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The bl4 group I intron binds directly to both its protein splicing partners, a tRNA synthetase and maturase, to facilitate RNA splicing activity

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ABSTRACT

The imported mitochondrial leucyl-tRNA synthetase (NAM2p) and a mitochondrial-expressed intron-encoded maturase protein are required for splicing the fourth intron (bl4) of the yeast *cob* gene, which expresses an electron transfer protein that is essential to respiration. However, the role of the tRNA synthetase, as well as the function of the bl4 maturase, remain unclear. As a first step towards elucidating the mechanistic role of these protein splicing factors in this group I intron splicing reaction, we tested the hypothesis that both leucyl-tRNA synthetase and bl4 maturase interact directly with the bl4 intron. We developed a yeast three-hybrid system and determined that both the tRNA synthetase and bl4 maturase can bind directly and independently via RNA–protein interactions to the large bl4 group I intron. We also showed, using modified two-hybrid and three-hybrid assays, that the bl4 intron bridges interactions between the two protein splicing partners. In the presence of either the bl4 maturase or the Leu-tRNA synthetase, bl4 intron transcribed recombinantly with flanking exons in the yeast nucleus exhibited splicing activity. These data combined with previous genetic results are consistent with a novel model for a ternary splicing complex (two protein: one RNA) in which both protein splicing partners bind directly to the bl4 intron and facilitate its self-splicing activity.

Keywords: *cob-box*; leucyl-tRNA synthetase; *NAM2*; three-hybrid; two-hybrid

INTRODUCTION

tRNA synthetases have essential cellular roles that extend beyond aminoacylation of cognate tRNAs (Martinis et al., 1999a, 1999b). For example, RNA splicing of some mitochondrial group I introns (Dujardin & Herbert, 1997; Lambowitz et al., 1999) requires certain tRNA synthetases. Although the mitochondrial Tyr-tRNA synthetase (TyrRS or CYT-18p) from *Neurospora crassa* (Akins & Lambowitz, 1987) is the most extensively characterized, tRNA synthetase-dependent splicing has also been demonstrated for a homologous TyrRS from the related organism *Podospora anserina* (Kämper et al., 1992), and mitochondrial leucyl-tRNA synthetase (LeuRS or NAM2p) from *Saccharomyces cerevisiae* (Herbert et al., 1988; Labouesse, 1990).

Yeast mitochondrial LeuRS promotes excision of the bl4 intron from the *cob* gene (Fig. 1A; Labouesse, 1990; Li et al., 1996) and also the al4 α intron from *cox1 α* (Dhawale et al., 1981; De La Salle et al., 1982; Labouesse et al., 1984). Expression of these processed genes, respectively, yields the electron transfer protein cytochrome b and the α subunit of cytochrome oxidase. Both are essential to respiration.

Previous data suggest that yeast LeuRS-mediated RNA splicing is distinct from that aided by TyrRS. Significantly, LeuRS lacks an obvious idiosyncratic sequence that is critical to TyrRS proteins that facilitate RNA splicing (Cherniack et al., 1990; Kittle et al., 1991; Kämper et al., 1992). In addition, TyrRS-dependent splicing activity is blocked when a peripheral RNA domain called P5abc is present in the group I intron (Mohr et al., 1994; Wallweber et al., 1997), whereas introns aided by LeuRS contain a form of the RNA P5abc domain (Fig. 2; Michel & Westhof, 1990). Finally, excision of the bl4 intron is the first example of a group I intron splicing mechanism that requires two effector proteins, the LeuRS and the mitochondrial bl4 maturase. Genetic and biochemical investigations support that CYT-18p promotes or stabilizes folding of the RNA active

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Abbreviations: 3-AT: 3-aminotriazole; Glu-ProRS: Glutamic acid-prolyl tRNA synthetase; IleRS: Isoleucyl-tRNA synthetase; IRE: iron response element; IRP: iron regulatory protein; LeuRS: Leucyl-tRNA synthetase; ORF: open reading frame; TyrRS or CYT-18 (CYT-18p): Tyrosyl-tRNA synthetase; X-gal: 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside.

FIGURE 1. Schematic of the *cob* gene and bl4 intron fusion. **A:** The mosaic of introns, exons, and ORFs in the *cob* gene are indicated as follows: exons (B), black; group I introns (b), shaded; group II introns (b), speckled; and maturase ORF (M), open. **B:** The bl4 intron construct used herein contains about 100 nt of each of the flanking 5' B4 exon and 3' fused B5/B6 exon.

site for catalysis (Lambowitz & Perlman, 1990). However, the presence of two essential cellular effectors suggests that the role of the LeuRS in the bl4 intron splicing reaction may be at least somewhat different from that of TyrRS.

FIGURE 2. Secondary structure prediction of the bl4 intron catalytic core. The secondary structure was drawn based in part on predictions by Michel and Westhof (1990). Arrows near the P1 and P9 helices indicate sites of cleavage. Boxed nucleotides represent P10. The large insert of 1018 nt at the end of helix P8 encodes, in part, the bl4 maturase.

As a first step to understanding the LeuRS function in the ribozyme-based splicing mechanism, we have tested hypotheses predicting putative RNA–protein or protein–protein interactions between the bl4 maturase, LeuRS, and the bl4 group I intron. Specifically, we designed experiments to determine if both bl4 maturase and LeuRS bind to the group I intron via RNA–protein interactions. Alternatively, only one or perhaps neither of the proteins may directly interact with the intron. We have developed a three-hybrid system (SenGupta et al., 1996; Zhang et al., 2000) to test for specific RNA–protein interactions in the bl4 intron splicing complex. We have also screened for protein–protein interactions between the two protein splicing factors via a two-hybrid assay (Fields & Song, 1989; Gyuris et al., 1993; Golemis et al., 1996; Vidal & Legrain, 1999). Our data support that the bl4 maturase and LeuRS bind directly and simultaneously to the intron to form a ternary complex. In addition, both proteins mediate self-splicing activity of the recombinant bl4 intron within the yeast nucleus.

