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Expression of the essential mRNA export factor Yra1p is autoregulated by a splicing-dependent mechanism

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ABSTRACT

Recent evidence supports the idea that pre-mRNA splicing and mRNA export are mechanistically coupled. In metazoans, this process appears to be mediated by a multicomponent complex, which associates with the spliced RNA upstream of the exon–exon junction. One of these components (Aly/REF) has a homolog in the budding yeast *Saccharomyces cerevisiae* known as Yra1p. The *YRA1* gene is essential for growth and required for mRNA export. Notably, *YRA1* is one of the only ~5% intron-containing genes in yeast. Moreover, the *YRA1* intron has several unusual features and is conserved in other budding yeast species. Previously, overexpression of intronless *YRA1* was shown to be toxic. We show here that overexpression of the intron-containing gene results in increased levels of unspliced pre-mRNA but normal levels of Yra1 protein; conversely, expression of the cDNA results in increased levels of protein and accumulation of nuclear poly(A)$^+$ RNA. Two additional lines of evidence suggest that expression of Yra1p is autoregulated: First, expression of excess Yra1p from a plasmid reduces the level of tagged, chromosomal Yra1p, and, second, this effect requires wild-type protein. Replacement of the *YRA1* intron with that of other *S. cerevisiae* genes cannot rescue the dominant-negative growth defect of intronless *YRA1*. We conclude that the level of Yra1p is negatively autoregulated by a mechanism that involves splicing of its unusual intron. Tight control of the levels of Yra1p might be necessary to couple the rates of pre-mRNA splicing and mRNA export.

Keywords: Aly; hnRNP; intron; SUB2

INTRODUCTION

Prior to their export into the cytoplasm, the primary transcripts of protein-encoding eukaryotic genes (pre-mRNAs) require extensive processing into mRNAs, including the removal of intronic sequences and the addition of a cap structure to the 5′ end and a poly(A) tail to the 3′ of the transcript. Presumably, cells have evolved mechanisms to coordinate these processing events to improve the efficiency of each step and to ensure that only fully and correctly processed transcripts are translated into protein. Early evidence for a coupling between splicing and downstream events came from the observation that the presence of an intron promotes expression of *trans*-genes in metazoan cells (Matsumoto et al., 1998). More recently, this effect has been attributed to an enhancement in the rate of export of mRNAs that have undergone splicing, relative to mRNAs derived from non-intron-containing genes (Luo & Reed, 1999). In the same study, it was proposed that the increased export efficiency was due to assembly of the mRNA with specific RNA-binding proteins as a result of splicing. Shortly thereafter, the identification of a multiprotein complex, referred to as the exon-junction complex (EJC) was reported. This complex is deposited in a sequence-independent, but position-specific manner ~20 nt upstream of exon–exon junctions concomitant with, or immediately following, in vitro splicing (Le Hir et al., 2000).

The EJC was suggested to serve as a marker for properly spliced and thus export-competent mRNAs. Consistent with this notion, one of the components was identified as Aly (also known as REF), a small RNA-binding protein implicated in mRNA export in metazoans (Le Hir et al., 2000; Stutz et al., 2000; Zhou et al., 2000). Aly/REF directly interacts with the conserved mRNA export factor TAP (Stutz et al., 2000). TAP in

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turn interacts with components of the nuclear pore complex, the site of transport of macromolecules between the nucleus and the cytoplasm (Kang & Cullen, 1999; Bachi et al., 2000). As expected for a bona fide mRNA export carrier, TAP continuously shuttles between these two compartments (Bear et al., 1999).

Another link between pre-mRNA splicing and export was uncovered when UAP56, a factor originally implicated in spliceosome assembly, was subsequently shown to be also required for efficient mRNA export (Gatfield et al., 2001; Luo et al., 2001). UAP56 is a member of the DEAD-box family of ATPases. These enzymes have been demonstrated to promote changes in RNA–RNA and, more recently, RNA–protein interactions that drive multistep reactions such as translation, pre-mRNA splicing, and ribosomal RNA processing (for review, see Staley & Guthrie, 1998; Linder et al., 2001). Strikingly, in vitro experiments demonstrated that UAP56 can interact directly with Aly, and that association of UAP56 with newly spliced mRNA can be detected prior to Aly in time-course experiments (Luo et al., 2001). In addition, mutant forms of Aly that are unable to interact with UAP56 also fail to associate with mRNA in these experiments (Luo et al., 2001). Taken together, these results suggest a model in which UAP56 recruits Aly (and possibly other components of the EJC) to the mRNA during splicing to provide an adapter for the export carrier TAP.

Like other basic cellular processes, the mechanisms of mRNA splicing and export are remarkably conserved between higher eukaryotes and the budding yeast *Saccharomyces cerevisiae*. Homologs of UAP56, TAP, and Aly/REF are encoded by the essential yeast genes *SUB2*, *MEX67*, and *YRA1*, respectively. Mutations in each of these genes have been shown to adversely affect mRNA export (Segref et al., 1997; Sträßer & Hurt, 2000, 2001; Jensen et al., 2001). In addition, protein–protein interactions observed between the metazoan factors are also largely conserved in yeast. Notably, however, Aly/REF is, at present, the only component of the vertebrate EJC that is clearly conserved in budding yeast.

Interestingly, *YRA1* is among the only ~5% of yeast genes that contains an intron (Portman et al., 1997). Furthermore, the *YRA1* intron and the first exon are unusually large, and the branchpoint sequence differs from the otherwise highly conserved consensus. We find that removal of the intron results in overexpression of the protein, a dominant-negative growth defect and constitutive accumulation of poly(A)$^+$ RNA in the nucleus in agreement with recent findings from other laboratories (Zenklusen et al., 2001; Rodriguez-Navarro et al., 2002). Importantly, here we present several lines of evidence demonstrating that Yra1p expression is negatively autoregulated at the level of splicing of its unusual intron. This finding has important implications for the integration of the splicing and export machineries.

RESULTS

Expression of a cDNA copy of *YRA1* leads to a dosage-dependent, dominant-negative growth defect, elevated protein levels, and nuclear accumulation of poly(A)$^+$ RNA

The *YRA1* gene was previously isolated from a cDNA library in a screen for transcripts that, when overexpressed from an inducible promoter, would arrest yeast cell growth (Espinet et al., 1995). To discover how expression of an intronless copy of *YRA1* under control of its own promoter would affect cell viability, we transformed the wild-type gene and a cDNA copy of *YRA1* (*YRA1-ΔIVS*) on both low- and high-copy-number plasmids into wild-type cells. Extra copies of intron-containing *YRA1* had no or little effect on cell growth, even when expressed from a high-copy-number plasmid (Fig. 1A). In contrast, expression of *YRA1-ΔIVS* from a low-copy-number vector significantly reduced cell growth at all temperatures tested. This effect was exacerbated when the cDNA construct was expressed from a high-copy-number plasmid (Fig. 1A; also see Fig. 6D). Thus, expression of *YRA1* devoid of its intron confers a dosage-dependent, dominant-negative growth defect.

To test whether these growth differences might be due to an effect on the level of protein expressed from the cDNA, we epitope-tagged both intron-containing and intronless Yra1p at their C-termini with two tandem IgG-binding domains (see Materials and Methods). The tags do not interfere with Yra1p function, as the tagged, intron-containing *YRA1* constructs fully complemented the chromosomal *YRA1* knockout, whereas tagged *YRA1-ΔIVS* conferred a slow growth phenotype (data not shown). Western blot analyses of extracts prepared from cells expressing tagged, intron-containing *YRA1* from either a low- or high-copy-number plasmid as the only copy of the gene revealed no detectable difference in the steady-state level of the protein, suggesting that Yra1p levels are subject to regulation (Fig. 1B). In stark contrast, even low-level transcription of the cDNA copy of *YRA1* resulted in several-fold higher expression of protein. Similar results were obtained with polyclonal antibodies against untagged Yra1p (see below; Figs. 5B, 6B; Zenklusen et al., 2001; Rodriguez-Navarro et al., 2002).

