An intron in the *YRA1* gene is required to control Yra1 protein expression and mRNA export in yeast

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Yra1p is an essential and conserved mRNA export factor in yeast. Strikingly, removal of the intron from *YRA1* causes a dominant-negative growth phenotype and a concomitant inhibition of mRNA export. However, both defects are neutralized by replacement of the intron of *YRA1* by a different intron. Significantly, Yra1p is overproduced in yeast when expressed from its intronless gene, but Yra1p levels are the same as the wild type when expressed from an intron-containing *YRA1* gene. Thus, an intron in *YRA1* controls Yra1p expression and mRNA export.

INTRODUCTION

In eukaryotes, the presence of the nuclear envelope necessitates transport between the nucleus and the cytoplasm. Specifically, mRNAs synthesized in the nucleus need to be exported to the cytoplasm before being translated. This mRNA export process is mediated by an evolutionarily conserved mRNA export machinery. The mRNA exporter Mex67p/Mtr2p in yeast and its metazoan homologue, TAP/p15, interact directly with their transport cargo mRNA and with FG repeat nucleoporins (Segref *et al.*, 1997; Bear *et al.*, 1999; Braun *et al.*, 1999; Kang and Cullen, 1999; Kang *et al.*, 1999; Katahira *et al.*, 1999; Bachi *et al.*, 2000; Sträßer *et al.*, 2000), which line the nuclear pore channel. Mex67p/Mtr2p also binds directly to Yra1p, an RNA-binding protein that is mainly located inside the nucleus (Sträßer and Hurt, 2000; Stutz *et al.*, 2000; Zenklusen *et al.*, 2001). Thus, it is thought that Yra1p serves as a coupling protein that mediates recruitment of Mex67p/Mtr2p to the mRNP. Interestingly, the lethality caused by deletion of *YRA1* in yeast can be rescued by heterologous expression of its murine homologue, Aly (Sträßer and Hurt, 2000). Aly was first identified to be a transcriptional coactivator (Bruhn *et al.*, 1997) and a protein chaperone (Virbasius *et al.*, 1999). More recently, Aly has been shown to be

involved in splicing-coupled mRNA export in metazoans (Le Hir *et al.*, 2000, 2001; Zhou *et al.*, 2000). Furthermore, Yra1p and Aly belong to a conserved protein family called RNA export factor binding (REF) proteins (Sträßer and Hurt, 2000; Stutz *et al.*, 2000). All REF proteins share a common domain organization. They possess a central RNA recognition motif (RRM), which is linked to a highly conserved N-terminal domain and a highly conserved C-terminal domain by variable linker regions. Recently, it has been shown that the recruitment of Yra1p/Aly to the mRNA is mediated by its direct interaction with Sub2p/UAP56 (Luo *et al.*, 2001; Sträßer and Hurt, 2001), which was first identified as a splicing factor (Fleckner *et al.*, 1997; Kistler and Guthrie, 2001; Libri *et al.*, 2001; Zhang and Green, 2001). Surprisingly, this conserved splicing factor Sub2p/UAP56 is needed for export of intron-containing, as well as intronless, transcripts (Gatfield *et al.*, 2001; Jensen *et al.*, 2001; Sträßer and Hurt, 2001). How Sub2p is recruited to mRNAs lacking introns is not yet known. Interestingly, Sub2p and Mex67p/Mtr2p compete for binding to Yra1p (Sträßer and Hurt, 2001). Thus, Sub2p might first recruit Yra1p to the mRNP and could then be displaced from the mRNA, when Mex67p/Mtr2p binds to Yra1p. All of this suggests that Yra1p is a key player in mRNA export.

Here, we show that deletion of the intron from *YRA1* causes a dominant-negative phenotype and an mRNA export defect. Both defects can be rescued by insertion of an heterologous intron in place of the *YRA1* intron. Yra1p is expressed at wild-type levels from *YRA1* genes containing the *RPL25* or *UBC8* intron, whereas Yra1p is overexpressed from the intronless *YRA1* gene. Thus, the 'toxicity' of intron removal is most likely due to the resulting overexpression of Yra1p. Taken together, an intron in *YRA1* is crucial for Yra1p expression, which in turn controls mRNA export.