RESULTS

Three-hybrid analysis of bl4 intron interactions with protein splicing partners

As a first step to test for direct interactions between the bl4 intron and LeuRS or maturase, we developed a three-hybrid system by employing the commercially available RNA–Protein Hybrid Hunter Kit. Two separate plasmids containing *TRP1* markers were constructed that fused the gene for either the LeuRS (pYESTrp2-LRS) or the bl4 maturase (pYESTrp2-mat) to B42-AD (activation domain) to yield the prey molecule. As shown in Figure 1B, the hybrid bait bl4 intron RNA (including about 100 bases of each of its flanking B4 and fused B5/B6 exons) is comprised of over 1,600 nt. It contains a downstream fusion of the two tandemly repeated units of MS2 RNA as well as an upstream RNase P leader sequence for processing and promoting transcript stability (SenGupta et al., 1996). The plasmid p3Mbl4R bearing the bl4 intron hybrid bait RNA and a *URA3* marker was first introduced into a yeast host strain (L40uraMS2) that was selected for growth on yeast synthetic medium (Ura[−]). The parent plasmid pRH3' and pRH3'/IRE that encodes the RNA iron response element (IRE), were also used in separate transformations for control experiments.

Yeast transformed with plasmid expressing the RNA bait hybrid were then transformed with plasmids bearing the gene for the prey fused to B42-AD, which included pYESTrp2-LRS expressing LeuRS and pYESTrp2-mat expressing the bl4 maturase. The parent plasmid pYESTrp2 and pYESTrp2/IRP that expresses an RNA binding protein were also introduced in separate transformations as controls. Transformed

cells were selected on yeast synthetic medium (Ura⁻, Trp⁻, His⁻) that incorporated 5 mM 3-AT. The His-deficient medium selected for expression of the *HIS3* reporter gene. Inclusion of 3-AT, a competitive inhibitor of the *HIS3* reporter protein, suppressed background growth at this step of up to about 200-fold. As shown in Figure 3, cell growth occurred for those isolated colonies where the bait RNA hybrid containing the fused bl4 intron was coexpressed with the bl4 maturase hybrid prey or the LeuRS hybrid prey, suggesting that LeuRS and bl4 maturase can interact with the bl4 intron.

Several controls were carried out to assess the specificity of the three-hybrid interaction between the bl4 intron and its protein-splicing partners. Appropriate negative controls included each of the tested vectors alone as well as combinations of the RNA-expressing parent vector pRH3' with either pYESTrp2-mat or pYESTrp2-LRS; and also p3Mbl4R with the parent prey plasmid, pYESTrp2 resulted in no cell growth on the His-deficient medium. A commercially provided positive control, which included pRH3'/IRE and pYESTrp2/IRP that respectively express IRE RNA and IRP protein and are known to interact, resulted in cell growth (SenGupta et al., 1996). Coexpression of the bl4 intron and IRP did not induce cell growth. Likewise, coexpression of either of the protein-splicing partners with IRE did not yield cell viability, supporting that the *in vivo* interaction between the bl4 intron and the bl4 maturase or LeuRS exhibits specificity.

FIGURE 3. Three-hybrid system detects bl4 intron RNA-protein interactions with both LeuRS and bl4 maturase. Transformed yeast cells were grown on solid agarose synthetic medium (Ura⁻, Trp⁻, His⁻) in the presence of glucose and 5 mM 3-AT. Each of the five transformants on the right expressed bl4 intron. Cell growth indicating positive three-hybrid reporter activity was only found for the bl4 intron in the presence of either LeuRS or bl4 maturase (lower right hand corner) and a positive control expressing IRE and IRP (upper left hand corner). Bait RNA (bl4 intron or IRE) and prey protein (LeuRS, bl4 maturase, or IRP) were expressed respectively from pRH3' and pYESTrp2. LeuRS "only" and bl4 maturase "only" were expressed in the presence of the bait RNA parent vector (pRH3'). The bl4 intron "only" control was expressed solely and in the presence of pYESTrp2, the prey protein parent vector. Additional details are described within the Materials and Methods and Results sections. A control duplication was grown in the presence of histidine and demonstrated cell viability for each colony (data not shown).

FIGURE 4. β -Galactosidase reporter assay confirms interactions of the bl4 intron with both bl4 maturase and LeuRS. Transformed yeast cells were grown on solid agarose synthetic medium (Ura⁻, Trp⁻) that contained 2% galactose, 1% raffinose, and 20 mg/mL X-gal. Bait RNA (bl4 intron or IRE) and prey protein (LeuRS, bl4 maturase, or IRP) were expressed respectively from the parent plasmids pRH3' and pYESTrp2. Blue color (shown as dark color) developed for both the positive control (IRE and IRP) and each of the yeast strains that expressed bl4 intron as bait and LeuRS or bl4 maturase as prey. The bl4 intron "only" control was expressed in the presence of pYESTrp2, the prey protein parent vector. Negative controls that expressed the bl4 intron in the absence of prey or in the presence of IRP did not yield significant color development.

Upon interaction of the bl4 intron and its partner proteins, the yeast three-hybrid system also expresses a *lacZ* gene that yields β -galactosidase as a reporter protein. We carried out a second screen by monitoring β -galactosidase activities to confirm RNA-protein interactions. Colonies containing the expressed bl4 intron and either of its protein-splicing partners developed blue color (shown as darker or black color in Fig. 4). Lysates from yeast cells that were selected during three-hybrid analysis were also isolated on filters. Upon incubation of the filter with appropriate enzymatic buffer and X-gal substrate, β -galactosidase activity further verified interaction of the bl4 intron with both the bl4 maturase and LeuRS (data not shown). Similar assays carried out in solution quantitatively measured β -galactosidase activity and demonstrated that the strength of the three-hybrid response between the bl4 intron and LeuRS or bl4 maturase was similar to that found for the IRE and IRP interaction (data not shown). These three-hybrid analyses support the hypothesis that both the tRNA synthetase and maturase can bind directly and independently to the self-splicing ribozyme.