Following a shift to the nonpermissive temperature, conditional mutants of *YRA1* cause a rapid accumulation of poly(A)$^+$ RNA inside the nuclei of most cells (Sträßer & Hurt, 2000; Zenklusen et al., 2001). Because depletion of wild-type *YRA1* under the control of a repressible promoter has a similar effect (Zenklusen et al., 2001), we wanted to know whether overexpression of wild-type Yra1p would also inhibit mRNA export. The localization of poly(A)$^+$ RNA was examined using a fluorescently labeled $(dT)_{50}$ probe in cells expressing either intron-containing *YRA1* or a cDNA copy

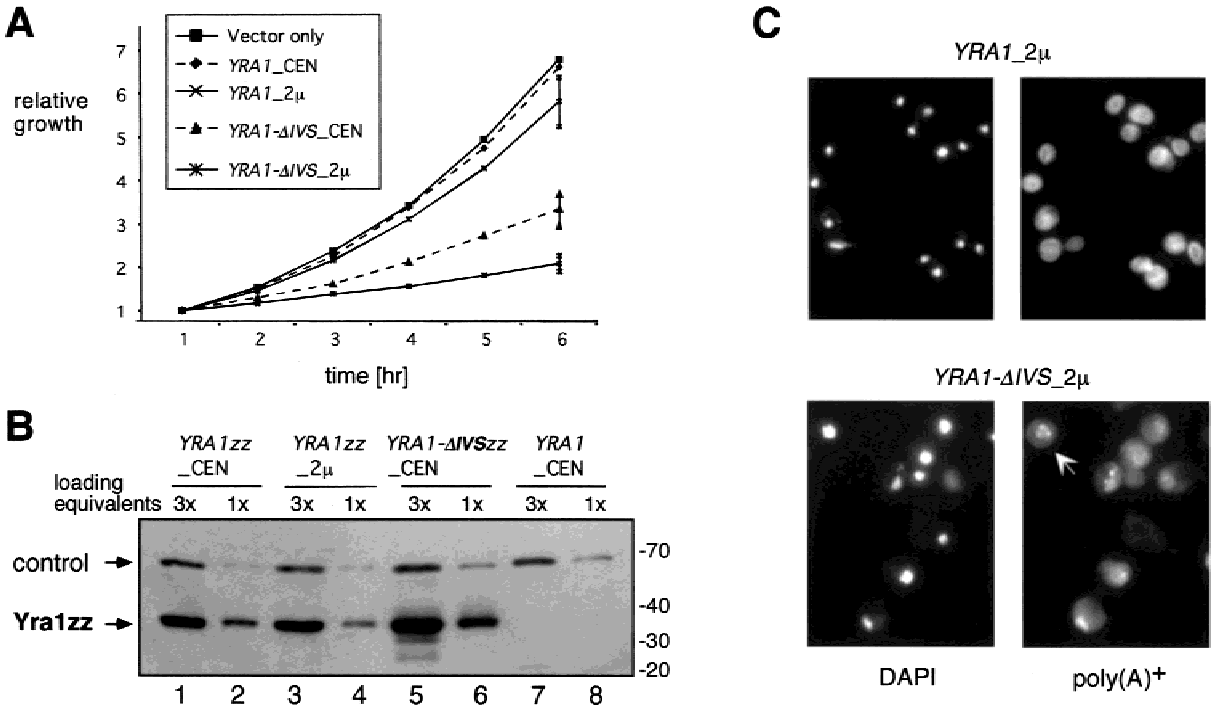

FIGURE 1. Phenotypic consequences of expression of a cDNA copy of *YRA1*. **A:** Dosage-dependent dominant growth inhibition. *YRA1* or an otherwise identical cDNA copy (*YRA1-ΔIVS*) on both low-copy-number (pRS314) and high-copy-number (pRS424) plasmids were transformed into a wild-type strain (PJP168-32C <pRS316-YRA1>) and growth was monitored in liquid minimal medium (SD-Trp-Ura) at 30 °C as increase in OD_{600} over time. Averages of two to three independent cultures are shown and were normalized to an arbitrary value of 1 at $t = 0$ min. Standard deviations >0.05 are depicted as vertical lines for the final time points only. **B:** Overexpression of Yra1p from intronless *YRA1*. Western blot analysis of PJP168-32C strains expressing various zz-tagged forms of the gene, as indicated on top. Two different amounts of extract from each strain were separated by electrophoresis on 10% polyacrylamide/SDS gels and blotted to nitrocellulose. Blots were probed with polyclonal antibodies against Gle1p ("control"), which also recognizes the zz-tag. Lanes 7 and 8 are from a control strain expressing untagged *YRA1* from plasmid pIA271. The position of protein markers (in kilodaltons) is indicated on the right. **C:** Nuclear accumulation of poly(A)$^+$ RNA in cells overexpressing Yra1p. PJP168-32C cells expressing either *YRA1* (pIA300, top panels) or *YRA1-ΔIVS* (pIA304, bottom panels) from a high-copy-number plasmid as the only copy of the gene were grown to mid-log phase, fixed, and hybridized with FITC-labeled $(dT)_{50}$ probe. The same cells were also stained with DAPI to visualize nuclear DNA (left panels).

of the gene on high-copy-number plasmids as the only copy of the gene. In contrast to cells expressing intron-containing *YRA1*, which showed a whole-cell distribution of mRNA, over 50% of cells overexpressing Yra1p from *YRA1-ΔIVS* exhibited a reproducible accumulation of mRNA in the nucleus (Fig. 1C). The mRNA frequently appeared to be concentrated in a few foci at the nuclear periphery (arrow in Fig. 1C). Double-labeling experiments with antibodies against Nsp1p, a component of the nuclear pore complex, confirmed the proximity of these foci to the nuclear envelope, but revealed only a limited degree of overlap (data not shown). The mRNA export defect was less penetrant in cells expressing *YRA1-ΔIVS* from a low-copy-number vector, with only 10–20% of cells showing nuclear accumulation (data not shown). At physiological concentrations, Yra1p localizes exclusively to the nucleus (Portman et al., 1997). Overexpression of Yra1-ΔIVS-GFP did not result in any detectable mislocalization of the fusion protein to the cytoplasm (data not shown). Thus, an excess of nuclear Yra1p is correlated with an mRNA export defect.

