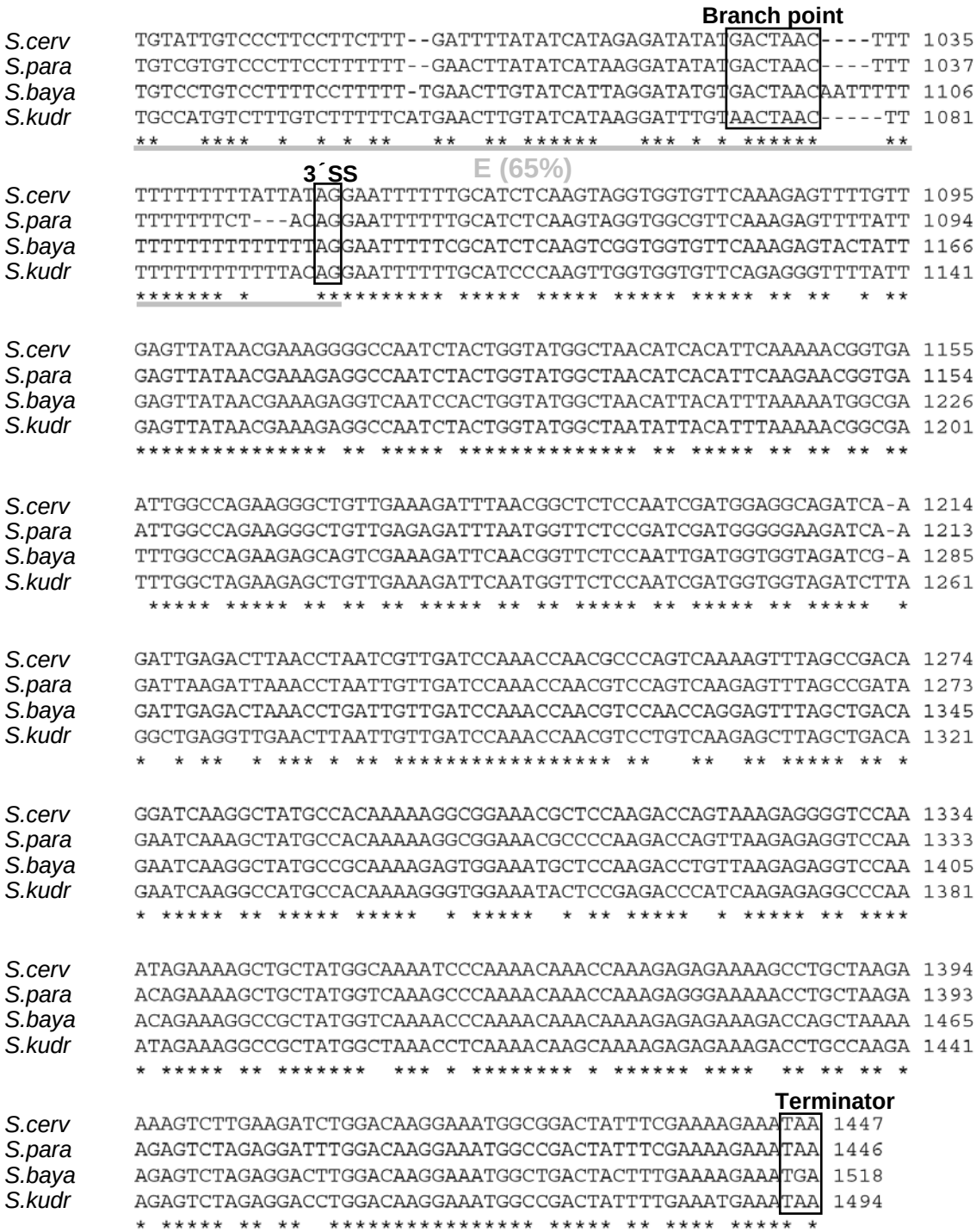
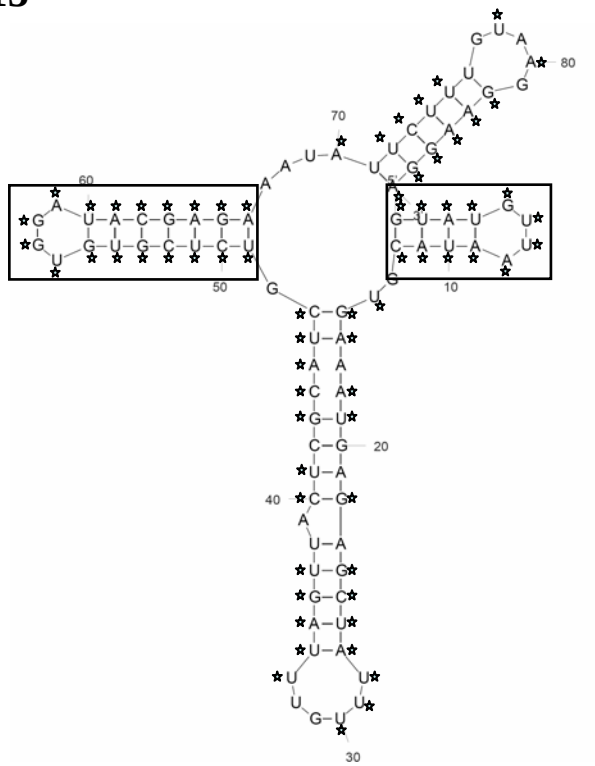