Two-hybrid analysis of bl4 maturase and LeuRS protein-protein interactions

It has been hypothesized that the bl4 intron functions within a ternary splicing complex that is dependent on both LeuRS and bl4 maturase. The results from the three-hybrid analysis clearly showed that the bl4 intron

FIGURE 5. Two-hybrid analysis of bl4 maturase and LeuRS in the presence and absence of the bl4 intron. Transformed yeast cells were grown on solid agarose synthetic medium (Ura⁻, His⁻, Trp⁻, Leu⁻) that contained 2% galactose. The colonies on the left contained bl4 maturase and LeuRS expressed respectively as LexA and B42 fusions, and the colonies on the right reversed the fusions and expressed LeuRS as a LexA fusion and bl4 maturase as a B42 fusion. The top row is a positive control as described in the Results section. The middle row displays yeast cells that exhibit a two-hybrid interaction between LeuRS and bl4 maturase when the bl4 intron RNA is expressed from p3Mbl4R. The bottom row shows a lack of growth by these yeast cells in the absence of the expressed bl4 intron RNA.

can interact with both of its protein-splicing partners. We next tested the hypothesis that the bl4 maturase and LeuRS interact via protein–protein interactions. We constructed a two-hybrid assay in which we fused the bl4 maturase to the LexA DNA-binding protein (pLexA-mat) as bait and the LeuRS to B42-AD (pJG4-5-LRS) as prey and screened for specific transcriptional activation of the reporter genes *LEU2* and *lacZ* within yeast cells (Fig. 5). Although a positive control reported interactions between two human tRNA synthetases (glutamic acid–proline and isoleucine) that had previously been shown to induce a two-hybrid response (Rho et al., 1996, 1999), multiple attempts to detect two-hybrid-based interactions between the bl4 maturase and LeuRS were unsuccessful. We also reversed the polarity of the system and fused bl4 maturase to B42-AD (pJG4-5-mat) as prey and LeuRS to LexA (pLexA-LRS) as bait, but these also failed to yield two-hybrid interactions.

Although the two-hybrid assay did not detect specific protein–protein interactions for the two protein-splicing partners, it is possible that they do interact, either weakly or upon binding the RNA intron in the splicing complex. We introduced the p3Mbl4R plasmid to express the bl4 intron within the two-hybrid host cells that also contained plasmids expressing the two protein fusions. In the presence of the bl4 intron and the two protein-

splicing partners, the two-hybrid cells grew on medium that lacked leucine, suggesting that the bl4 maturase and the LeuRS were in close enough association for the split transcriptional factors to interact. Two-hybrid interactions in the presence of the bl4 intron were detected when the bl4 maturase and Leu-tRNA synthetase was expressed as bait and prey or vice versa.

Cell lysates were isolated to test for β -galactosidase activity. β -Galactosidase shows significant reporter activity for the cells that expressed both the bl4 intron and the two protein-splicing partners, whereas the cells that lacked the bl4 intron had no detectable activity (Fig. 6). Because reporter activity was only found in the presence of the bl4 intron, it appears that the two protein-splicing partners may be bridged by the RNA and in at least close proximity in a potential ternary complex.

MS2-independent three-hybrid analysis of the bl4 intron ternary splicing complex

We modified our original three-hybrid assay to further confirm bridging RNA interactions of the two protein-splicing partners. An MS2-independent three-hybrid assay was developed that could directly test ternary complex assembly of the bl4 intron splicing complex with both its protein-splicing partners. Specifically, we replaced the MS2-binding protein encoding sequence

FIGURE 6. β -Galactosidase reporter activity reports two-hybrid interaction between the bl4 maturase and LeuRS in the presence of the bl4 intron. Transformed yeast cells were grown on solid agarose synthetic medium (Ura⁻, His⁻, Trp⁻) which contained 2% galactose, 1% raffinose, and 20 mg/mL X-gal. The colonies on the left contained bl4 maturase and LeuRS expressed respectively as LexA and B42 fusions, and the colonies on the right reversed the fusions and expressed LeuRS as a LexA fusion and bl4 maturase as a B42 fusion. The top row is a positive control as described in the Results section. The middle row displays yeast cells that exhibit a two-hybrid interaction between LeuRS and bl4 maturase when the bl4 intron RNA is expressed from p3Mbl4R. The bottom row shows a lack of growth by these yeast cells in the absence of the expressed bl4 intron RNA.

that is fused to the LexA DNA-binding domain (see Materials and Methods) with either the gene encoding bl4 maturase or LeuRS. Rather than relying on the MS2-binding protein interactions with the MS2 RNA, either the expressed bl4 maturase or the LeuRS (fused to LexA DNA binding domain) would be predicted to anchor the bl4 intron hybrid RNA for interactions with the prey protein-splicing partner that is fused to the B42-AD.

We anchored the bl4 intron hybrid bait RNA via interactions with the bl4 maturase-LexA fusion and found three-hybrid-dependent cell growth when the LeuRS prey B42-AD fusion was introduced (data not shown). We also determined that three-hybrid host cells exhibited reporter activity in the opposite polarity where LeuRS fused to LexA anchored the bl4 intron hybrid RNA and maturase (fused to B42-AD) was incorporated as prey. These results were confirmed by detection of β -galactosidase activity within the three-hybrid cells that expressed each of the three proposed bl4 intron splicing complex components including the bl4 intron, bl4 maturase, and LeuRS (Fig. 7). Systematic substitution of each of the three-hybrid plasmids by the relevant parent plasmid abolished β -galactosidase reporter activity. Thus, similar to the two-hybrid analysis that requires all three components for reporter activity, MS2-independent three-hybrid analysis also depends on both protein-splicing partners and the bl4 intron. These combined results support that the bl4 intron can assemble into a ternary complex with both the LeuRS and bl4 maturase protein-splicing partners.

Protein-dependent splicing activity

Expression of the bl4 intron and its two protein-splicing partners within the yeast nucleus provided a unique

opportunity to probe for protein-dependent bl4 ribozyme splicing activity that is uncoupled from cell viability. We isolated RNA from yeast cell nuclei that demonstrated a positive two-hybrid response for the bl4 maturase and Leu-tRNA synthetase in the presence of the bl4 intron. Primers targeting the flanking B4 and B5 exons were incorporated into RT-PCR reactions to probe for spliced product. We amplified a PCR product at the expected length of 250 bp for spliced B4-B5 exons as well as unspliced RNA at about 1.6 kb (Fig. 8A). The isolated 250-bp PCR product was cloned and sequenced to confirm that its identity was fused B4-B5 exons. In the absence of the bl4 maturase and LeuRS, only the 1.6-kb band representing unspliced RNA could be detected (lane 2 of Fig. 8A). We also tested for background RNA contamination from mitochondria. Specifically, RT-PCR analysis of RNA samples isolated from yeast nuclei in the absence of the nuclear-expressed bl4 intron failed to yield any PCR products (lane 3 of Fig. 8A) supporting that spliced B4-B5 exons originated from nuclear RNA.