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RESULTS AND DISCUSSION

YRA1 belongs to the small group of yeast genes that contain an intron. The intron of *YRA1* is unusual, as it is the second largest intron of *Saccharomyces cerevisiae* (766 nucleotides). In addition, it is located far downstream of the start codon (in the sequence coding for the RRM domain; for domain organization of Yra1, see Sträßer and Hurt, 2000), and its branchpoint sequence does not correspond to the consensus motif (Figure 1A). In the course of studying the function of Yra1p, we noticed that the intron in *YRA1* is important for yeast cell growth (Figure 1B). Specifically, an intronless version of *YRA1* (*yra1-Δi*; Figure 1A) complements the lethal *yra1* null mutant, but cells grow significantly slower than cells expressing the corresponding intron-containing version of *YRA1* (Figure 1B). To test whether *yra1-Δi* causes a dominant-negative phenotype, the *YRA1* shuffle strain was transformed with a plasmid-borne copy of either *yra1-Δi* or *YRA1*. This revealed that an extra copy of *yra1-Δi*, but not of *YRA1*, inhibits cell growth in yeast (Figure 1C). Thus, removal of the intron from *YRA1* causes a dominant-negative growth defect.

Since Yra1p is essential for mRNA export, we tested whether removal of the *YRA1* intron, which impairs cell growth, also causes an mRNA export defect. Hence, localization of poly(A)⁺ RNA was detected in the *YRA1* and *yra1-Δi* strains by *in situ* hybridization. As shown in Figure 2, poly(A)⁺ RNA accumulates in the nucleus of cells expressing *YRA1* from the intronless gene but not in the nucleus of cells expressing Yra1p from the intron-containing *YRA1* gene. Thus, the intron of *YRA1* is necessary for efficient mRNA export.

Since the dominant-negative phenotype and the mRNA export defect are caused solely by intron removal from *YRA1* (note that the deduced amino acid sequence of *yra1-Δi* is not altered; see Figure 1A), we next sought to test whether a foreign intron can substitute for the *YRA1* intron. We therefore replaced exactly the intron of *YRA1* with an intron derived from the *RPL25* gene, which encodes the ribosomal protein Rpl25p. The *RPL25* intron is also comparably large (414 nucleotides) but exhibits a branch-point sequence that matches the consensus (Figure 1A). Strikingly, insertion of the *RPL25* intron into *yra1-Δi* (Figure 1A) restored cell growth (Figure 1B; *yra1-L25i*) and the mRNA export (Figure 2; *yra1-L25i*). However, the intron of *RPL25* did not fully substitute for the intron of *YRA1*, as cells expressing *yra1-L25i* grew slightly more slowly at elevated temperatures (e.g. 37°C; Figure 1B). In addition, the mRNA export defect was not fully abolished, as a small number of cells (10%) accumulate poly(A)⁺ RNA in the nucleus (Figure 2; *yra1-L25i*). The introns of *YRA1* and *RPL25* are both large. To test whether the growth defect can also be abolished by the presence of a small intron in *YRA1*, the intron of *UBC8* was inserted in the coding region of *YRA1* (Figure 1A). The *UBC8* intron is very small (123 nucleotides) and also contains a non-canonical branchpoint. As observed for insertion of the *RPL25* intron, insertion of the *UBC8* intron also restores cell growth to wild-type levels (Figure 1B; *yra1-UBC8i*). Therefore, the presence of a foreign intron within the *YRA1* coding region restores the function of *YRA1* to a large extent. We conclude that an intron within the *YRA1* gene can regulate the function of *YRA1*.

To find out how the presence of an intron in *YRA1* can affect cell growth and mRNA export, we sought to test whether Yra1p

Fig. 1. Removal of the intron of *YRA1* results in a dominant-negative growth defect. (A) A schematic showing the *YRA1* gene, *yra1-Δi*, *yra1-L25i* and *yra1-UBC8i* (exons and an intron). The two exons are indicated in grey and the intron in white. Furthermore, the DNA sequence at the 5' and 3' splice site and the branchpoint sequence are shown. The deduced amino acid sequence is indicated below the boxes. (B) Cells expressing *YRA1* from the wild-type gene (*YRA1*), the intronless version (*yra1-Δi*), the *YRA1* gene containing *RPL25* (*yra1-L25i*) or the *UBC8* (*yra1-UBC8i*) intron instead of the *YRA1* intron were spotted in 1:10 dilutions on YPD plates, and growth was assessed after 2 days incubation at 23, 30 and 37°C. (C) The *YRA1* shuffle strain ($\Delta yra1$ + pRS316-*YRA1*) was transformed with *ARS/CEN* plasmids containing the *LEU2* marker and a copy of either *YRA1* or *yra1-Δi*. Transformants were spotted on SDC (-ura/-leu) plates to select for both plasmids, and growth was assessed after 3 days incubation at 30°C.