Expression of *YRA1* is regulated at the level of splicing

Because Yra1p levels are increased when the gene's intron is deleted, we speculated that *YRA1* expression is regulated at the level of splicing. To test this hypothesis, we isolated total RNA from cells harboring *YRA1* on either a high- or low-copy-number vector. From quantitation of dot-blot hybridization experiments, we estimate the total level of *YRA1* transcripts (mRNA and pre-mRNA) expressed from the high-copy-number vector to be approximately three times that expressed from the low-copy-number vector (data not shown). Following primer-extension reactions with radiolabeled oligonucleotides complementary to either the 3′ exon or the intron of *YRA1*, the reaction products were resolved by gel electrophoresis (Fig. 2). As a specificity control we included a strain expressing *YRA1* harboring a small deletion within the intron (*YRA1-IVSΔ179*) on a high-copy-number plasmid. This deletion removes the region complementary to one of the intron-specific oligonucleotides but does not appear to affect regula-

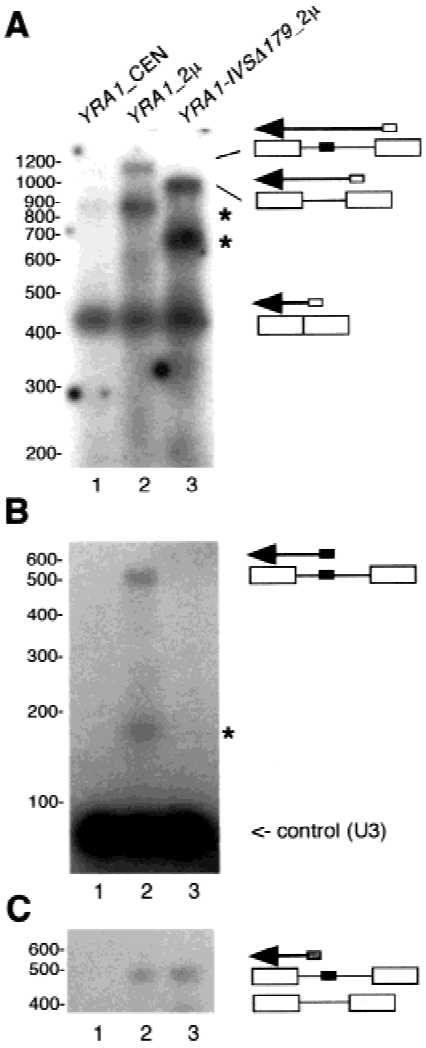

FIGURE 2. *YRA1* expression is regulated at the level of splicing. **A:** Pre-mRNA accumulates in cells transcribing *YRA1* at high levels. Equal amounts of total RNA isolated from PJP168-32C cells transformed with either pIA271 (pRS31-YRA1; lane 1), pIA300 (pRS424-YRA1; lane 2), or pIA310 (pRS424-YRA1-IVSΔ179; lane 3) were subjected to primer extension analyses with an end-labeled oligonucleotide (SFO131, depicted as an open box), complementary to the 3′ exon. Reaction products were resolved on a denaturing 1.5% agarose gel and exposed to a phosphorimager. The positions of the spliced and unspliced RNAs are indicated on the right. The asterisks indicate the position of reaction products derived from a strong reverse transcriptase stop. The migration of marker fragments, in number of nucleotides, is indicated on the left. **B:** The same RNAs as in **A** were in parallel subjected to primer extension analysis with an intron-specific oligonucleotide (SFO160, filled box) to visualize pre-mRNA only and an oligonucleotide specific for U3-mRNA (oAK185). Note that the region complementary to SFO160 has been deleted in *YRA1-IVSΔ179* (lane 3). Reaction products were visualized as in **A**. **C:** Primer-extension analysis with an oligonucleotide (SFO159, grey box) complementary to intronic sequences upstream of the deletion in *YRA1-IVSΔ179* was done as in **A** and **B**.

tion (data not shown). Primer extension with an oligonucleotide complementary to the 3′ exon revealed that all three strains contained similar amounts of spliced mRNA when compared to an internal control (Fig. 2A,B). In contrast, fragments with lower electrophoretic mobility accumulated when either *YRA1* or *YRA1-IVSΔ179* was carried on a high-copy-number plasmid (Fig. 2A; lanes 2, 3). The mobility of the largest fragments was in good agreement with that expected from the unspliced pre-mRNAs. *YRA1* pre-mRNA also accumulated from chromosomally encoded *YRA1* in a *prp28-1* strain, which is impaired in the first step of splicing (data not shown). Additional shorter species (labeled by asterisks in Fig. 2A,B) are likely the result of a strong reverse transcriptase stop site near the end of the first exon. When using the intron-specific oligonucleotides, pre-mRNA accumulation was only detected in cells bearing the high-copy *YRA1* plasmids (Fig. 2B,C; note that the region complementary to one of the two intron-specific oligonucleotides has been deleted in *YRA1-IVSΔ179*). We conclude that increased transcriptional activity results in little or no increase in mature mRNA, but instead in accumulation of unspliced pre-mRNA, indicative of regulated splicing.

Heterologous introns from other *S. cerevisiae* genes cannot rescue the dominant-negative growth defect of *YRA1-ΔIVS*

To test whether regulation specifically requires the *YRA1* intron, we precisely replaced it with introns from five different *S. cerevisiae* genes (*ACT1*, *APS3*, *PHO85*, *SEC14*, and *SFT1*) ranging in size from 77 to 408 nt. To allow for regulation of expression, we placed the hybrid genes as well as wild-type and intronless *YRA1* under the control of the inducible/repressible *GAL1* promoter on plasmids and transformed these constructs into cells harboring intron-containing, wild-type *YRA1*. As expected, when cells were grown on glucose-containing medium, no effect on growth was observed for any of the construct (Fig. 3, left panels). In stark contrast, upon induction of the *GAL1* promoter on medium containing galactose, growth was strongly impaired in cells expressing intronless *YRA1*, as compared to cells expressing the intron-containing form of the gene. This difference was further enhanced at 37 °C (Fig. 3, right panel). None of the five heterologous introns could fully restore wild-type growth; instead their growth phenotypes ranged from being identical to that of cells expressing *YRA1-ΔIVS* (e.g., *SEC14* and *APS3*) to intermediate (*ACT1*). This suggests that *YRA1* regulation does not simply require the process of splicing per se, but rather that the conserved *YRA1* intron plays a more specific role in regulation.

Yra1p expression is negatively autoregulated

The results presented above strongly argue that *YRA1* splicing is regulated by a negative feedback loop. Thus, we predicted that the level of Yra1p expressed from the

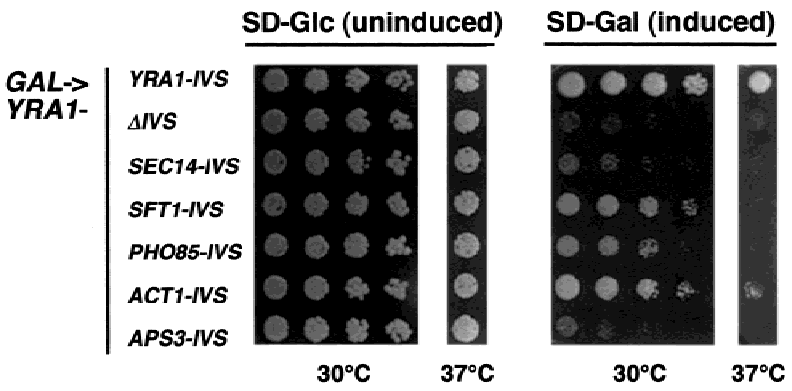

FIGURE 3. Introns from unrelated *S. cerevisiae* genes cannot rescue the dominant-negative growth defect of *YRA1-ΔIVS*. The effects of overexpressing wild-type or intronless *YRA1* (*YRA1-ΔIVS*) were compared to those of overexpressing hybrid genes consisting of the two *YRA1* exons separated by introns from each of five unrelated *S. cerevisiae* genes. All constructs were under the control of the *GAL1* promoter on plasmids and transformed into the *YRA1* shuffle strain PJP168-32C <pRS316-YRA1>. Transformants were grown in glucose-containing medium lacking tryptophane to select for the presence of the plasmids. Serial dilutions of an equal number of cells were applied to plates lacking tryptophane and containing either glucose ("uninduced," left panels) or galactose ("induced," right panels), and the plates were incubated for 3 to 4 days at 30 °C and 37 °C, as indicated below each panel.

chromosome would decrease in cells that also express excess Yra1p from a plasmid. To be able to distinguish between chromosomally and plasmid-encoded protein, we constructed a reporter strain in which the chromosomal copy of *YRA1* was tagged at the C-terminus (Yra1zz; see Materials and Methods). After transformation with either a plasmid bearing intron-containing, untagged *YRA1* or the empty vector, the levels of Yra1zz were analyzed by western blotting. Figure 4 shows that Yra1p expressed from a plasmid reduces Yra1zz levels two- to threefold *in trans* (lanes 3–6). The levels of Yra1zz were further reduced when two different *YRA1* plasmids were simultaneously expressed, indicating that the effect of Yra1p on Yra1zz expression is dosage dependent (Fig. 4, lanes 7, 8). Taken together, these data suggest an intron-dependent negative feedback loop regulating Yra1p expression.