We also tested nuclear RNA from our binary three-hybrid assays to determine if the individual splicing partners could separately induce splicing activity. Interestingly, whether just the Leu-tRNA synthetase (Fig. 8B, lane 3) or the bl4 maturase (Fig. 8C, lane 4) was expressed in the nucleus with the bl4 intron bait, spliced B4-B5 product was also detected. It is possible that other RNA-binding proteins could bind in the absence of the missing splicing partner and facilitate either Leu-tRNA synthetase or bl4 maturase-dependent splicing activity. However, as no spliced product was detected in the absence of both proteins, these putative nuclear factors are not sufficient to confer splicing activity. These results suggest that both the bl4 maturase and LeuRS can play a direct role in en-

FIGURE 7. MS2-independent three-hybrid analysis reports ternary complex assembly of LeuRS and bl4 maturase with the bl4 intron. Transformed yeast cells were grown on solid agarose synthetic medium (Ura⁻, Trp⁻) that contained 2% galactose, 1% raffinose, and 20 mg/mL X-gal. Colonies on the left contained LeuRS and bl4 maturase expressed respectively as LexA and B42 fusions, and the colonies on the right reversed the fusions and expressed bl4 maturase as a LexA fusion and LeuRS as a B42 fusion. The protein bait is further highlighted by an asterisk. The bottom row contains plasmids expressing bl4 maturase, LeuRS, and bl4 intron. Controls included substitution of the protein bait expression plasmid with parent plasmid pHybLex/Zeo (top row), bl4 intron RNA expression plasmid with the parent plasmid pRH3' (upper middle row), and prey expression plasmid with the parent plasmid pYESTrp2 (lower middle row).

FIGURE 8. RT-PCR detects spliced B4-B5 exons in the presence of either bl4 maturase or Leu-tRNA synthetase. RNA was isolated from three- or two-hybrid cells and amplified by RT-PCR as described in Materials and Methods. The PCR products were analyzed on a 1% agarose gel. DNA markers (Stratagene) were included in lane 1 of each gel. A band at 1.6 kb represents unspliced RNA that includes flanking exons and the intron. A band at 250 bp indicates ligated B4-B5 exons. A diffuse band near the bottom of each gel is due to excess primers. **A:** RNA was isolated from two-hybrid cells that contained bl4 intron expressed from p3Mbl4R in the absence (lane 2) and presence (lane 4) of both LeuRS and bl4 maturase expressed respectively from pJG4-5-LRS and pLexA-mat. The control in lane 3 lacked bl4 intron, but contained LeuRS and bl4 maturase. **B:** RNA was isolated from three-hybrid cells that expressed bl4 intron from p3Mbl4R in the absence (lane 2) and presence (lane 3) of LeuRS expressed from pYESTrp2-LRS. **C:** RNA was isolated from three-hybrid cells that expressed bl4 intron from p3Mbl4R in the absence (lane 2) and presence (lane 4) of bl4 maturase expressed from pYESTrp2-mat. A control in lane 3 omitted reverse transcriptase in the RT-PCR amplification to test for DNA contamination in the RNA preparation. This control was carried out for all samples (data not shown).

ablating ribozyme-based splicing activity. Because either appears to be capable of stimulating splicing activity, it is possible that they may collaborate to enhance splicing efficiency.

DISCUSSION

Protein-dependent splicing activity of the bl4 intron

The *cob* gene encoding the essential respiratory protein cytochrome *b* comprises a mosaic of exons and introns (Fig. 1A; Jacq et al., 1982; Labouesse et al., 1984; Wenzlau et al., 1989; Goguel et al., 1992; Henke et al., 1995). The bl4 maturase is expressed from the fourth intron (bl4) and subsequently facilitates removal of the group I intron by an unknown mechanism that involves the LeuRS. In the absence of a functional bl4 maturase, LeuRS has also been suggested to activate latent maturase activity of the al4 α endonuclease that is encoded in the fourth intron of the mitochondrial *cox1 α* gene (Herbert et al., 1988). The al4 α endonuclease is highly homologous to the bl4 maturase. Both belong to a family of proteins characterized by two conserved LAGLIDADG sequences that are separated by about 100 amino acids (Lambowitz et al., 1999). Minimal amino acid changes interconvert the maturase to an endonuclease and vice versa (Goguel et al., 1992; Henke et al., 1995). The collaborative splicing roles between LeuRS and either bl4 maturase or al4 α endonuclease remain unclear.

Because mitochondrial LeuRS is also essential to protein synthesis, including translation of the bl4 maturase, confirmation of the protein's direct contribution to RNA splicing required uncoupling its dual splicing and aminoacylation activities (Li et al., 1996). Specifically, although it was conceivable that LeuRS mutations or its altered expression levels could effect maturase/tRNA synthetase-dependent splicing of the bl4 intron, it was also possible that mutant leucine enzymes could simply impact maturase expression via its protein synthesis role in the mitochondria. To circumvent the latter possibility, mitochondrially expressed maturase was inactivated and a functional maturase with the proper codon conversions was expressed in the yeast cytoplasm and then imported into the mitochondria (Labouesse, 1990; Li et al., 1996). Cytoplasmic coexpression of maturase and LeuRS and their mutant forms demonstrated unequivocally that bl4 intron excision requires both proteins.

Analysis of spliced product isolated from our three-hybrid assays suggested that either bl4 maturase or Leu-tRNA synthetase were sufficient to induce bl4 ribozyme splicing activity. In the absence of its splicing partner in the three-hybrid system, either protein co-factor may be assisted in the yeast nucleus by other available, but nonphysiological and nonspecific, RNA-binding proteins. However, these nuclear factors could not facilitate splicing activity in the absence of the bl4 maturase or Leu-tRNA synthetase. Alternatively, it is also possible that the sensitive RT-PCR assay detected low amounts of splicing activity that are pro-

moted by a single protein cofactor, but these levels may not be sufficient to sustain mitochondrial activity. In this latter scenario, the two protein-splicing partners may collaborate under physiological conditions to enhance or even regulate splicing activity to control expression of the critical *cox1* and *cob* genes.