Branch point
S.cerv TGTATTGTCCCTTCCTTCTTT--GATTTTATATCATAGAGATATATGACTAAC----TTT 1035
S.para TGTCGTGTCCCTTCCTTTTTT--GAACTTATATCATAAGGATATATGACTAAC----TTT 1037
S.baya TGTCCTGTCCTTTTCCTTTTT-TGAACTTGTATCATTAGGATATGTGACTAACAATTTTT 1106
S.kudr TGCCATGTCTTTGTCTTTTTCATGAACTTGTATCATAAGGATTTGTAACTAAC-----TT 1081
3´SS
E (65%)
S.cerv TTTTTTTTTATTATAGGAATTTTTTGCATCTCAAGTAGGTGGTGTTCAAAGAGTTTTGTT 1095
S.para TTTTTTTCT---ACAGGAATTTTTTGCATCTCAAGTAGGTGGCGTTCAAAGAGTTTTATT 1094
S.baya TTTTTTTTTTTTTTAGGAATTTTTCGCATCTCAAGTCGGTGGTGTTCAAAGAGTACTATT 1166
S.kudr TTTTTTTTTTTTTACAGGAATTTTTTGCATCCCAAGTTGGTGGTGTTCAGAGGGTTTTATT 1141
S.cerv GAGTTATAACGAAAGGGGCCAATCTACTGGTATGGCTAACATCACATTCAAAAACGGTGA 1155
S.para GAGTTATAACGAAAGAGGCCAATCTACTGGTATGGCTAACATCACATTCAAGAACGGTGA 1154
S.baya GAGTTATAACGAAAGAGGTCAATCCACTGGTATGGCTAACATTACATTTAAAAATGGCGA 1226
S.kudr GAGTTATAACGAAAGAGGCCAATCTACTGGTATGGCTAATATTACATTTAAAAACGGCGA 1201
S.cerv ATTGGCCAGAAGGGCTGTTGAAAGATTTAACGGCTCTCCAATCGATGGAGGCAGATCA-A 1214
S.para ATTGGCCAGAAGGGCTGTTGAGAGATTTAATGGTTCTCCGATCGATGGGGGAAGATCA-A 1213
S.baya TTTGGCCAGAAGAGCAGTCGAAAGATTCAACGGTTCTCCAATTGATGGTGGTAGATCG-A 1285
S.kudr TTTGGCTAGAAGAGCTGTTGAAAGATTCAATGGTTCTCCAATCGATGGTGGTAGATCTTA 1261
S.cerv GATTGAGACTTAACCTAATCGTTGATCCAAACCAACGCCCAGTCAAAAGTTTAGCCGACA 1274
S.para GATTAAGATTAAACCTAATTGTTGATCCAAACCAACGTCCAGTCAAGAGTTTAGCCGATA 1273
S.baya GATTGAGACTAAACCTGATTGTTGATCCAAACCAACGTCCAACCAGGAGTTTAGCTGACA 1345
S.kudr GGCTGAGGTTGAACTTAATTGTTGATCCAAACCAACGTCCTGTCAAGAGCTTAGCTGACA 1321
S.cerv GGATCAAGGCTATGCCACAAAAAGGCGGAAACGCTCCAAGACCAGTAAAGAGGGGTCCAA 1334
S.para GAATCAAAGCTATGCCACAAAAAGGCGGAAACGCCCCAAGACCAGTTAAGAGAGGTCCAA 1333
S.baya GAATCAAGGCTATGCCGCAAAAGAGTGGAAATGCTCCAAGACCTGTTAAGAGAGGTCCAA 1405
S.kudr GAATCAAGGCCATGCCACAAAAGGGTGGAAATACTCCGAGACCCATCAAGAGAGGCCCAA 1381
Terminator
S.cerv ATAGAAAAGCTGCTATGGCAAAATCCCAAAACAAACCAAAGAGAGAAAAGCCTGCTAAGA 1394
S.para ACAGAAAAGCTGCTATGGTCAAAGCCCAAAACAAACCAAAGAGGGAAAAACCTGCTAAGA 1393
S.baya ACAGAAAGGCCGCTATGGTCAAAACCCAAAACAAACAAAAGAGAGAAAGACCAGCTAAAA 1465
S.kudr ATAGAAAGGCCGCTATGGCTAAACCTCAAAACAAGCAAAAGAGAGAAAGACCTGCCAAGA 1441
S.cerv AAAGTCTTGAAGATCTGGACAAGGAAATGGCGGACTATTTCGAAAAGAAATAA 1447
S.para AGAGTCTAGAGGATTTGGACAAGGAAATGGCCGACTATTTCGAAAAGAAATAA 1446
S.baya AGAGTCTAGAGGACTTGGACAAGGAAATGGCTGACTACTTTGAAAAGAAATGA 1518
S.kudr AGAGTCTAGAGGACCTGGACAAGGAAATGGCCGACTATTTTGAAATGAAATAA 1494

FIGURE 3.15

A



B

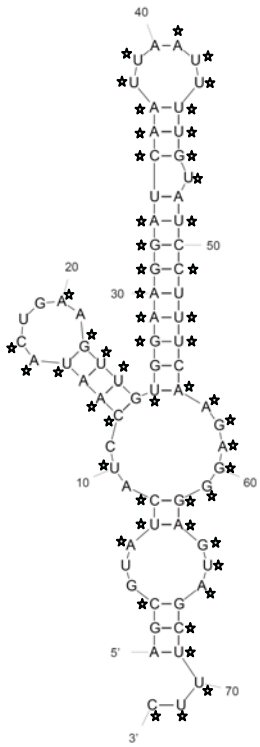


TABLE 3.1. Nucleotide Conservation of *YRA1* DNA Sequence in 4 *Saccharomyces* Species

Sequences	identity *
Exon1	81% (230/285)
A	78% (68/87)
B	56% (64/115)
C	76% (55/72)
D	43% (85/198)
E	65% (71/109)
Exon2	64% (254/396)
BetweenA-B	20% (37/186)

* The "identity" is calculated by the percentage of the numbers of nucleotide identical in 4 *Saccharomyces* species to the total numbers of nucleotide in the relative region.

TABLE 3.2. Yeast Strains Used in this Study

Strain name	Genotype
Y114	*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 UPF1 NMD2 UPF3*
Y871	*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 upf1::HIS3 NMD2 UPF3*
CFY25	*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 UPF1 NMD2 UPF3 edc3::URA3*
SYY158	*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 UPF1 NMD2 UPF3 edc3::URA3 upf1::HIS3*
CFY13	*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 UPF1 NMD2 UPF3 prt1-1*
Y1218	*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 UPF1 NMD2 UPF3 sup45-2*