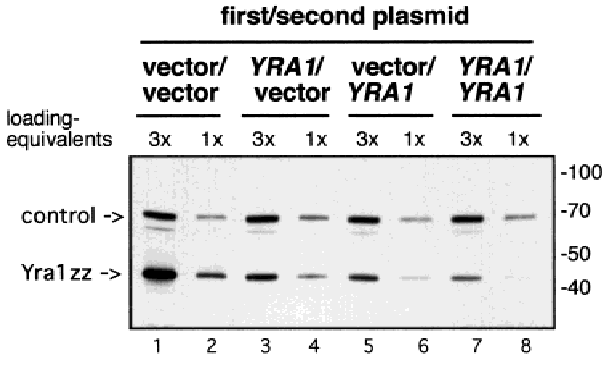

FIGURE 4. Exogenous *YRA1* can suppress expression of endogenous *YRA1*. A strain (PJP174) in which the chromosomal copy of *YRA1* was epitope-tagged was simultaneously transformed with two different plasmids, both either with or without wild-type, intron-containing *YRA1*, as indicated. The amount of tagged, chromosomally encoded Yra1zz was determined by western blotting as in Figure 1B. Lanes 1, 2: pRS315 and pRS314; lanes 3, 4: pIA272 and pRS314; lanes 5, 6: pRS315 and pIA271; lanes 7, 8: pIA272 and pIA271. Gle1p served as a loading control.

Yra1p mutations in a region interacting with the splicing factor Sub2p are deficient for regulation and confer a dominant-negative growth defect

The experiment described above (Fig. 4) provided us with an assay to screen for mutations in *YRA1* that would affect autoregulation. We first transformed the Yra1zz reporter strain with a plasmid containing the temperature-sensitive *yra1-1* allele, which has a strong mRNA export defect at the nonpermissive temperature (Sträßer & Hurt, 2000). Strikingly, unlike wild-type Yra1p, plasmid-encoded yra1-1p failed to suppress expression of the Yra1zz reporter protein (data not shown). The yra1-1p mutant harbors five amino acid substitutions. After separating the individual missense mutations from each other, we were able to show that a single Phe-to-Ser change at position 223 of the 226-amino-acid protein was necessary and sufficient for the temperature-sensitive (ts) phenotype in our strain background (Fig. 6C). Moreover, the *yra1-F223S* allele also failed to down-regulate Yra1zz expression in trans (Fig. 5A; compare lanes 3 and 5, and 4 and 6).

An immediate prediction from this result was that *yra1-F223S* would also fail to regulate its own expression, thus resulting in higher levels of the mutant protein. As shown in Figure 5B, when present as the only copy, *yra1-F223S* is expressed several-fold higher than wild-type *YRA1* and similar to the level of protein expressed from *YRA1-ΔIVS*. During the course of this study, Sträßer & Hurt (2001) reported that the conserved C-terminus of Yra1p, encompassing Phe223, interacts with Sub2p, a protein previously implicated in splicing (see Discussion). In addition, a 17-amino-acid deletion of the C-terminus causes a strong ts phenotype (Zenklusen et al., 2001). We constructed a *YRA1* allele, *yra1-ΔC11*, lacking the C-terminal 11 amino acids of Yra1p. Like *yra1-F223S*, the *yra1-ΔC11* allele is ts for growth (Fig. 6C), fails to down-regulate endogenous Yra1zz (data not shown), and is overexpressed when

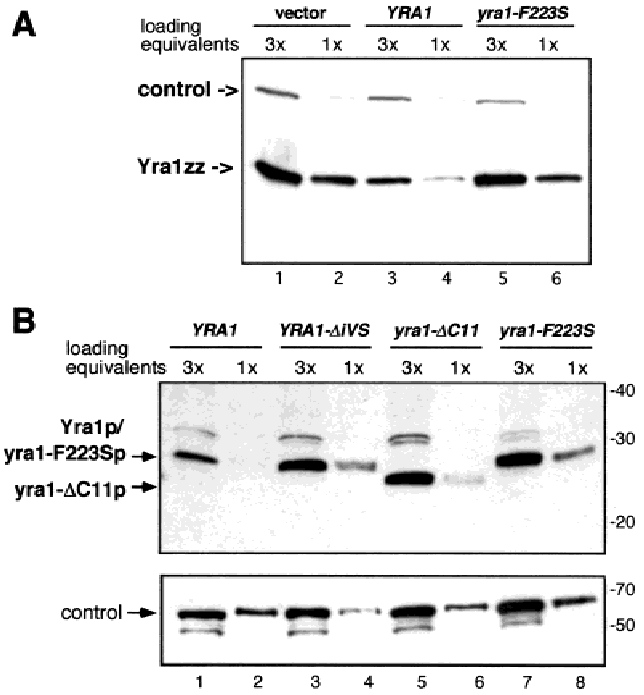

FIGURE 5. *Yra1* mutants are deficient for autoregulation. **A:** *Yra1-F223S* mutants fail to suppress expression of endogenous *YRA1*. Strain PJP174 was transformed with either *YRA1* or *yra1-F223S* on a plasmid or the empty vector, and the amount of chromosomally encoded, tagged Yra1zz was determined by western blotting as in Figures 1B and 4. **B:** *Yra1* mutant protein is overexpressed when present as the only copy in the cell. Plasmid-borne *YRA1*, *YRA1-ΔIVS*, *yra1-ΔC11*, or *yra1-F223S*, as indicated on the top, was introduced into a *YRA1*-shuffle strain (PJP168-32C). After loss of the residual wild-type plasmid, extracts were prepared from cells grown to mid-log phase in liquid medium. Proteins were separated by electrophoresis on 12% polyacrylamide/SDS gels and blotted to nitrocellulose. The blot was probed simultaneously with polyclonal antibodies raised against Yra1p and Gle1p. The top panel shows an exposure of the region of the blot where the various forms of Yra1p are migrating, and the lower panel shows a shorter exposure of the same blot depicting Gle1p ("control").

present as the only copy within the cell (Fig. 5B, lanes 5 and 6). Unlike *yra1-F223S*, however, growth of *yra1-ΔC11* is also affected at low and intermediate temperatures (Fig. 6C).