Other group I introns have been shown *in vivo* to require more than one protein cofactor for excision. For example, splicing activity of the yeast mitochondrial $\alpha 5\beta$ intron in the *cox1* gene is effected by up to four protein partners (Costanzo et al., 1986; Séraphin et al., 1988; Valencik et al., 1991; Bousquet et al., 1990; Conrad-Webb et al., 1990; Johnson & McEwen, 1997). Because the *bl4* intron utilizes two defined proteins for splicing activity in the mitochondria (Li et al., 1996), it provides a novel model to characterize splicing activities that require one or more protein cofactors. Although it has been firmly established via genetic approaches that both the *bl4* maturase and *LeuRS* play a role in the *bl4* intron activity (Labouesse, 1990; Li et al., 1996), the actual functions of these two splicing partners in the ribozyme-based mechanism remain unclear. Elucidation of each protein's molecular role has been hampered by lack of an *in vitro* splicing assay. The development of a suitable splicing assay may be hindered in part by accurate folding *in vitro* of the very large 1.4 kb *bl4* intron RNA.

Three-hybrid analysis of the large *bl4* intron

In the absence of a protein-dependent *in vitro* splicing assay, we sought alternative methods to test hypotheses about macromolecular interactions that may be important to assembly and activity of the splicing complex. Specifically, we developed three-hybrid and two-hybrid approaches that rely on intracellular mechanisms for RNA processing and folding of the *bl4* intron to facilitate transition of the nascent RNA molecule to a competent conformation. Three-hybrid analysis of RNA–protein interactions has been suggested to be limited to RNA molecules that are less than a couple of hundred nucleotides (Zhang et al., 2000). In addition, although it is context dependent, oligouridine tracts of four or more uridines may terminate RNA polymerase transcription. The *bl4* intron bait RNA was greater than 1,600 nt and included multiple sites of at least four successive uridines (see Fig. 2). RT-PCR analysis demonstrates that the very large RNA molecule is stably produced in the three-hybrid system under standard conditions that have been utilized for much smaller RNAs (Fig. 8). Because the RT-PCR method is not quantitative, it is possible that low amounts of the nuclear expressed *bl4* intron were amplified for detection. However, these quantities (even if somewhat diminished due to *bl4* intron splicing activity) were sufficient to detect three- and two-hybrid responses with the group I intron's protein-splicing partners. The strength

of these interactions was similar to that of the well-characterized IRE and IRP three-hybrid response (data not shown).

The three-hybrid and RNA-dependent two-hybrid *in vivo* assays can now be exploited to map and define specific molecular interactions between the *bl4* ribozyme molecules and its protein-splicing partners that are important to complex assembly. Moreover, these assays can be used to distinguish and identify important molecular sites on the RNA and protein that influence protein-dependent splicing activity of the *bl4* ribozyme. Significantly, three-hybrid and RNA-dependent two-hybrid analyses can be used to initially and rapidly screen RNA and/or protein variants that may be more suitable for incorporation into a functional *in vitro* splicing assay. For example, minimized *bl4* group I introns that target reduction of domains of the 1.4 kb RNA that are peripheral to the catalytic core may be more likely to fold into a competent ribozyme *in vitro*. Engineered RNA molecules that retain interactions with both of their protein-splicing partners and splicing activity in the *in vivo* three-hybrid and two-hybrid assays could be used to more efficiently develop an *in vitro* splicing assay for protein-dependent structure–function analysis of the ternary complex splicing activity.

Macromolecular arrangement of the ternary *bl4* intron splicing complex

Herein, we asked whether the *bl4* intron ribozyme interacts via RNA–protein interactions with either or both the *LeuRS* or *bl4* maturase. Alternatively, one of the protein-splicing partners could recruit the second protein factor to the ribozyme splicing complex via protein–protein interactions. We have shown, using three-hybrid analysis, that both proteins bind independently and directly to the RNA. Although it is possible that they also interact via protein–protein interactions in the splicing complex, our two-hybrid analyses in the absence of the *bl4* intron suggest that each protein primarily interacts with the RNA. Moreover, in the presence of the RNA, the *bl4* intron appears to bridge the two protein-splicing partners in a ternary complex.

Although group I introns are autocatalytic and self-splice *in vitro*, protein factors are required as effectors for splicing activity *in vivo* (Lambowitz & Perlman, 1990; Cech, 1993; Weeks, 1997; Lambowitz et al., 1999). These factors include “maturases” which are encoded by the intron itself (Lazowska et al., 1980) and/or other proteins encoded by nuclear genes. Only a limited number of protein-dependent ribozyme activities have been characterized allowing specific RNA–protein interactions that are important to splicing activity to be identified. These include an *in vitro* assay for an *Aspergillus nidulans* maturase-dependent ribozyme activity (Ho et al., 1997; Ho & Waring, 1999) as well as group I ribozymes that depend on the yeast nuclear-encoded CBP2p

(Gampel et al., 1989; Gampel & Cech, 1991; Lewin et al., 1995) or CYT-18p from *N. crassa* (Akins & Lambowitz, 1987). Interestingly, in the latter cases, CBP2p is completely dedicated to RNA splicing, whereas CYT-18p also functions as a TyrRS in mitochondrial protein synthesis. Each protein-splicing partner has been clearly shown to aid in assembly or stabilize secondary or tertiary interactions required for the active ribozyme (Gampel & Cech, 1991; Mohr et al., 1992; Shaw & Lewin, 1995; Weeks & Cech, 1995a, 1995b, 1996; Caprara et al., 1996b; Saldanha et al., 1996; Shaw et al., 1996; Ho & Waring, 1999).

It is intriguing to utilize these 1 protein:1 RNA splicing complexes as a model to consider how the bl4 intron might simultaneously interact with both the maturase and LeuRS to facilitate splicing. Extensive RNA footprinting and crosslinking studies have shown that CBP2p (Shaw & Lewin, 1995; Weeks & Cech, 1995b) and CYT-18p (Caprara et al., 1996a, 1996b) bind specifically to their respective group I intron partners, but on opposite sides of the conserved tertiary structure in a 1:1 protein:RNA binary complex (Fig. 9A; Weeks, 1997; Lambowitz et al., 1999). We propose that the LeuRS may

interact with the group I intron analogous to CYT-18p (TyrRS) while the bl4 maturase binds to the opposite side similar to CBP2p (Fig. 9B).