levels are controlled by the *YRA1* intron. It has been reported previously that overexpression of *YRA1* from a cDNA construct (i.e. lacking the intron) is toxic for the cells (Espinete *et al.*, 1995). In addition, it has been noted that expression from an intronless *YRA1* construct results in overproduction of Yra1p (Zenklusen *et al.*, 2001). We therefore tested whether an intron in *YRA1* can modulate Yra1p expression by western blot analysis using anti-Yra1p antibodies. Strikingly, the Yra1p is overexpressed (~3-fold) in yeast when expressed from the *yra1-Δi* construct, but it is normal when expressed from either

Fig. 2. An intron in *YRA1* is crucial for mRNA export. *YRA1*, *yra1-Δi* and *yra1-L25i* cells were grown at 30°C, and localization of poly(A)⁺ RNA was assessed by *in situ* hybridization with a fluorescence-labelled oligo(dT) probe. The DNA was stained with 4'-6-diamidine-2-phenylindole (DAPI).

the intact *YRA1* gene or the *yra1-L25i* and *yra1-UBC8i* constructs (Figure 3A). We conclude that the authentic intron or a foreign intron inserted into the *YRA1* gene is responsible for downregulation of Yra1p expression.

These findings prompted us to test whether the intron-dependent downregulation of Yra1p expression depends on the promoter that drives *YRA1* transcription. Therefore, we placed the *YRA1*, *yra1-Δi* and *yra1-L25i* constructs under control of the strong *GAL1* promoter, which is switched on by galactose and switched off by glucose (Guarente, 1984). Unexpectedly, cells expressing Yra1p from the *GAL1::YRA1* or *GAL1::yra1-L25i* constructs grew normally in galactose-containing medium (Figure 3B). However, *GAL1::yra1-Δi* cells grew significantly slower in galactose-containing medium (Figure 3B). In contrast, all three constructs, *GAL1::YRA1*, *GAL1::yra1-L25i* and *GAL1::yra1-Δi*, did not affect cell growth in glucose-containing medium (data not shown). To find out whether growth inhibition in galactose-containing medium observed for the *GAL1::yra1-Δi* construct results from Yra1p overproduction, western blot analysis was performed using anti-Yra1p antibodies. As seen in Figure 3C, the amount of Yra1p is significantly higher in *GAL1::yra1-Δi* cells than in *GAL1::YRA1* and *GAL1::yra1-L25i* cells when grown in galactose-containing medium. Thus, the presence of an intron in *YRA1* can downregulate Yra1p expression to physiological, i.e. non-toxic, levels even when its expression is driven from the strong *GAL1* promoter.

This last result also suggests that Yra1p production is not transcriptionally regulated. Therefore, Yra1p expression appears to be regulated post-transcriptionally. To assess whether the amount of *YRA1* mRNA is altered in *YRA1*, *yra1-Δi* and *yra1-L25i* cells, we performed northern blot analysis of poly(A)⁺ RNA isolated from these three different strains. The levels of

Fig. 3. An intron within the *YRA1* gene downregulates expression of Yra1p. (A) Equivalent amounts of whole-cell lysates of *yra1-Δi* (lane 1), *yra1-L25i* (lane 2), *yra1-UBC8i* (lane 3) and *YRA1* (lane 4) cells were analysed by SDS-PAGE and western blotting using Yra1p and Arc1p antibodies. (B) Growth curve of wild-type yeast cells (RS453) transformed with plasmids encoding *GAL1::YRA1*, *GAL1::yra1-Δi*, *GAL1::yra1-L25i* or *GAL1*. Transformants were grown in galactose-containing medium and the optical density was determined at 600 nm. (C) Whole-cell lysates of *GAL1::yra1-Δi* (lane 1), *GAL1::yra1-L25i* (lane 2) and *GAL1::YRA1* (lane 3) cells were analysed by SDS-PAGE and western blotting using Yra1p and Arc1p antibodies. (D) Northern blot analysis of *YRA1* mRNA in *yra1-Δi* (lane 1), *yra1-L25i* (lane 2) and *YRA1* cells (lane 4). Two bands corresponding to the spliced (*YRA1* s) and unspliced (*YRA1* u) mRNA were detected with a *YRA1* probe. After quantification, an increase of 15% of spliced *YRA1* mRNA for the *yra1-Δi* was detected. The value was obtained after normalization with *ACT1* mRNA levels as a loading control.

mature YRA1 mRNA were not strikingly different in these strains, although *yra1-Δi* had the tendency to exhibit slightly enhanced amounts of YRA1 mRNA (Figure 3D). Moreover, unspliced YRA1 pre-mRNA could be detected in the YRA1 and *yra1-L25i* strains. Taken together, northern and western blotting data revealed that a post-transcriptional regulatory mechanism that depends on splicing downregulates Yra1p expression.