TABLE 3.3. Plasmids Used in this Study

Name	Allele	Description
SYE74	pRS314-YRA1(XhoI-SacI)	Contains wild-type YRA1allele as a 2.2 kb XhoI-SacI fragment
SYE444	pRS314-YRA1-N-942(F16N)	Same as SYE74 but contains an internal deletion from nt 943 to the 3' end of the YRA1 intron
SYE495	pRS314-YRA1-N-852(NR9-F16N)	Same as SYE74 but contains an internal deletion from nt 853 to the 3' end of the YRA1 intron
SYE493	pRS314-YRA1-N-771(NR7-F16N)	Same as SYE74 but contains an internal deletion from nt 772 to the 3' end of the YRA1 intron
SYE521	pRS314-YRA1-N-753(R6A-F16N)	Same as SYE74 but contains an internal deletion from nt 754 to the 3' end of the YRA1 intron
SYR568	pRS314-YRA1-N-744(R6C-F16N)	Same as SYE74 but contains an internal deletion from nt 745 to the 3' end of the YRA1 intron
SYE491	pRS314-YRA1-N-711(NR5-F16N)	Same as SYE74 but contains an internal deletion from nt 712 to the 3' end of the YRA1 intron
SYE487	pRS314-YRA1-N-543(NR1-F16N)	Same as SYE74 but contains an internal deletion from nt 544 to the 3' end of the YRA1intron
SYE401	pRS314-YRA1-N-399(F16)	Same as SYE74 but contains an internal deletion from nt 400 to the 3' end of the YRA1 intron
SYE436	pRS314-YRA1-N-372(F16-R3)	Same as SYE74 but contains an internal deletion from nt 373 to the 3' end of the YRA1 intron
SYE434	pRS314-YRA1-N-342(F16-R2)	Same as SYE74 but contains an internal deletion from nt 343 to the 3' end of the YRA1 intron
SYE403	pRS314-YRA1-N-312(F16-R1)	Same as SYE74 but contains an internal deletion from nt 313 to the 3' end of the YRA1 intron
SYE442	pRS314-YRA1-C-397(R4)	Same as SYE74 but contains an internal deletion from the 5' end of the YRA1 intron to nt 396
SYE497	pRS314-YRA1-C-544(R4-F1)	Same as SYE74 but contains an internal deletion from the 5' end of the YRA1 intron to nt 543
SYE648	pRS314-YRA1-C-549(R4-F1C)	Same as SYE74 but contains an internal deletion from the 5' end of the YRA1 intron to nt 548
SYE564	pRS314-YRA1-C-553(R4-F1A)	Same as SYE74 but contains an internal deletion from the 5' end of the YRA1 intron to nt 552
SYE650	pRS314-YRA1-C-559(R4-F1D)	Same as SYE74 but contains an internal deletion from the 5' end of the YRA1 intron to nt 558
SYE566	pRS314-YRA1-C-565(R4-F1B)	Same as SYE74 but contains an internal deletion from the 5' end of the YRA1 intron to nt 564
SYE653	pRS314-YRA1-C-574(R4-F1E)	Same as SYE74 but contains an internal deletion from the 5' end of the YRA1 intron to nt 573
SYE611	pRS314-YRA1-C-625(R4-F14A)	Same as SYE74 but contains an internal deletion from the 5' end of the YRA1 intron to nt 624
SYE614	pRS314-YRA1-C-637(R4-F14B)	Same as SYE74 but contains an internal deletion from the 5' end of the YRA1 intron to nt 636
SYE503	pRS314-YRA1-C-649(R4-F15)	Same as SYE74 but contains an internal deletion from the 5' end of the YRA1 intron to nt 648
SYE616	pRS314-YRA1-C-661(R4-F15A)	Same as SYE74 but contains an internal deletion from the 5' end of the YRA1 intron to nt 660
SYE618	pRS314-YRA1-C-673(R4-F15B)	Same as SYE74 but contains an internal deletion from the 5' end of the YRA1 intron to nt 672
SYE654	pRS314-YRA1-C-679(R4-F15C)	Same as SYE74 but contains an internal deletion from the 5' end of the YRA1 intron to nt 678
SYE477	pRS314-YRA1-C-685(R4-F2)	Same as SYE74 but contains an internal deletion from the 5' end of the YRA1 intron to nt 684
SYE479	pRS314-YRA1-C-715(R4-F5)	Same as SYE74 but contains an internal deletion from the 5' end of the YRA1 intron to nt 714
SYE481	pRS314-YRA1-C-775(R4-F7)	Same as SYE74 but contains an internal deletion from the 5' end of the YRA1 intron to nt 774
SYE483	pRS314-YRA1-C-853(R4--F9)	Same as SYE74 but contains an internal deletion from the 5' end of the YRA1 intron to nt 852
SYE485	pRS314-YRA1-C-943(R4-F12)	Same as SYE74 but contains an internal deletion from the 5' end of the YRA1 intron to nt 942
SYE830	pRS314-YRA1-AR3-F2-NR6C	Same as SYE74 but contains an internal deletions from nts 373-672 and nt 744 to the 3' end of the YRA1 intron
SYE558	pRS314-YRA1-I-F1A-R6A	Same as SYE74 but contains an internal deletions from the 5' end of the YRA1 intron to nt 552 and nt 754 to the 3' end of the YRA1 intron
SYE764	pRS314-YRA1-I-F1-F16N	Same as SYE74 but contains an internal deletions from the 5' end of the YRA1 intron to nt 542 and nt 942 to the 3' end of the YRA1 intron
SYE646	pRS314-YRA1-I-F15B-F16N	Same as SYE74 but contains an internal deletions from the 5' end of the YRA1 intron to nt 672 and nt 942 to the 3' end of the YRA1 intron
SYE585	pRS314-YRA1-SL31	Same as SYE74 but contains a stem-loop structure 31 nucleotides downstream form the YRA1 transcription start site
SYE125	pRS314-YRA1-F7	Same as SYE74 but contains an internal deletions from nts 400-774
SYE589	pRS314-YRA1-SL31-F7	Same as SYE125 but contains a stem-loop structure 31 nucleotides downstream form the YRA1 transcription start site
SYE591	pRS314-YRA1-SL31-N-399(F16)	Same as SYE401 but contains a stem-loop structure 31 nucleotides downstream form the YRA1 transcription start site
SYE603	pRS314-YRA1-SL31-C-775(R4-F7)	Same as SYE487 but contains a stem-loop structure 31 nucleotides downstream form the YRA1 transcription start site