CYT-18p binds on the backside of the active site cleft (Fig. 9A) to a tRNA-like moiety of the folded intron catalytic core (Caprara et al., 1996a, 1996b) in a bi-phasic manner (Saldanha et al., 1995). The first fast-binding step targets the initially formed P4-P6 coaxial stacked helices (Caprara et al., 1996b) that comprise the nucleus of the largely unfolded RNA intron (Zarrinkar & Williamson, 1994). A second slow step requires a conformational change aided by the protein to induce tertiary structure formation, including the P3-P9 domain that forms the other part of the ribozyme active site cleft. The resultant very tight protein-RNA complex of CYT-18 with K_d s in the pM range (Guo & Lambowitz, 1992) stabilizes the conformation of the intron to promote splicing (Caprara et al., 1996b).

It is striking that the tRNA-like moiety of the group I intron superimposes to a class II tRNA (Caprara et al., 1996a) that is marked by an extra-long variable loop and is found in tyrosine, leucine, and also serine tRNAs. Thus, it is possible that LeuRS binds similarly to a tRNA-like moiety on the backside of the bl4 group I intron. Because a P5abc domain has been shown to be inhibitory to CYT-18 (Mohr et al., 1994; Wallweber et al., 1997) and is present in the bl4 intron (Fig. 2), by comparison, conformational changes in the RNA or alterations in the orientation of LeuRS interactions with the group I intron may facilitate formation of the RNA-protein complex. Alternatively, significant differences between the protein structures of monomeric LeuRS and dimeric CYT-18 may simply allow LeuRS interactions with a group I intron that contains a P5abc domain.

In contrast to CYT-18p, CBP2p binds to the opposite face near the active site cleft of the bl5 group I intron (see Figs. 1 and 9A) and is essential to the ordered assembly of the ribozyme catalytic core (Gampel & Cech, 1991; Shaw & Lewin, 1995; Weeks & Cech, 1995a, 1995b, 1996). Crosslinking and mutagenesis studies have identified a short 12 amino acid RNA-binding motif comprised of alternating basic and aromatic residues that is critical for the CBP2p-assisted splicing activity (Tirupati et al., 1999). CBP2p appears to bind to the P4-P6 domain and helps induce secondary structure formation (Shaw & Lewin, 1995; Shaw et al., 1996). It also specifically targets the 5' splice site where it captures a transiently folded RNA tertiary domain (Weeks & Cech, 1996). At this step, CBP2p appears to sequester the P1-P2 domain of the bl5 intron between the P5-P4-P6 and P8-P3-P7 domains and stabilizes tertiary interactions required for ribozyme activity (Shaw & Lewin, 1995; Weeks & Cech, 1995a, 1995b, 1996).

Similar to CBP2p, the more recently characterized *Aspergillus* maturase also appears to enable docking of the P1 helix to the folded catalytic core of the group I intron (Ho et al., 1997). Interestingly, the *Aspergillus*

A. Known Binary Ribozyme Complexes

B. Proposed bl4 Intron Ternary Complex

FIGURE 9. Proposed model of ternary bl4 splicing complex. **A:** As described in the Discussion section, CBP2p (light gray) binds near the active site cleft of its group I intron partner in a binary complex and CYT-18p (black) binds to the backside of the active site cleft of its respective splicing partner (adapted from Weeks, 1997). **B:** This bl4 intron model hypothesizes that LeuRS (black) binds to the backside of group I intron similar to CYT-18p while the bl4 maturase (light gray) interacts with the opposite face near the active site cleft in a ternary complex.

and bl4 maturases share two similar dodecapeptide motifs known as the LAGLIDADG sequences that are also conserved in a family of DNA homing endonucleases (Belfort & Roberts, 1997; Lambowitz et al., 1999). Although the native bl4 maturase does not function as a DNA endonuclease, minimal amino acid changes within the conserved motif can confer alternate splicing or maturase activities (Delahodde et al., 1989; Wenzlau et al., 1989; Goguel et al., 1992). Thus, the much better characterized LAGLIDADG-containing endonucleases that bind to DNA can potentially be used as a model for LAGLIDADG-containing maturases that interact with RNA. DNA footprinting and molecular modeling based on a solved *PI-SceI* crystal structure suggest that the endonuclease interacts via the 5' and 3' exon DNA that would respectively encode the P1 and P10 helices of the group I intron (Duan et al., 1997; Heath et al., 1997; Gimble et al., 1998; He et al., 1998; Hu et al., 1999). Because of the high similarity between the LAGLIDADG-containing maturase and endonucleases, it has been proposed that, by analogy, the maturase may bind to the P1 and P10 helices that lie at the intron–exon junctions (Lambowitz et al., 1999). This site would overlap on the same face of the intron that binds CBP2p. Importantly, this also suggests a potential binding site for the bl4 maturase that is distinct from the putative LeuRS site of interaction on the backside of the ribozyme.

Summary

We have presented data supporting the hypothesis that the bl4 intron binds directly to each of its protein-splicing partners. Our results suggest a unique group I ribozyme splicing complex that is comprised of two proteins and one RNA. In addition, we have demonstrated that each of the protein-splicing factors, bl4 maturase and LeuRS, directly facilitates splicing activity. The novel bl4 intron ternary complex provides an excellent system to map and identify critical and potentially synergistic RNA–protein interactions in an elementary splicing complex that is dependent on two proteins. Moreover, in vivo characterization of ternary complex assembly and its molecular interactions may facilitate development of a protein-dependent in vitro RNA splicing assay. Significantly, this ternary complex may serve as an important evolutionary model in which functional ribozymes became increasingly dependent on proteins and recruited multiple protein cofactors to maintain, enhance, or possibly regulate its splicing activity.

MATERIALS AND METHODS

Materials

Unless otherwise specified, all reagents and materials were purchased from standard commercial sources. The RNA–

protein Hybrid Hunter Kit, Zeocin, and plasmid pHybLex/Zeo were purchased from Invitrogen. All PCR amplifications were carried out using *Pfu* polymerase (Stratagene) according to the commercial protocol. Plasmids pG3bl4 and pGEX-2Tmat2 were generously provided by Dr. A. Delahodde (Ecole Normale Supérieure, Paris). The origins of these cloned genes were from yeast strain 777-3A. Yeast genomic DNA was kindly provided by Professor M. Benedik (University of Houston). Standard yeast molecular biology procedures and media preparation were carried out as previously described (Lundblad, 1995).