Because removal of the intron from wild-type *YRA1* or a mutation in the C-terminus of intron-containing *YRA1* both result in overexpression of the protein and mRNA export defects, it seemed possible that the primary defect of the *yra1* mutants was a failure to regulate their own expression. To investigate the relationship between *YRA1-ΔIVS* and *yra1* mutants further, we first asked whether overexpression of mutant *yra1-1* would also cause a dominant-negative growth phenotype. Because chromosomally encoded wild-type Yra1p is likely able to down-regulate expression of intron-containing mutant yra1p, we further boosted expression by putting the mutant under control of the strong, inducible *GAL1* promoter on a plasmid. For comparison, we also inserted the *GAL1* promoter in front of wild-type *YRA1* and *YRA1-ΔIVS*. Under noninducing conditions, Yra1zz strains transformed with each of the three constructs grew equally well on solid growth medium. When transcription was induced by inclusion of galactose, however, strains expressing either *YRA1-ΔIVS* or *yra1-1* grew more slowly (Fig. 6A). As expected, this effect was more pronounced for the *GAL1-YRA1-ΔIVS* construct (also see Fig. 3). To verify whether the growth defects correlated with expression levels from the various plasmids, western analysis was performed on all three strains grown in liquid media before and after induction. Expression from *GAL1*-driven *YRA1*, *yra1-1*, and *YRA1-ΔIVS* constructs resulted in relatively low, intermediate, and high levels of protein expression, respectively (Fig. 6B). Over the course of the experiment (4 h), no change in growth rate or in the level of endogenous Yra1zz was detected in any of the three strains. (Note that the presence of two IgG-binding domains on chromosomally encoded Yra1zz prohibits any direct comparison between the levels of tagged and untagged protein.) These results demonstrate that overexpression of mutant yra1p causes a dominant-negative growth defect. In addition, we found the growth defect of *YRA1-ΔIVS* cells to be exacerbated at higher temperatures, both as the only copy in the cell and in the presence of wild-type *YRA1* (Fig. 6C,D).

Taken together, the striking phenotypic similarities between *YRA1-ΔIVS* and *yra1* mutant cells support the hypothesis that the failure of *yra1* mutants to regulate their own expression contributes to their growth and mRNA export defects. To investigate this possibility further, we constructed intronless versions of the *yra1-F223S* and *yra1-ΔC11* mutants on centromeric (low-copy-number) plasmids and tested their growth phenotype both in the presence and absence of intron-containing wild-type *YRA1*. The expectation was that, if the primary defect of the conditional *yra1* mutants is a failure to regulate themselves, removal of the intron should have no additional effect. In that case, the intronless mutant would be predicted to grow as well as cells containing either the wild-type cDNA or the intron-containing mutants. Figure 6C shows that in the presence of a wild-type copy of *YRA1*, the *yra1-F223S-ΔIVS* and *yra1-ΔC11-ΔIVS* confer a dominant negative growth defect very similar to that observed in *YRA1-ΔIVS* cells (right panel). However, in the absence of wild-type *YRA1*, *yra1-F223S-ΔIVS* failed to support cell growth at any temperature, whereas *yra1-ΔC11-ΔIVS* cells grew significantly worse than intron-containing *yra1-ΔC11* cells at 30 °C. Curiously, *yra1-ΔC11* cells are also cold sensitive. This phenotype, however, is not exacerbated by removal of the intron (Fig. 6C, left panel).

Finally, neither intron-containing *yra1-F223S* nor *yra1-ΔC11* mutants were able to support growth when expressed from a high-copy-number vector as the only

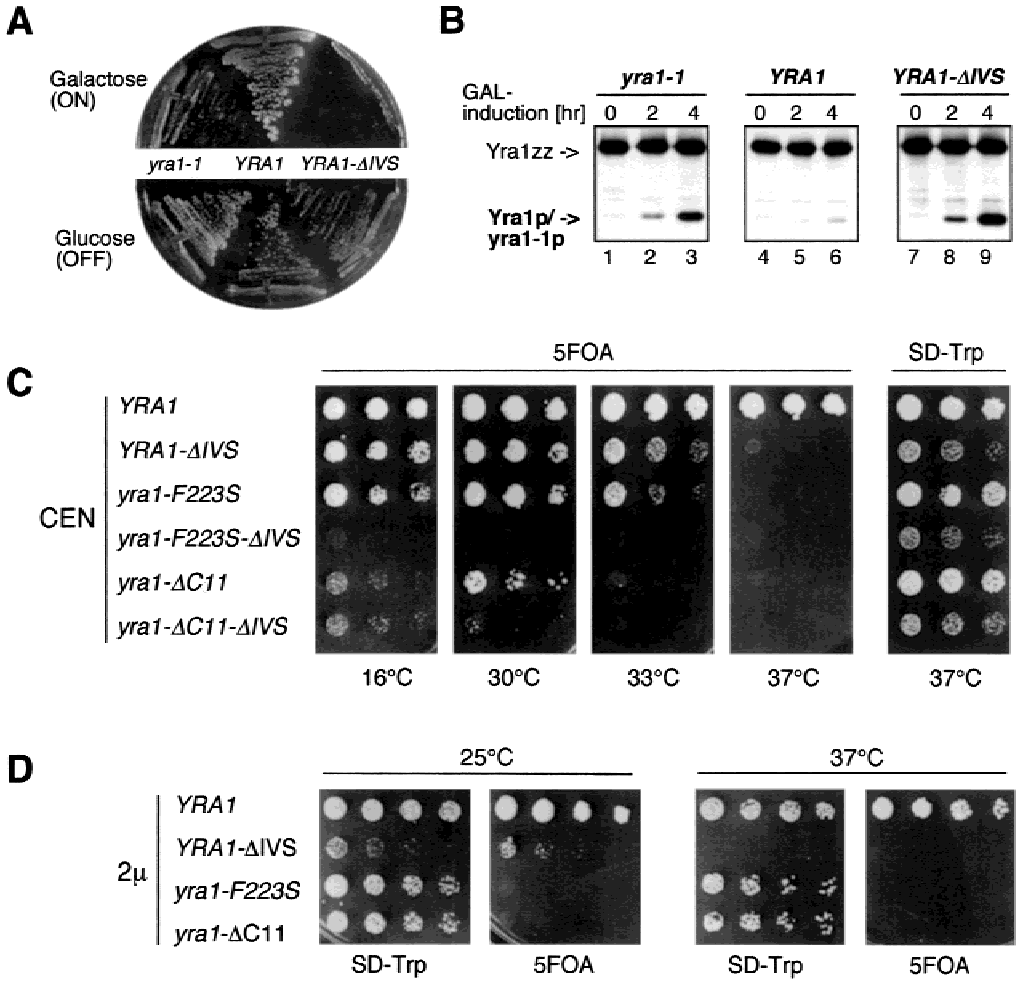

FIGURE 6. Growth phenotypes of cells expressing intron-containing mutant *yra1* or intronless mutant and wild-type *YRA1*. **A:** High-level transcription of mutant *yra1* causes a dominant negative growth defect. YRA1zz (PJP174) strains were transformed with plasmids harboring wild-type, *yra1-1*, or *YRA1-ΔIVS* under control of the inducible *GAL1* promoter and streaked out on synthetic medium lacking tryptophane and supplemented with either 2% glucose or 2% galactose, as indicated. Plates were incubated at 30 °C for 3 days. **B:** The same strains as in **A** were grown at 30 °C in synthetic liquid medium containing 1% raffinose and 1% sucrose and lacking tryptophane to select for the presence of the plasmid. At time 0, transcription from the *GAL1* promoter was induced by addition of galactose to a final concentration of 3%, and an aliquot was removed immediately (lanes 1, 4, 7) or following growth for another 2 and 4 h (lanes 2, 5, 8 and 3, 6, 9, respectively). The amount of untagged Yra1p or yra1-1p was determined by western blotting with polyclonal antibodies against recombinant Yra1p. Note that the amount of tagged, endogenous Yra1p remains unchanged over the course of the experiment. **C:** Intronless, mutant *yra1* does not support growth under conditions where both the intronless wild-type gene or the intron-containing *yra1* mutant do. The shuffle strain PJP168-32C, containing wild-type *YRA1* on an URA3-marked plasmid (pRS316-YRA1) was transformed with the constructs indicated to the left on a low-copy-number plasmid (pRS314). Serial dilutions of cells grown in selective medium were applied to either synthetic medium lacking tryptophane or onto the same medium supplied with 5-FOA, to select for the absence of the *URA3*-marked wild-type plasmid (right panel) and incubated at the temperatures indicated below each panel. **D:** The same strain as in **C** was transformed with wild-type, intronless, or mutant *YRA1* on a high-copy-number plasmid (pRS424), and growth was monitored in the presence or absence of pRS316-YRA1 at 25 °C and 37 °C as described above.

copy in the cell (Fig. 6D). In summary, both removal of the intron from *yra1* mutants or high-level transcription of the mutant genes results in nonviable cells. This suggests that conditional *yra1* mutants are impaired in at least one other function in addition to the failure to regulate their own splicing and/or that the mutant cells retain some capability of regulating their own expression in an intron-dependent way.