SYE374	pRS314-YRA1-R1-F7	Same as SYE74 but contains internal deletions from nts 312-774
SYE710	pRS314-YRA1-F7-MS2	Same as SYE125 but contains two MS2 binding sites between the intron deletion sites
SYE389	pRS314-YRA1-R1-F7-MS2	Same as SYE374 but contains two MS2 binding sites between the intron deletion sites
SYE385	pRS315-ADH1p-MS2p-Crm1p	Coding sequences of Crm1p fused to MS2-coat protein under the control of the ADH1 promoter
SYE383	pRS315-ADH1p-MS2p-Edc3p	Coding sequences of Edc3p fused to MS2-coat protein under the control of the ADH1 promoter
SYE393	pRS315-ADH1p-MS2p-Mex67p	Coding sequences of Mex67p fused to MS2-coat protein under the control of the ADH1 promoter
SYE454	pRS315-ADH1p-MS2p-Sub2p	Coding sequences of Sub2p fused to MS2-coat protein under the control of the ADH1 promoter
SYE354	pRS315-ADH1p-MS2p-Yra1p	Coding sequences of Yra1p fused to MS2-coat protein under the control of the ADH1 promoter
SYE541	pRS314-YRA1-I-R4-NR6A	Same as SYE74 but contains an internal deletions from nts 286-394 and nt 754 to the 3' end of the YRA1 intron
SYE543	pRS314-YRA1-I-R4-NR6B	Same as SYE74 but contains an internal deletions from nts 286-394 and nt 734 to the 3' end of the YRA1 intron
SYE539	pRS314-YRA1-I-R4-NR5	Same as SYE74 but contains an internal deletions from nts 286-394 and nt 714 to the 3' end of the YRA1 intron
SYE537	pRS314-YRA1-I-R4-NR2	Same as SYE74 but contains an internal deletions from nts 286-394 and nt 684 to the 3' end of the YRA1 intron
SYE535	pRS314-YRA1-I-R4-NR1	Same as SYE74 but contains an internal deletions from nts 286-394 and nt 544 to the 3' end of the YRA1 intron
SYE549	pRS314-YRA1-I-F1-R6A	Same as SYE74 but contains an internal deletions from nts 286-542 and nt 754 to the 3' end of the YRA1 intron
SYE560	pRS314-YRA1-I-F1B-R6A	Same as SYE74 but contains an internal deletions from nts 286-564 and nt 754 to the 3' end of the YRA1 intron
SYE547	pRS314-YRA1-I-F13-R6A	Same as SYE74 but contains an internal deletions from nts 286-577 and nt 754 to the 3' end of the YRA1 intron
SYE107	pRS314-YRA1-R1	Same as SYE74 but contains internal deletions from nts 312-394
SYE171	pRS314-YRA1-R2	Same as SYE74 but contains internal deletions from nts 342-394
SYE173	pRS314-YRA1-R3	Same as SYE74 but contains internal deletions from nts 372-394
SYE99	pRS314-YRA1-F1	Same as SYE74 but contains internal deletions from nts 400-542
SYE175	pRS314-YRA1-F13	Same as SYE74 but contains internal deletions from nts 400-577
SYE177	pRS314-YRA1-F14	Same as SYE74 but contains internal deletions from nts 400-612
SYE179	pRS314-YRA1-F15	Same as SYE74 but contains internal deletions from nts 400-647
SYE101	pRS314-YRA1-F2	Same as SYE74 but contains internal deletions from nts 400-682
SYE121	pRS314-YRA1-F5	Same as SYE74 but contains internal deletions from nts 400-712
SYE123	pRS314-YRA1-F6	Same as SYE74 but contains internal deletions from nts 400-743
SYE127	pRS314-YRA1-F8	Same as SYE74 but contains internal deletions from nts 400-797
SYE780	pRS314-YRA1-A-F2-NR7	Same as SYE74 but contains an internal deletions from nts 400-682 and nt 773 to the 3' end of the YRA1 intron
SYE761	pRS314-YRA1-A-F14B-NR6C	Same as SYE74 but contains an internal deletions from nts 400-636 and nt 744 to the 3' end of the YRA1 intron
SYE922	pRS314-YRA1-dA-C	Same as SYE74 but contains an internal deletions from the 5' end of the YRA1 intron to nt 394 and nts 673-743
SYE525	pRS314-YRA1-I-R4-F16N	Same as SYE74 but contains an internal deletions from the 5' end of the YRA1 intron to nt 394 and nt 943 to the 3' end of the YRA1 intron
SYE576	pRS314-YRA1-I-R4-NR10	Same as SYE74 but contains an internal deletions from the 5' end of the YRA1 intron to nt 394 and nt 884 to the 3' end of the YRA1 intron
SYE574	pRS314-YRA1-I-R4-NR9	Same as SYE74 but contains an internal deletions from the 5' end of the YRA1 intron to nt 394 and nt 854 to the 3' end of the YRA1 intron
SYE570	pRS314-YRA1-I-R4-NR8	Same as SYE74 but contains an internal deletions from the 5' end of the YRA1 intron to nt 394 and nt 799 to the 3' end of the YRA1 intron
SYE545	pRS314-YRA1-I-R4-NR7	Same as SYE74 but contains an internal deletions from the 5' end of the YRA1 intron to nt 394 and nt 774 to the 3' end of the YRA1 intron
SYE541	pRS314-YRA1-I-R4-NR6A	Same as SYE74 but contains an internal deletions from the 5' end of the YRA1 intron to nt 394 and nt 754 to the 3' end of the YRA1 intron
SYE770	pRS314-YRA1-I-F14-F16N	Same as SYE74 but contains an internal deletions from the 5' end of the YRA1 intron to nt 612 and nt 943 to the 3' end of the YRA1 intron
SYE644	pRS314-YRA1-I-F14A-F16N	Same as SYE74 but contains an internal deletions from the 5' end of the YRA1 intron to nt 625 and nt 943 to the 3' end of the YRA1 intron
SYE664	pRS314-YRA1-F15B-NR13	Same as SYE74 but contains an internal deletions from the 5' end of the YRA1 intron to nt 672 and nt 983 to the 3' end of the YRA1 intron
SYE666	pRS314-YRA1-F15B-NR14	Same as SYE74 but contains an internal deletions from the 5' end of the YRA1 intron to nt 672 and nt 1023 to the 3' end of the YRA1 intron
SYE668	pRS314-YRA1-F15B-NR15	Same as SYE618 but contains a NcoI site at the 3' end of the YRA1 intron