Methods

Three-hybrid systems have been designed to identify RNA–protein interactions (Putz et al., 1996; SenGupta et al., 1996; Zhang et al., 2000) based on a modification of its predecessor, the two-hybrid system, which is an in vivo approach to detect specific protein–protein interactions (Fields & Song, 1989; Gyuris et al., 1993; Golemis et al., 1996; Vidal & Legrain, 1999). We enlisted a yeast three-hybrid system that relies on two protein fusions (SenGupta et al., 1996). A DNA-binding protein LexA is linked to an RNA-binding protein called MS2 coat protein. The second protein hybrid includes a transcriptional activation domain (AD) called B42-AD that is fused to the protein hypothesized to interact with a particular RNA molecule. A third component incorporates a hybrid RNA comprised of the well-characterized MS2 RNA (Uhlenbeck et al., 1983; Bardwell & Wickens, 1990; Witherell et al., 1990) fused to the RNA of interest (SenGupta et al., 1996). The RNA hybrid is anchored by interactions between its MS2 RNA end and the LexA–MS2 binding protein fusion and acts as bait to test for interactions with the protein prey fused to the B42-AD. Respective RNA–protein interactions between the bait and prey are detected by specific transcriptional activation of reporter genes in yeast that amplify the initial signal manyfold and has proven successful in multiple cases (Putz et al., 1996; SenGupta et al., 1996; Wang et al., 1996; Martin et al., 1997; Zhang et al., 1997).

Plasmid constructions

PCR amplifications of the bl4 intron gene with its flanking B4 (102 bp) and fused B5/B6 exons (84 bp) were carried out using pG3bl4 as template (Delahodde et al., 1989). Two fragments were amplified to introduce an internal *Bam*HI site. Fragment I was comprised of DNA encoding a portion of the B4 exon and 363 bp of the 5' end of the intron using primer Exon 4-F [5'-CCG GAA TTC ATG CAT TCA TAC TTT ATT TTT-3'], which incorporated a 5' *Eco*RI restriction site, and primer Exon 4-R [5'-CCG GGA TCC TTT ATT TTC TAG TGT GCG TGT-3'], which introduced a *Bam*HI site. Fragment I was digested and cloned into *Eco*RI and *Bam*HI-digested plasmid pGEM-3Zf vector (Promega) yielding plasmid pB4M96. Fragment II was generated using a 5' primer containing a *Bam*HI site (Intron 4-F [5'-CCG GGA TCC ATT TTC TCC GTA TTC ATT ATT-3']) and a reverse primer, Exon 6-R, targeting the sequence encoding the B6 exon and introduced a *Sal*I 3' site [5'-CCG GTC GAC TCA TAA AAT AGC ATA GAA TGG-3']. The fragment II PCR product was digested and cloned into *Bam*HI and *Sal*I-digested pB4M96 yielding plasmid pGEM-bl4. The isolated

pGEM-bl4 plasmid was used as a PCR template to amplify the bl4 intron with primers targeting the DNA sequence encoding the flanking B4 [5'-CGT TTA AAC ATG CAT TCA TAC TTT ATT TTT-3'] and B6 exons [5'-CCG CCT AGG TCA TAA AAT AGC ATA GAA TGG-'] and introducing respective 5' *PmeI* and 3' *AvrII* sites. This PCR product was digested and cloned into *PmeI* and *AvrII*-cleaved plasmid pRH3' (Invitrogen) yielding plasmid p3Mbl4R.

The mitochondrial LeuRS gene was amplified via PCR from yeast genomic DNA. The 3' primer, LIC3-Scm [5'-GAG GAG AAG CCC GGT TTA CTT GTG GAA TAA GAA AC-3'], targeted the stop codon region of the nuclear-encoded gene. The 5' primer, LIC5-Scm [5'-GAC GAC GAC AAG ATG CTG TCT CGA CCT TCA AG-3'] annealed just downstream of the DNA encoding the mitochondrial import sequence, thus deleting it from the final PCR product. The gel-purified 3.1-kb PCR product was cloned into the pET-32 LIC vector by ligation-independent cloning followed by transformation of NovaBlue competent cells exactly as described by the commercial protocol (Novagen). DNA was isolated from transformants and screened using a *BamHI* restriction digestion that yields a doublet at 1.1 kb for a positive clone. A colony containing plasmid pYM3-17 was identified and sequenced completely, confirming the sequence of the cloned mitochondrial LeuRS gene.

Plasmids expressing the bl4 maturase or LeuRS as prey linked to B42-AD for three-hybrid analysis were constructed by cloning restriction-digested PCR products for each gene into plasmid pYESTrp2 (Invitrogen). Specifically, the LeuRS gene was amplified from pYM3-17 using ymtLeuRS-F (*HindIII*) primer [5'-CCG AAG CTT ATG CTG TCT CGA CCT TCA AGC-3'], which introduced a 5' *HindIII* site, and ymtLeuRS-R (*SphI*) primer [5'-CCG GCA TGC TTA CTT GTG GAA TAA GAA ACT-3'], which incorporated a 3' *SphI* site. The purified PCR product was digested and cloned into *HindIII* and *SphI* digested pYESTrp2 yielding plasmid pYESTrp2-LRS. The bl4 maturase gene was amplified from a pGEX-2Tmat2 template (Delahodde et al., 1989) that contains 15 base changes to adapt the mitochondrial-expressed gene to the universal genetic code. The forward primer Maturase-F (*KpnI*) [5'-CCG GGT ACC AGA TCT AAA ATG AAT CAA ATT-3'] introduced a 5' *KpnI* site and the reverse primer Maturase-R (*XhoI*) [5'-CCG CTC GAG CTA ATT ATT CCA TTT TTC-3'] incorporated a 3' *XhoI* site. The purified digested PCR product was cloned into *KpnI* and *XhoI* digested pYESTrp2 to produce plasmid pYESTrp2-mat.