DISCUSSION

YRA1, under its alias *SHE11*, was originally isolated from a cDNA library in a screen for transcripts that would arrest yeast cell growth when overexpressed from a strong promoter (Espinet et al., 1995). More recently, Zenklusen et al. (2001) and Rodriguez-Navarro et al. (2002) reported that Yra1p is present at several-fold

higher levels in cells expressing a cDNA copy of the gene as compared to cells expressing intron-containing *YRA1*. We have now shown that *YRA1* expression is subjected to autoregulation and that regulation occurs, at least in part, at the level of splicing of its unusual intron.

Expression of Yra1p is regulated on the level of splicing

The *YRA1* intron is unusual in size, location within the coding region, and in the sequence of one of the consensus splicing signals (Portman et al., 1997). Consistent with a biological relevance of these features, the intron and its main characteristics are conserved in all known budding yeast homologs of *YRA1* (P.J. Preker & C. Guthrie, unpubl. results). In further support of a specific requirement for the *YRA1* intron, its function in autoregulation cannot be substituted for by the introns of at least five unrelated *S. cerevisiae* genes (Fig. 3). These findings are in apparent conflict with results reported while this article was in preparation (Rodriguez-Navarro et al., 2002). In a set of very similar experiments, these authors showed that the introns of the yeast *UBC8* and *RPL25* genes can functionally substitute for the *YRA1* intron under most conditions tested. The only exception noted by Rodriguez-Navarro et al. (2002) was that cells expressing *YRA1* containing the *RPL25* intron from the *YRA1* promoter had a slight dominant-negative growth defect at 37 °C. Intriguingly, some of the introns we have tested, notably that of *ACT1*, could partially complement the growth defect of *YRA1*-Δ*IVS*. It thus seems that the *YRA1* intron shares some characteristics that are relevant for autoregulation with a subset of introns from unrelated yeast genes. These observations will likely facilitate the identification of *cis*-acting regulatory elements in the *YRA1* transcript. For example, like the *YRA1* intron, the *RPL25* and *ACT1* introns are unusually large (414 and 408 nt, respectively), whereas both the *UBC8* and *YRA1* introns have branchpoint sequences that differ from the normally highly conserved consensus at the first position (CAC UAAC and GACUAAC, respectively). In addition, the discrepancies between our findings and those of Rodriguez-Navarro et al. (2002) might, at least in part, also reflect subtle differences in the experimental details involved, such as different strain backgrounds or plasmid-copy numbers.

Even though we do not know at this point which of the peculiarities of the *YRA1* intron mentioned above are involved in autoregulation, each of these characteristics has been shown to be important in the few reports of regulated splicing in yeast. One prominent example is the meiosis-specific splicing of *MER2* pre-mRNA. During mitotic growth, basal splicing efficiency is lowered by a noncanonical 5′ splice site and an unusually large 5′ exon (Engebrecht et al., 1991; Nandabalan & Roeder, 1995). Activation of splicing during meiosis requires a splicing enhancer located downstream of the 5′ splice site (Spingola & Ares, 2000) and the product of the *MER1* gene that is only expressed during meiosis and specifically binds to the *MER2* splicing enhancer (Nandabalan & Roeder, 1995; Spingola & Ares, 2000). Another well-studied example is the ribosomal protein L30, which regulates splicing of the transcript of its own gene, *RPL30*. Binding of L30 to an RNA structure formed by nucleotides surrounding the noncanonical 5′ splice site of *RPL30* inhibits splicing prior to the first step (Eng & Warner, 1991; Vilardell & Warner, 1994). Finally, autoregulation of *DBP2,* a member of the DEAD-box family of putative RNA helicases, depends on the presence of a 1,002-nt intron, the largest in *S. cerevisiae* (Barta & Iggo, 1995).

To our knowledge, *YRA1* is the first example of a yeast gene that causes a dramatic growth defect when its intron is removed. No effect on growth has been observed as a result of *DBP2* overexpression from a cDNA copy (Barta & Iggo, 1995). Moreover, a cocultivation assay capable of revealing subtle differences in biological fitness was required to show that mutations in *RPL30* that abolish autoregulation lead to detectably slower growth only over the course of many generations (Li et al., 1996).

Mechanism of *YRA1* autoregulation

How might Yra1p inhibit splicing of its own pre-mRNA? Like L30 and possibly Dbp2p, Yra1p might have adapted its RNA-binding capabilities to recognize specific sequences within its own transcript. Intriguingly, in the case of L30 autoregulation, the structure required for regulation mimics the binding site of L30 in the 60S ribosomal subunit (Vilardell et al., 2000). In support of this notion, we find that Yra1p preferentially interacts with its own mRNA and, more importantly, pre-mRNA in vivo (K.S. Kim, P.J. Preker, & C. Guthrie, in prep.). We do not yet know whether this interaction is direct or mediated by other factors.

Yra1p has been identified as the major yeast RNA-annealing activity in vitro (Portman et al., 1997). Strikingly, the substrate used in that study was derived from the region surrounding the 3′ splice site of the adenovirus-2 first leader intron. Even though it was later shown that nonintronic sequences can also serve as substrate for Yra1p annealing activity (Sträßer & Hurt, 2000), it is tempting to speculate that this activity might be relevant for autoregulation. For example, Yra1p might act to promote a secondary structure in its pre-mRNA that interferes with efficient splicing, conceivably by promoting base pairing of the 3′ splice site and/or the branchpoint region to an as yet unidentified complementary region of the transcript.

In a different but not mutually exclusive scenario, autoregulation might involve inhibition of the activity of

splicing factors by Yra1p. Notably, mutations in a region of Yra1p that has previously been shown to mediate a direct interaction with Sub2p (Sträßer & Hurt, 2001) largely abolish the ability of the protein to regulate its own expression (Fig. 5). Sub2p has been shown to promote spliceosome formation (Kistler & Guthrie, 2001; Libri et al., 2001; Zhang & Green, 2001) and bears the hallmarks of ATP-dependent "resolvases" (Staley & Guthrie, 1998). Thus Sub2p might possess an unwinding activity that could be used to resolve an RNA structure unfavorable for *YRA1* splicing. Alternatively, Sub2p might promote splicing by displacing an inhibitory factor from the *YRA1* pre-mRNA. In the light of recent data from our laboratory, we consider the latter possibility more likely (Kistler & Guthrie, 2001). In that study, it was shown that a deletion of Mud2p, the yeast homolog of vertebrate U2AF65, bypasses the requirement for Sub2p. Because Mud2p interacts with the branchpoint region at an early stage of pre-mRNA recognition, these data can be best explained by a requirement of Sub2p to remove Mud2p from the pre-mRNA and to allow spliceosome formation to proceed. In our working model (Fig. 7), *YRA1* autoregulation is achieved in part by direct binding of Yra1p to Sub2p, thereby affecting Sub2p's ability to remove an inhibitory factor from the pre-mRNA. Further experiments are required to elucidate the *cis*- and *trans*-acting elements involved in Yra1p regulation. For example, it will be interesting to test whether mutations in Sub2p, Mud2p, or other factors involved in recognition of the branchpoint and/or 3′ splice site affect *YRA1* regulation.