TABLE 3.4. Oligonucleotides Used in this Study

Name	Sequences
YRA1-3	ACTTGAAGAACTATAAAAGGCCGC
YRA1-4	GTGTGCCATATCCTTCCTTACAAA 3'
YRA1-5	GTATGTTAATACGTGAAATGAGAGCT
YRA1-6	GAGTTGCCAAGCTCTTGGACACCACTA
YRA1-7	TCATGAAAGACATGTTTCCCATAGCTA
YRA1-BglII-F	ACCTAGATCTGCTAAATGTCTGCTAACTTAGATA
YRA1-StyI-R	TGCTTAATGTCCCTTGGCAAACCTTCG
YRA1-BglI-R	TAGCAGATCTAGGTATTTTCTTAATATGGATTTCTTA
YRA1-DU-R1	TAGCAGATCTAGTTTCAAATAGAACGAGGGAGATTTT
YRA1-DU-R2	TAGCAGATCTTTATCTATTTGTCTTCGATAGTTTTCG
YRA1-DI-5'	GATCGAATTCCTCCTTGATTGTTTGTGTATTGTCC
YRA1-DI-F1	GATCGAATTCAGAATGGGTTTGTTCTTATTGGCTG
YRA1-DI-F1A	GATCGAATTCTGTTCTTATTGGCTGGGTTAACTCAAAT
YRA1-DI-F1B	GATCGAATTCCTGGGTTAACTCAAATCAATCTGACTAT
YRA1-DI-F1C	GATCGAATTCGGGTTTGTTCTTATTGGCTGGGTTAAC
YRA1-DI-F1D	GATCGAATTCTATTGGCTGGGTTAACTCAAATCAATC
YRA1-DI-F1E	GATCGAATTCAACTCAAATCAATCTGACTATCTGCTA
YRA1-DI-F2	GATCGAATTCCCAATACTGAAGTTGTGGAAGGATC
YRA1-DI-F3	GATCGAATTCTCGCTTCATTTTTTTATTATGATAG
YRA1-DI-F4	GATCGAATTCTTTATATCATAGAGATATATGACTA
YRA1-DI-F5	GATCGAATTCATTTTGTATCCTTTCAAGAGGGAGT
YRA1-DI-F6	GATCGAATTCTCAGTTTCGAATGGCACCATTTTCA
YRA1-DI-F7	GATCGAATTCTAGCTATGGGAAACATGTCTTTCAT
YRA1-DI-F8	GATCGAATTCGAACGTTCGAAAAGATATGAGTGCA
YRA1-DI-F9	GATCGAATTCAAAATATTTGATTTGAGACACCTAA
YRA1-DI-F10	GATCGAATTCCCAAAGAGGAACTCACCCATCGTGG
YRA1-DI-F11	GATCGAATTCGTTTATTTGAGGGTTTATTTTTCCT
YRA1-DI-F12	GATCGAATTCATGGTATACTTTTAATACTCTCAA
YRA1-DI-F13	GATCGAATTCAATCAATCTGACTATCTGCTAATCT
YRA1-F13-UAA	GATCGAATTCTAAAATCAATCTGACTATCTGCTAATCT
YRA1-DI-F14	GATCGAATTCCATTTTTAAGCTGGCGTATTGTGTC
YRA1-F14-UAA	GATCGAATTCTAACATTTTTAAGCTGGCGTATTGTGTC
YRA1-DI-F14A	GATCGAATTCGGCGTATTGTGTCGCCTCGACCGTGATA
YRA1-DI-F14B	GATCGAATTCCGCCTCGACCGTGATAGTTAGTTCCTAT
YRA1-DI-F15	GATCGAATTCTGATAGTTAGTTCCTATTTTTAATA
YRA1-DI-F15A	GATCGAATTCCCTATTTTTAATAAGCGTATCATCCAAT
YRA1-DI-F15B	GATCGAATTCAAGCGTATCATCCAATACTGAAGTTGTG
YRA1-DI-F15C	GATCGAATTCATCATCCAATACTGAAGTTGTGGAAGG
YRA1-DI-F16N	TACGCCATGGGAATTTTTTGCATCTCAAGTAGGTGGTG
YRA1-DI-F16	GATCGAATTCGAATTTTTTGCATCTCAAGTAGGTGGTG
YRA1-DI-3'	AATCTTGATCTGCCTCCATCGATTG
YRA1-DI-GF1	TTTGCCAAGGGACATTAAGCAGGATGCTGTAAGAGTATGTATTTGTTTAGTTACTCGCATCGTCT
YRA1-DI-GF2	TTTGCCAAGGGACATTAAGCAGGATGCTGTAAGAGTATGTCGTCTCGTGTGGATACGAGAAATAT
YRA1-DI-GF3	TTTGCCAAGGGACATTAAGCAGGATGCTGTAAGAGTATGTAATATTCTTTGTAAGGAAGGATATG
YRA1-DI-GF4	TTTGCCAAGGGACATTAAGCAGGATGCTGTAAGAGTATGTTATGGCACACGCTTTTGATCAAGAA
YRA1-DI-R1	GATCGAATTCAGCTCTCATTTCACGTATTAACATA
YRA1-DI-R2	GATCGAATTCTTAGTTACTCGCATCGTCTCGTGTG
YRA1-DI-R3	GATCGAATTCGAGAAATATTCTTTGTAAGGAAGGA
YRA1-DI-R4	GATCGAATTCTCTTACAGCATCCTGCTTAATGTCC
YRA1-R4-BamHI	TACGGGATCCTCTTACAGCATCCTGCTTAATGTCC
YRA1-DI-GR1	GATACAAAATTAATTGATCCTTCCACAACTTCAGTATTGGCGGTCGAGGCGACACAATACGCCAG
YRA1-DI-GR2	GATACAAAATTAATTGATCCTTCCACAACTTCAGTATTGGTAAGATTGACAGATTAGCAGATAGT
YRA1-DI-GR3	GATACAAAATTAATTGATCCTTCCACAACTTCAGTATTGGTGAGTTAACCCAGCCAATAAGAACA
YRA1-DI-GR4	GATACAAAATTAATTGATCCTTCCACAACTTCAGTATTGGCAGTGGTAGCTGGTAATTCCAACCT
YRA1-EcoR1-F	GATCAAGAATTCTCTTTTTAGAGATGAA
YRA1-NcoI-F	TACGCCATGGTATACTTTTAATACTCTCAACTC
YRA1-NcoI-R	TATACCATGGCGTAGGAAAAATAAACCCTCAAATAA
YRA1-NcoI-R9	TACGCCATGGTGTAACTATCATAATAAAAAAATGAAG
YRA1-NcoI-R7	TACGCCATGGTTTTTTTGAAAATGGTGCCATTCGAAAC
YRA1-NcoI-R5	TACGCCATGGTAATTGATCCTTCCACAACTTCAGTAT
YRA1-NcoI-R2	TACGCCATGGATGATACGCTTATTAAAAATAGGAACT
YRA1-NcoI-R1	TACGCCATGGCAGTGGTAGCTGGTAATTCCAACCTTA
YRA1-NcoI-R6A	TACGCCATGGTCGAAACTGAAAGCTACTCCCTCTTGA
YRA1-NcoI-R6B	TACGCCATGGCTCTTGAAAGGATACAAAATTAATTGA
YRA1-NcoI-R6C	TACGCCATGGAAGCTACTCCCTCTTGAAAGGATACAAA
YRA1-NcoI-R3	TACGCCATGGTGCACTCATATCTTTTCGAACGTTCAT
YRA1-NcoI-R8	TACGCCATGGATGAAAGACATGTTTCCCATAGCTATT

YRA1-NcoI-R10	TACGCCATGGTTTCATTAGGTGTCTCAAATCAAATAT
YRA1-NcoI-R11	TACGCCATGGATGTACCACGATGGGTGAGTTCCTCTT
YRA1-Nco-R13	TACGCCATGGACAAACAATCAAGGAGTTGAGAGTA
YRA1-Nco-R14	TACGCCATGGTATCTCTATGATATAAAATCAAAGA
YRA1-Nco-R15	TACGCCATGGCTATAATAAAAAAAAAAAAGTTAGT
YRA1-SL31-F	GATCGGATCCCGCGGTTCGCCGCGGGATCTAAAAATCTCCCTCGTTCTATTTGAAACTTT
YRA1-SL31P	GCGGCGAACCGCGGGATCTTTATCTAT
YRA1-MS2-I-R	GATCGAATTCGATGGATCCAGCTCTCATTTCACGTATTAACATACTCTT
MS2-BamH-EcoR-F	GATCCAAAGTTGAGGATCACCCAACTGTGCAGGTCGAGACTAGAAAAGTTGAGGATCACCCAACTG
MS2-BamH-EcoR-R	AATTCAGTTGGGTGATCCTCAACTTTTCTAGTCTCGACCTGCACAGTTGGGTGATCCTCAACTTTG
YRA1-BamHI-R	GATCGGATCCTTGATCAAAAGCGTGTGCCATATCCTTC
YRA1-MS2-F	GATCGCGGCCGCGCTAGCATGTCTGCTAACTTAGATAAATCCTTAG
EDC3-MS2-F	GATCGCGGCCGCGCTAGCATGTCACAATTTGTTGGTTTCGGAGTAC
EDC3-MS2-R	ACGCCCTCACTTTGAAAGCTTGAGATTT
MS2-MEX67-F	GATCGCGGCCGCGCTAGCATGAGCGGATTTCACAATGTTGGAAATA
MS2-MEX67-R	GATCGTCGACGAGCTCATCAAAAGCTCTCTTAGATCTTTGAA
CRM1-MS2-F	GATCGCGGCCGCGCTAGCATGGAAGGAATTTTGGATTTTTCTAACG
CRM1-MS2-R	GATCGTCGACGGATCCAAGCTTTAGCTTGTGTCATTTGTTCCGC
SUB2-MS2-F	GATCGCGGCCGCGCTAGCATGTCACACGAAGGTGAAGAAGATTTAT
SUB2-MS2-R	GATCGTCGACTCTAGATTCCAAAAATCTTACCGTTAAGAA
YRA1-m1	GATGCAAAAAATTCTCTTACAGCATCC
YRA1-p1	AGCTCTCATTTCACGTATTAACATAC
yra1-2-r	CGCCATTTCCTTGTCCAGATCTTC