Plasmids encoding either bl4 maturase or LeuRS for two-hybrid analysis were constructed using plasmid pJG4-5 and pLexA to respectively express prey and bait proteins (Gyuris et al., 1993; Rho et al., 1998, 1999). The LeuRS gene was amplified from pYM3-17 using ymtLeuRS-F (*EcoRI*) primer [5'-CCG GAA TTC ATG CTG TCT CGA CCT TCA AGC-3'], which introduced a 5' *EcoRI* site, and ymtLeuRS-R (*SalI*) primer [5'-CCG GTC GAC TTA CTT GTG GAA TAA GAA ACT-3'], which incorporated a 3' *SalI* site. The purified PCR product was cleaved with *EcoRI* and *SalI* and cloned into restriction-digested pJG4-5 (*EcoRI* and *XhoI*) and pLexA (*EcoRI* and *SalI*) yielding respectively plasmids pJG4-5-LRS and pLexA-LRS. The bl4 maturase gene was amplified from pGEX-2Tmat2 (Delahodde et al., 1989) using a forward primer, Maturase-F (*EcoRI*) [5'-CCG GAA TTC AGA TCT AAA ATG AAT CAA ATT-3'], which introduced a 5' *EcoRI* site, and

reverse primer Maturase-R (*XhoI*) as described above. The purified digested PCR product was cloned into *EcoRI* and *XhoI*-digested pJG4-5 to obtain plasmid pJG4-5-mat. The *EcoRI*-*XhoI* PCR fragment was also cloned into *EcoRI* and *SalI* restriction-digested pLexA to produce pLexA-mat.

Plasmids expressing either bl4 maturase or LeuRS as bait for MS2 protein-independent three-hybrid analysis were constructed using plasmid pHybLex/Zeo (Invitrogen). Plasmid pLexA-LRS was restriction-digested with *EcoRI* and *SalI* and the gene encoding LeuRS was cloned into the *EcoRI* and *SalI* sites of pHybLex/Zeo to produce plasmid pHybLex-LRS. Likewise, the *EcoRI* and *XhoI* DNA fragment from pJG4-5-mat was isolated and cloned into pHybLex/Zeo, which was restriction digested with *EcoRI* and *SalI*, yielding pHybLex-mat. Construction of pHybLex-mat eliminated the *XhoI* and *SalI* sites, but cleavage with *EcoRI* and *PstI* yielded a fragment for the cloned maturase gene with the expected size of 0.77 bp.

Three-hybrid and two-hybrid analyses

Three-hybrid analysis was carried out as described by Sen-Gupta et al. (1996) and the RNA-protein Hybrid Hunter Kit commercial protocol (Invitrogen). Introduction of plasmids expressing the bait and prey constructs into the yeast L40uraMS2 host strain (Invitrogen) were carried out in two successive steps. The first step selected for the bait-encoding plasmid p3Mbl4R on yeast synthetic dextrose (SD) medium (Ura⁻). In separate transformations, the parent bait vector pRH3' and plasmid pRH3'/IRE (Invitrogen; encodes the IRE RNA) were also introduced into the yeast L40uraMS2 host strain for control experiments. Cells that contained the appropriate bait-encoding plasmid or parent vector were then transformed with plasmid expressing prey (pYESTrp2-LRS or pYESTrp2-mat) and selected on yeast SD medium (Ura⁻, Trp⁻, His⁻). Five millimoles 3-AT were included to suppress high background (Klopotowski & Wiater, 1965; Struhl & Davis, 1977). Control plasmids were also introduced in separate transformations and included the parent vector pYESTrp2 and pYESTrp2/IRP (Invitrogen), which expresses the IRP that binds IRE.

MS2-independent three-hybrid analysis was carried out in three successive transformation steps of yeast L40 host strain (Invitrogen). Standard procedures are described by the commercial protocol. In the first step, transformants that contained either pHybLex-mat, pHybLex-LRS, or pHybLex/Zeo (control) were selected on yeast YPD medium that contained the antibiotic Zeocin (50 µg/mL; Invitrogen). Cells that contained the bait protein-expressing plasmid or its parent vector were then transformed with p3Mbl4R or pRH3' and selected on yeast SD medium (Ura⁻). Finally, plasmid expressing prey (pYESTrp2-LRS or pYESTrp2-mat or control plasmid pYESTrp2) was introduced and selected on yeast SD medium (Ura⁻, Trp⁻) that contained 5 mM 3-AT, 1% raffinose, and 20 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal).

Two-hybrid selection and analysis were carried out as described previously in yeast host strain EGY48 [*MATa*, *his3*, *trp1*, *ura3-52*, *leu2::pLeu2-LexAop6/pSH 18-34* (*LexAop-lacZ* reporter)] using LexA DNA binding protein (pLexA) and B42-AD (pJG4-5) fusion proteins (Rho et al., 1996, 1998, 1999). Plasmids introducing bait protein included pLexA-LRS

or pLexA-mat. The prey was expressed from pJG4-5-mat or pJG4-5-LRS. The bI4 intron was introduced using the plasmid p3Mbl4R. A positive control was also performed using plasmids LexA-IRS and B42-EPRS that respectively expressed human isoleucyl-tRNA synthetase (IleRS) as bait and glutamic acid-prolyl tRNA synthetase (Glu-ProRS) as prey and were shown previously to interact in a two-hybrid assay (Rho et al., 1996, 1999).

β -galactosidase reporter assays

Yeast cells that were selected for three-hybrid or two-hybrid interactions were grown in liquid culture using yeast synthetic medium containing 2% glucose. At mid-log phase (0.5–0.6), the OD₆₀₀ was measured. The cells were lysed and β -galactosidase activity assays were carried out exactly as described before using a filter assay (Bartel et al., 1994; Rho et al., 1996).

RT-PCR analysis

Glass beads were used to lyse yeast cells followed by isolation of nuclear RNA (Lundblad, 1995). The RNA was denatured for 1 min at 90 °C and primers targeting the B4 and B5 exons were annealed at 48 °C for 45 min. Reverse transcription was carried out using AMV-RT (Promega) at 37 °C for 1 h. The cDNA was denatured and amplified by PCR (30 cycles: 94 °C, 1 min; 50 °C, 1 min; 72 °C, 2 min). Controls carried out in the absence of reverse transcriptase yielded no PCR products, thus indicating that amplification was not due to DNA contaminants.

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