Our observation that intronless *yra1-F223S* cannot support growth suggests that there may be additional levels of intron-dependent regulation (Fig. 6C). This is reminiscent of L30 expression, which is regulated by feedback inhibition of splicing as well as of translation of its own mRNA (Dabeva & Warner, 1993). Interestingly, both forms of L30 autoregulation depend on very similar *cis*-acting sequences in the RNA. Because overexpression of Yra1p causes nuclear accumulation of poly(A)$^+$ mRNA, it seems possible that, at physiological concentrations, Yra1p modulates the nuclear export of specific mRNAs through nuclear retention. Such an activity could conceivably serve as an additional mechanism for Yra1p autoregulation by inhibiting the export of *YRA1* mRNA at elevated Yra1p levels (Fig. 7). Alternatively, Yra1p might also affect the transcription of its own gene. Circumstantial evidence in support of this hypothesis comes from the fact that its mammalian homolog has previously been identified as a transcriptional coactivator (Bruhn et al., 1997; Virbasius et al., 1999). More recently, Yra1p was found to be associated with a protein complex involved in transcription elongation in yeast (Sträßer et al., 2002). Finally, in light of accumulating evidence that the processes of transcription, pre-mRNA splicing, and mRNA export are coordinated (Bentley, 2002), it seems possible that the expression of Yra1p is achieved by a combination of mechanisms that act at each of these steps of gene expression (see below).

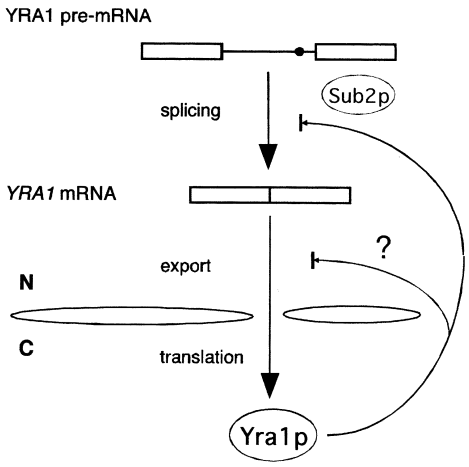

FIGURE 7. Model for *YRA1* autoregulation. *YRA1* splicing is regulated though negative feedback inhibition. The involvement of Sub2p in regulation is largely speculative. Possible additional levels of *YRA1* regulation, conceivably during export of *YRA1* mRNA, are also alluded to. See Discussion for more details.

The tight control of Yra1p levels is required for efficient mRNA export and cell viability

We have shown here that expression of Yra1p is autoregulated through negative feedback inhibition and requires the presence of the *YRA1* intron as well as functional Yra1p. A failure to autoregulate results in increased Yra1p levels, a dominant-negative growth defect and the inhibition of mRNA export (Fig. 1; also see Rodriguez-Navarro et al., 2002). Accumulation of poly(A)$^+$ RNA inside the nucleus is also observed in cells that have either been genetically depleted of Yra1p or that carry conditional *yra1* alleles (Sträßer & Hurt, 2000; Zenklusen et al., 2001). Thus the level of functional Yra1p is critical for efficient mRNA export as well as cell growth. In the light of accumulating evidence linking Yra1p/Aly to the mRNA export machinery (see Introduction), we favor the hypothesis that the effect of Yra1p overexpression reflect a direct role for this protein in mRNA export rather than an indirect consequence of perturbations in some other cellular process.

We suggest that overexpression inhibits mRNA export through the titration of essential export factor(s). In view of a reported direct interaction between Yra1p and Sub2p (Sträßer & Hurt, 2001), it might be significant that a sizeable fraction of cells overexpressing Yra1p accumulate mRNA in a granular pattern within the nucleus (Fig. 1C), similar to the pattern previously observed in a variety of *sub2* mutants (Jensen et al.,

2001; J. Pan & C. Guthrie, unpubl. data). It thus seems possible that overexpression of Yra1p and mutations in *sub2* affect mRNA export by a similar mechanism. Intriguingly, Sub2p overexpression is also inhibitory to both cell growth and mRNA export (Espinet et al., 1995; Sträßer & Hurt, 2001). Moreover, injection of excess UAP56, the vertebrate ortholog of Yra1p, into oocyte nuclei specifically blocks mRNA export, and this block can be relieved by coinjection of excess Aly (Zhuo et al., 2000). Nonetheless, we have been unable to restore wild-type growth by simultaneous overexpression of Sub2p and Yra1p (data not shown). This negative result might be reconciled if cells are extremely sensitive to the relative levels of these two proteins or if overexpression of either of the proteins affects additional processes. Another mRNA export factor that is engaged in a direct and evolutionarily conserved interaction with Yra1p is Mex67p. However, as with Sub2p, high-copy expression of Mex67p did not relieve the growth defect of *YRA1*-Δ*IVS* cells (data not shown).

It thus remains to be seen how excess Yra1p leads to an mRNA export block and whether this is in fact the primary cause for the observed growth defect. Because Aly has also been identified as a transcriptional coactivator (see above), it seems likely that Yra1p overexpression also affects additional aspects of gene expression. Intriguingly, very recent results tie Yra1p (as well as Sub2p) to the transcription elongation machinery in yeast, raising the possibility that Yra1p might participate in coupling transcription and mRNA export (Sträßer et al., 2002). Using genome-wide microarray analysis, we have found that Yra1p interacts in vivo with a large number of gene transcripts (K.S. Kim, P.J. Preker, & C. Guthrie, in prep.). Contrary to expectations raised from the initial studies of Aly/REF in mammals (Le Hir et al., 2000; Zhou et al., 2000), these include intronless as well as intron-containing RNAs.

Future experiments will determine to what extent the regulation of Yra1p expression and its function in gene expression overlap. For now, it seems likely that these biochemically separable aspects of mRNA metabolism are but different faces of a single biological process. In this light, it is interesting to speculate that Yra1p levels might also serve to couple the relative rates of transcription and pre-mRNA splicing to mRNA export. For example, under conditions where transcription or splicing is generally limiting, less Yra1p would be available, resulting in a slowed rate of mRNA export.

MATERIALS AND METHODS

Strains and plasmids constructions

Yeast media and manipulations were done following standard protocols (Guthrie & Fink, 1991). G418, and 5-fluoroorotic acid (5-FOA) were included in solid growth media at 0.2 and 1 mg/mL, respectively, where indicated.

A strain (PJP174) in which the chromosomal copy of *YRA1* is tagged with two tandem IgG-binding (zz) domains was created by the method of Longtine et al. (1998). Strain BMA64-1A, a derivative of W303 (*MATa, ade2, his3, leu2, trp1, ura3*; Baudin-Baillieu et al., 1997) was transformed with a PCR fragment encoding two IgG-binding domains followed by the kanamycin resistance gene as a selectable marker. The PCR fragment was generated using pFa6-TEVzz-kanMX6 (P.J. Preker & C. Guthrie, unpubl. data) as a template primers SFO119 (oligonucleotide sequences not provided in this article are available on request) and oKD254. The *YRA1* shuffle strain (*MATa, ade2, his3, leu2, trp1, ura3, yra1::HIS3* <pRS316-YRA1>), a gift from K. Sträßer and E. Hurt (Biochemie-Zentrum Heidelberg, Germany), was back-crossed several times to generate strain PJP168-32C.