CHAPTER IV

General Discussion

Main findings

The work of this thesis demonstrates that autoregulation of the *YRA1* gene is accomplished through the coordination of multiple steps in its expression, particularly the splicing, nuclear export, and cytoplasmic degradation of its pre-mRNA. My work was the first to show that a yeast endogenous intron-containing pre-mRNA is exported to the cytoplasm through a Crm1p-dependent pathway and to identify an endogenous intron containing pre-mRNA that is degraded in the cytoplasm through a non-NMD mechanism despite the presence of multiple in-frame PTCs in the intron sequence.

In this thesis, microarray analysis together with other approaches revealed that Edc3p specifically mediates the degradation of only two yeast transcripts, *YRA1* pre-mRNA and *RPS28B* mRNA (Chapter II). Edc3p-mediated *YRA1* pre-mRNA degradation was shown to occur in the cytoplasm (Chapter II) and to be independent of translation (Chapter III). Further work found that Edc3p-mediated *YRA1* pre-mRNA degradation is a component of the negative feedback loop involved in *YRA1* autoregulation (Chapter II). The roles of specific *trans*-regulating factors (Edc3p, Yra1p, Mex67p and Crm1p) involved in the autoregulation of *YRA1* and Edc3p-mediated *YRA1* pre-mRNA decay were then characterized (Chapter II and III). The *cis*-acting elements regulating *YRA1* pre-mRNA splicing and decay were also delineated (Chapter II and III). Additional work presented in the thesis indicated that Edc3p-mediated *YRA1* pre-mRNA degradation is accomplished through the cooperative effects of *cis*- and *trans*-regulation (Chapter III).

The major findings of this thesis include:

1) Edc3p directly controls *YRA1* pre-mRNA degradation.
2) *YRA1* pre-mRNA degradation occurs through a 5′ to 3′ decay mechanism that requires the Dcp1p/Dcp2p decapping enzyme and the Xrn1p 5′ to 3′ exoribonuclease.
3) Edc3p-mediated *YRA1* pre-mRNA degradation occurs in the cytoplasm.
4) *YRA1* pre-mRNA is translationally repressed and Edc3p-mediated *YRA1* pre-mRNA degradation is independent of translation.
5) Edc3p activates but does not catalyze decapping of the *YRA1* pre-mRNA.
6) Edc3p-mediated *YRA1* pre-mRNA degradation is a component of the negative feedback loop involved in *YRA1* autoregulation.
7) *YRA1* autoregulation involves splicing inhibition, nuclear export, and cytoplasmic degradation of its pre-mRNA.
8) Yra1p autoregulates its own expression by inhibiting *YRA1* pre-mRNA splicing and committing the pre-mRNA to nuclear export.
9) *YRA1* exon1 sequences inhibit its pre-mRNA splicing in a size-dependent but sequence independent manner.
10) *YRA1* exon1 in *cis*, and Yra1p in *trans*, inhibit *YRA1* pre-mRNA splicing at or before the first step of the splicing reaction.
11) Crm1p is required for *YRA1* pre-mRNA nuclear export.
12) Mex67p functions in both nuclear export and cytoplasmic degradation of *YRA1* pre-mRNA.

13) The *YRA1* intron contains five functionally different but interdependent modules required for efficient Edc3p-mediated *YRA1* pre-mRNA degradation. Two modules are required for triggering an Edc3p-response and the other three modules are required for translational repression, thus inhibiting NMD of the transcript.

14) Mex67p appears to interact with sequences in the *YRA1* intron to inhibit NMD, most likely through translational repression, and to enhance the Edc3p response of *YRA1* pre-mRNA.

Since most of these findings have already been discussed in Chapters II and III I will just propose our current model of *YRA1* autoregulation and discuss the potential roles of Edc3p-mediated *YRA1* pre-mRNA degradation based on our findings. Then I will explore questions raised by these findings and the larger issues that remain unanswered in Edc3p-mediated transcript specific RNA degradation.

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A model for *YRA1* autoregulation and Edc3p-mediated *YRA1* pre-mRNA degradation

To integrate all of our data, we propose the following model for *YRA1* autoregulation, in which Edc3-mediated *YRA1* pre-mRNA degradation plays a key role (Figure 4.1): When nuclear Yra1p levels are low, *YRA1* is transcribed into pre-mRNA. U1 snRNP binds to the 5′ SS by a base-pairing interaction to initiate the formation of spliceosome. Then,

YRA1 pre-mRNA is spliced to mRNA. The mature mRNA is exported to the cytoplasm, translated to generate Yra1p, and the latter is imported into the nucleus, recruited to elongating RNAPII, and then packaged into nascent mRNPs to serve a function in general mRNA export (Figure 4.1A). However, when nuclear Yra1p levels reach a threshold, Yra1p is likely recruited to the *YRA1* gene by THO and Sub2p during an early phase of transcription elongation. Recruitment of Yra1p inhibits formation of spliceosome and promotes export of *YRA1* pre-mRNA. Yra1p then recruits Mex67p, inhibiting its pre-mRNA splicing and committing the pre-mRNA to export by the Crm1p-dependent pathway. Once in the cytoplasm, the *YRA1* pre-mRNA is degraded immediately through an Edc3p-mediated pathway without engaging the translation apparatus. Edc3p interacts with Crm1p and is recruited to the Edc3p response elements localized in the *YRA1* intron. Mex67p in the exported *YRA1* pre-mRNP is also involved in the degradation of *YRA1* pre-mRNA by repressing translation of *YRA1* pre-mRNA, thus inhibiting NMD and enhancing Edc3p's function (Figure 4.1B). Edc3p ultimately recruits the Dcp1p/Dcp2p decapping enzyme and this leads to decapping activation followed by 5′ to 3′ decay of *YRA1* pre-mRNA independent of deadenylation.