In this study, we have shown that Yra1p expression in yeast is regulated by the intron in YRA1. Strikingly, removal of the intron from the YRA1 gene causes overexpression of Yra1p, which results in a dominant-negative phenotype and an mRNA export defect. Thus, Yra1p expression must be tightly regulated in cells, otherwise excess Yra1p might titrate a factor(s) involved in mRNA export. Candidate proteins that may require a balanced concentration of Yra1p are the essential mRNA export factors Mex67p/Mtr2p and Sub2p, which form separate complexes with Yra1p (Sträßer and Hurt, 2000, 2001; Stutz *et al.*, 2000). However, overexpression of Sub2p or Mex67p from a high copy plasmid in the *yra1-Δi* strain did not suppress the dominant-negative phenotype (data not shown).

The finding that downregulation of Yra1p expression is not transcriptionally regulated and does not correlate with the amount of mRNA could point to a translationally controlled process. In higher eukaryotes, it is known that the nuclear history of a pre-mRNA can determine the translational activity of cytoplasmic mRNAs (Matsumoto *et al.*, 1998). Accordingly, the position of an intron within a pre-mRNA determines the translational efficiency of the mature mRNA in the cytoplasm. When the intron is close to the 5' end of the transcript, expression is stimulated. On the other hand, when the intron is close to the 3' end of the transcript, translation is repressed. In analogy, the intron in YRA1, which is located far downstream of the start codon (286 nucleotides), could repress translation. In contrast to YRA1, most yeast pre-mRNAs have the intron close to the 5' end of the open reading frame, which may stimulate translation (Fink, 1987; Woolford, 1989; Rymond and Rosbash, 1992). However, there could also be other ways to control Yra1p expression *in vivo* at the level of translation, e.g. by autoregulation of the splicing-derived YRA1 transcript upon Yra1p binding. Further work is required to unravel the exact mechanism that controls Yra1p expression and therefore mRNA export in yeast.

METHODS

Yeast strains and plasmids. *Saccharomyces cerevisiae* strains were grown in YPD (1% yeast extract, 2% peptone and 2% glucose) or minimal SC medium [0.67% yeast nitrogen base without amino acids (Difco) supplemented with the appropriate 'drop-out mix' (Difco)] containing raffinose or galactose as a carbon source. Growth curves of strains expressing YRA1 from the GAL1 promoter were obtained after transformation of strain RS453 with plasmids YCplac22G-YRA1, YCplac22G-*yra1-Δi* or YCplac22G-*yra1-L25i*. Cells were grown in selective raffinose-containing medium, washed and resuspended in SC medium containing galactose as a carbon source.

Wild-type RS453 and YRA1 shuffle strains were described previously (Sträßer and Hurt, 2000). DNA recombinant work such as restriction analysis, ligation, PCR amplification and *Escherichia coli* transformation were performed according to Maniatis *et al.* (1982).

The plasmid pUN100-YRA1 has been described previously (Sträßer and Hurt, 2000). pRS315-*yra1-Δi*, pRS315-*yra1-L25i* and pRS315-*yra1-UBC8i* were constructed by fusion PCR resulting in an exact deletion of the intron or its exact replacement by the intron of RPL25 or UBC8, respectively. GAL1::YRA1, GAL1::*yra1-Δi* and GAL1::*yra1-L25i* were cloned by amplifying the YRA1 coding region from pUN100-YRA1, pRS315-*yra1-Δi* or pRS315-*yra1-L25i*, respectively, creating BamHI and Pst I sites and cloning into the same sites of plasmid YCplac22G.

Northern blot, western blot and fluorescence microscopy. Isolation of poly(A)⁺ mRNA was performed following the recommendations of the provider (PolyAtract System 1000, Promega). Electrophoresis, blotting, radioactive probe labelling and signal detection were performed as described in Maniatis *et al.* (1982).

SDS-PAGE and western blot analysis were performed according to Siniosoglou *et al.* (1996). Poly(A)⁺ RNA export was analysed by *in situ* hybridization (Santos-Rosa *et al.*, 1998).

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