DNA recombinant work was done according to standard protocols (Sambrook et al., 1989). Enzymes were purchased from New England Biolabs (Beverly, Massachusetts) and Roche (Indianapolis, Indiana). The pRS series of shuttle vectors was used for expression of *YRA1* in yeast (Christianson et al., 1992). pRS314, pRS315, and pRS316 are centromeric (low-copy-number) vectors and are marked by the *TRP1*, *LEU2*, and *URA3* genes, respectively. pRS424 is a 2μ-based (high-copy-number) vector marked by *TRP1*. Plasmids pRS316-YRA1 and pRS314-yra1-1 have been published previously (Sträßer & Hurt, 2000).

The *YRA1* gene, including ~300 bp of both the 5′ and 3′ untranslated regions, was amplified by PCR from genomic DNA with primers SFO099 and SFO100 and inserted into the pCR2.1 cloning vector (Invitrogen, Carlsbad, California). A *Bam*HI/*Xho*I fragment carrying the gene was excised from this construct and introduced into the same sites of pRS314 and pRS315 to create pIA271 and pIA272, respectively. To generate intronless *YRA1*-Δ*IVS*, the cDNA was amplified from a library (Liu et al., 1992) with primers SFO107 and SFO108. Next, a *Sty*I/*Bgl*II fragment of pIA271 containing the intron and flanking exonic sequences was replaced with the corresponding fragment of the *YRA1* cDNA to generate pIA286 (pRS314-YRA1-ΔIVS). DNA sequencing confirmed that the *YRA1* coding sequence of pIA286 remained unchanged relative to that of pIA271, but also revealed a variation from the published sequence leading to a Leu to Pro change at position 48 within the variable region of the protein. Because this variation is upstream of the *Sty*I site, it is present on all of our *YRA1* constructs and appears to be phenotypically neutral.

The *yra1-F223S* alleles were generated by replacing *Bgl*II/*Sac*I fragments from pIA271 and pIA286 with the corresponding fragment from pRS314-yra1-1, yielding pIA297 (pRS314-yra1-F223S) and pIA296 (pRS314-yra1-F223S-ΔIVS), respectively. Subcloning of *Xho*I/*Sac*I fragments from pIA271, pIA286, and pIA297 into the same sites of pRS424 generated pIA300, pIA304, and pIA309, respectively. The *yra1-*Δ*C11* alleles were created by ligation of two copies of an oligonucleotide (GATCTG**TAG**AATTCTACA) containing an in-frame stop codon (bold) into the *Bgl*II sites of pIA271, pIA286, and pIA300. Deletion of a *Bcl*I/*Hpa*I fragment from the *YRA1* intron on pIA300 yielded pIA310 (pRS424-*YRA1-IVS*Δ*179*).

C-terminally tagged versions of *YRA1* were generated by homologous recombination between plasmids linearized near the termination codon with *Bgl*II and the same PCR fragment used to create PJP174 (see above). Transformants that contained the recombinant plasmid were selected on medium

lacking tryptophane and containing kanamycin. Plasmids were recovered from yeast, verified by restriction-enzyme mapping and retransformed into PJP168-32C.

The *GAL1* promoter was introduced in front of *YRA1*, *YRA1-ΔIVS*, and *yra1-1* by homologous in vivo recombination of pIA271, pIA286, and pRS314-yra1-1, respectively, that had been linearized with *Xho*I and *Kpn*I in the polylinker sequence upstream of the *YRA1* gene and a PCR fragment containing the *GAL1* promoter preceded by the kanamycin resistance gene. The PCR fragment was generated using pFa6-kanMX6-PGAL1 (Longtine et al., 1998) as a template and oligonucleotides SFO152 and SFO132. Recombinants were identified as above, recovered into *Escherichia coli* and retransformed into PJP174.

The *YRA1* intron was precisely replaced with introns from unrelated *S. cerevisiae* genes by homologous recombination in yeast. For that PJP168-32C <pRS316-YRA1> cells were cotransformed with pGAL-271 (see above) that had been linearized within the intron with *Hpa*I and PCR fragments containing any of five heterologous introns. The PCR fragments were generated using genomic DNA as a template and oligonucleotides introducing 30 to 40 base pairs of homology to the flanking exon sequences of *YRA1*. The following oligonucleotide combinations were used: SFO172/173 (*ACT1*), SFO197/198 (*APS3*), SFO199/200 (*PHO85*), SFO196/197 (*SEC14*), and SFO193/194 (*SFT1*). Recombinants were selected for growth on synthetic growth medium lacking tryptophane and confirmed by whole-cell PCR.

Antibodies and western blot analysis

For the production of polyclonal antiserum against Gle1p, an N-terminal ~30-kDa fragment of Gle1p was expressed in *E. coli*, purified, and injected into a rabbit (P.J. Preker & C. Guthrie, unpubl. data). Affinity purified polyclonal antibodies against Yra1p were a kind gift from A. Kashyap and D. Kellogg (University of California, Santa Cruz).

To prepare protein extracts, pellets from logarithmically growing cells were resuspended in buffer (20 mM Tris/HCl, pH 7.4, 50 mM ammonium acetate, 2 mM EDTA). After addition of glass beads and trichloroacetic acid to a final concentration of 10% (v/v), cells were lyzed by vortexing for ~5 min in the cold. Proteins were precipitated by centrifugation, washed with 80% acetone, and resuspended in SDS-PAGE loading buffer. Equivalent amounts of extract (normalized to OD_{600} of the starting cultures) were separated by electrophoresis on SDS-polyacrylamide gels, blotted to nitrocellulose membrane, and probed with antibodies against Gle1p and Yra1p at dilutions of 1:25,000 and 1:4,000, respectively. Secondary antibodies (goat anti-rabbit; Biorad, Hercules, California) conjugated to horseradish peroxidase were used at a dilution of 1:2,000 and detected by enhanced chemiluminescence (Amersham, Piscataway, New Jersey) and exposure to film.

FISH and immunofluorescence

Localization of poly(A)$^+$ RNA was done by FISH essentially as described (Duncan et al., 2000). In brief, exponentially growing cells were fixed by adding formaldehyde to the growth medium to a final concentration of 5% (v/v) and spheroplasted. mRNA was detected by in situ hybridization with a dioxigenin-tailed $(dT)_{50}$ probe followed by staining with FITC-coupled goat anti-dioxigenin Fab fragments (1:100; Roche). Cells were mounted in glycerol/gelatin containing DAPI (0.5 μg/mL). Images were collected with an Olympus BX-60 microscope outfitted with a Sensys CCD camera (Photometrics) and processed using IP Lab imaging software.

Primer extension analysis

Primer extension analysis was performed essentially as described (Kistler & Guthrie, 2001). In brief, 200 pmol of 5′-labeled oligonucleotides SFO131 (CGGTACCAGTAGATT GGCCCCTTTC), SFO160 (TTCTTCATCTCTAAAAAGAG AATT), SFO159 (CCTTACAAAGAATATTTCTCGTATCC), and/or oAK185 (CCAAGTTGGATTCAGTGGCTC; see legend to Fig. 2) were annealed to 9 μg of total RNA prepared from logarithmically growing cells by hot phenol extraction. cDNA was synthesized with AMV reverse transcriptase (Roche) at 42 °C for 30 min and separated by electrophoresis on a 1.5% agarose gel in denaturing buffer (0.05 N NaOH, 1 mM EDTA). The gel was dried under vacuum and subjected to analysis on a phosphorimager.

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