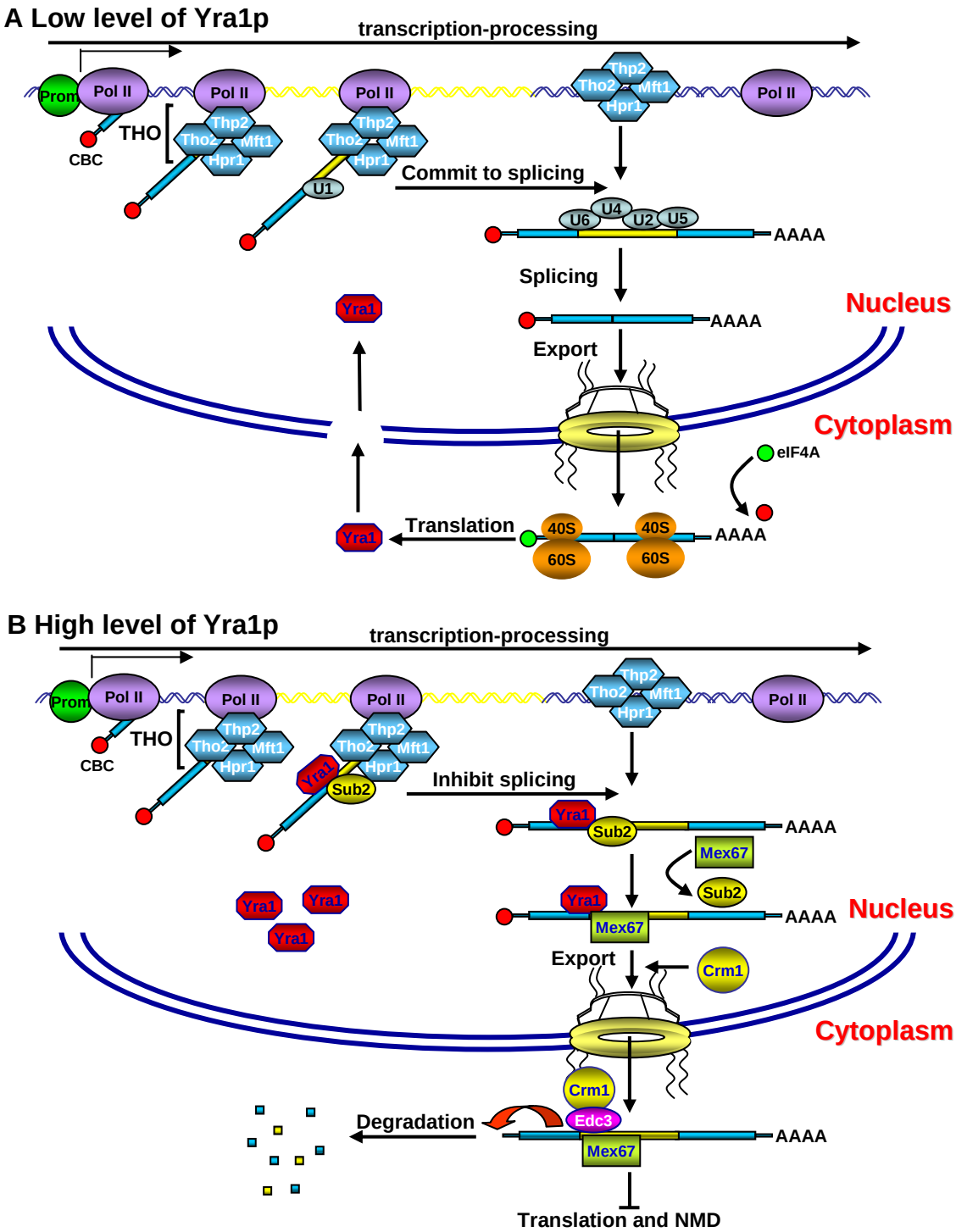

Figure 4.1 A model for *YRA1* autoregulation and Edc3p-mediated *YRA1* pre-mRNA degradation. Details are described in the text.

Transcript-specific decay mediated by Edc3p

Our results, combined with those from the Jacquier group (Badis et al., 2004), strongly indicate that Edc3p is a transcript-specific decapping activator. In turn, this conclusion suggests that there must be unique features of the *YRA1* pre-mRNP and the *RPS28B* mRNP that render them Edc3p-responsive. However, the interactions of Edc3p with its two substrates are very different: First, the *cis*-regulatory elements involved in Edc3p-mediated *RPS28B* mRNA and *YRA1* pre-mRNA degradation show no sequence similarity and are not interchangeable. Second, Edc3p-mediated *RPS28B* mRNA and *YRA1* pre-mRNA degradation also have different functional requirements for Edc3p. The former requires the function of full-length Edc3p and the latter only requires the function of the N-terminal 70 amino acids of Edc3p (data not shown in this thesis). Third, Edc3p-mediated *RPS28B* mRNA degradation requires translation (data not shown in this thesis). In contrast, Edc3p-mediated *YRA1* pre-mRNA degradation is independent of translation. These observations indicate that, even for transcripts regulated by the same decay factor or pathway, the decay mechanisms can be still distinct. We speculate that Edc3p may utilize different domains to identify different features of its mRNP substrates.

Possible regulatory functions of the Edc3p-mediated mRNA decay pathway

Our genome-wide microarray analysis has identified only two transcripts regulated by Edc3p, the *YRA1* pre-mRNA and the *RPS28B* mRNA. The existence of a specific cytoplasmic pathway dedicated to only two transcripts raises an important question of the biological function of this pathway, i.e., does the Edc3p-mediated decay pathway have additional regulatory functions in yeast cells? In support of the notion that Edc3p may have broader functions, there are observations that Edc3p physically interacts with the Dcp1p/Dcp2p decapping enzyme (Gavin et al., 2002; Ho et al., 2002; Ito et al., 2001; Uetz et al., 2000) and that Edc3p appears to have a comparable number of molecules per cell as that of Dcp1p (Gavin et al., 2002; Ho et al., 2002; Huh et al., 2003). In addition, biochemical evidence from both yeast and human cells suggests that Edc3p appears to be a core component of a large decapping enzyme complex (Fenger-Gron et al., 2005; Gavin et al., 2002; Ho et al., 2002; Huh et al., 2003). Third, deletions of *EDC3* and mutations in *DCP1* or *DCP2* exhibit synthetic or addictive effects on both cell growth and decapping of general reporter mRNAs (Kshirsagar and Parker, 2004). The prediction that Edc3p might have additional regulatory functions and the reasons that we identified only two transcripts regulated by the Edc3p-mediated pathway in this work might be:

1) Edc3p might regulate additional mRNAs during different stages of cell cycle progression. Since our microarray studies utilized asynchronous cells, some potential Edc3p substrates subject to cell cycle regulation could have been missed. In fact, two observations suggest that Edc3p may have a role in cell cycle regulation. First, *EDC3* exhibits a synthetic lethal interaction with *NDD1*, whose product is involved in G2/M specific gene transcription (Badis et al., 2004; Koranda et al., 2000). Second,

overexpression of *YRA1*, which is regulated by Edc3p, results in G1 arrest (Espinet et al., 1995). Yra1p is required for S phase entry and plays a role in DNA replication (Swaminathan et al., 2007). Therefore, additional Edc3p substrates might be identified in synchronous cells arrested in different cell cycle stages.

2) Edc3p might regulate additional mRNAs under different growth conditions. Another possibility is that Edc3p functions as a regulatory subunit of the decapping enzyme and its function in mRNA decapping is dispensable under normal growth conditions but becomes essential under different growth conditions. Our data indicate that Edc3p-mediated *YRA1* pre-mRNA degradation is functionally linked to Crm1p-mediated pre-mRNA nuclear export. The fact that Edc3p and Crm1p also interact suggests that the nuclear mRNA export pathway may impart specificity to this mRNA decay pathway, i.e., that transcripts exported by the Crm1p-mediated decay pathway are exclusively degraded by the Edc3p-mediated decay pathway. Since mammalian mRNAs exported by the Crm1p pathway largely encode factors involved in "early" or stress responses (Brennan et al., 2000; Gallouzi et al., 2000; Higashino et al., 2005; Kimura et al., 2004; Schutz et al., 2006), it is possible that a fraction of yeast mRNA is also exported by the Crm1p-mediated export pathway under certain stress conditions such as heat shock, osmotic stress, and oxidative stress. This group of mRNAs might also be controlled by Edc3p-mediated pathway under the stress conditions.

3) Edc3p might control the degradation of a limited class of intron-containing pre-mRNAs that enter the cytoplasm. Most intron-containing pre-mRNAs are retained in the nucleus to be spliced. However, it has previously been shown that

several inefficiently spliced pre-mRNAs enter the cytoplasm where they are degraded by the NMD pathway (He et al., 1993; Vilardell et al., 2000). It is possible that the Edc3p-mediated decay pathway is another mRNA surveillance system that targets a different class of intron-containing pre-mRNAs, such as those whose intron is localized far from the translation initiation codon or whose branch point region deviates from the consensus motif. These pre-mRNAs may be intrinsically low in abundance and thus under the limit of microarray analysis. The potential pre-mRNAs regulated by Edc3p might include those that have large introns or have similar splicing signals as those in *YRA1* pre-mRNA, and those whose intron is located far from the translation initiation codon. In addition, splicing defective pre-mRNAs and intermediates are also exported to the cytoplasm (Hilleren and Parker, 2003). Interestingly, these pre-mRNAs are at least partially degraded by a decapping-dependent mechanism and appear to be resistant to NMD (Hilleren and Parker, 2003), much as we observed for *YRA1* pre-mRNA. It is possible that Edc3p also targets these splicing defective pre-mRNAs and their intermediates.

4) Edc3p might function in a step that is not rate-limiting or be functionally redundant to other protein(s). As a part of an emerging set of proteins functioning in decapping, Edc3p might assist decapping in a non-rate-limiting manner. This is supported by the fact that deletion of *EDC3* in wild-type cells has almost no phenotype, but deletion of *EDC3* in strains with compromised temperature-sensitive alleles of *dcp1* and *dcp2* exaggerates the defect of general reporter mRNA degradation even at the permissive temperature. Another possibility is that there are other functionally redundant proteins for Edc3p in cells. As reported by the Jacquier group, deletion of *SCD6*, a gene encoding

another Lsm-like domain containing protein, is synthetically lethal with deletion of *EDC3*. Scd6p also interacts with Edc3p physically and shares a number of physical interacting partners involved in decapping (Zemam K, 2007). These findings suggest that the two Lsm domain-containing proteins share a redundant function in mRNA decapping.

Physiological Significance

This *YRA1* pre-mRNA regulation provides a further illustration of the extensive coupling of transcription, RNA processing, export, and cytoplacmic degradation. It will thus be very informative to elucidate the molecular mechanism of gene expression regulation in detail. In addition, 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, 2003a). This observation suggests that Rev may target the same cellular component or pathway as Yra1p to promote nuclear pre-mRNA export. Therefore, an understanding of the molecular mechanism involved in *YRA1* autoregulation may well shed light on the regulation of retroviral gene expression, possibly leading to novel anti-viral molecules.

Future directions

Our data have shown that the regulation of Edc3p-mediated *YRA1* pre-mRNA degradation requires multiple sequence segments in *cis* and two factors in *trans*. The two

factors, Edc3p and Mex67p, appear to have distinct functions. Edc3p acts as a decapping activator and is thus likely to be involved in both substrate recognition and recruitment of the decapping enzyme. Mex67p is likely to be a component of the cytoplasmic *YRA1* pre-mRNP and to function as a repressor of *YRA1* pre-mRNA translation by interacting with a *cis*-element(s) in the *YRA1* intron. However, it is still unknown if Edc3p and/or Mex67p directly bind to the intronic *cis*-elements. In addition, the observation that inactivation of Mex67p switches *YRA1* pre-mRNA from an Edc3p substrate to an NMD substrate also raises the question of whether Mex67p dictates the Edc3p substrate specificity or whether it just performs an essential function (i.e., translation repression) that is epistatic to the decapping activation function of Edc3p. Given the complexity of the *cis*-acting elements of the *YRA1* intron, and the observation that tethering Mex67p to the *YRA1* intron only largely but not completely restores the Edc3p response of a partial Edc3p substrate, there might be more factors besides Mex67p involved in Edc3p-mediated *YRA1* pre-mRNA degradation. Finally, it is still a mystery as to how Edc3p executes its function. Edc3p contains an Lsm domain at its N-terminus, an FDF domain in its middle, and an YjeF-N domain at its C-terminus. Our data indicate that the 70 amino acid N-terminal Lsm domain of Edc3p is sufficient for Edc3p-mediated *YRA1* pre-mRNA degradation. In addition, the Lsm domain of EDC3 in *Drosophila* mediates DCP1 binding and P-body targeting (Tritschler et al., 2007). However, it is unclear if Edc3p uses the same domain to recognize the substrate and recruit the decapping enzyme. To answer these molecular mechanism questions and to understand the possible regulatory function of Edc3p-medated mRNA degradation, future work should consider:

1) Defining the functions and the functional relationships of Edc3p and Mex67p in Edc3p-mediated *YRA1* pre-mRNA degradation.

2) Identifying other *trans*-acting factors that bind to the *YRA1* intron and evaluating the roles of these factors.

3) Dissecting the molecular events and interactions that lead to decapping activation of *YRA1* pre-mRNA.

4) Identifying other substrates of Edc3p-mediated mRNA degradation.

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