A tyrosyl-tRNA synthetase binds specifically to the group I intron catalytic core

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The *Neurospora* CYT-18 protein, the mitochondrial tyrosyl-tRNA synthetase, functions in splicing group I introns in mitochondria. Here, we show that CYT-18 binds strongly to diverse group I introns that have minimal sequence homology and recognizes highly conserved structural features of the catalytic core of these introns. Inhibition experiments indicate that the intron RNA and tRNA^{Tyr} compete for the same or overlapping binding sites in the CYT-18 protein. Considered together with functional analysis, our results indicate that the CYT-18 protein promotes splicing by binding to the intron core and stabilizing it in a conformation required for catalytic activity. Furthermore, the specific binding of the synthetase suggests that the group I intron catalytic core has structural similarities to tRNAs, which could reflect either convergent evolution or an evolutionary relationship between group I introns and tRNAs.

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Although group I introns use RNA-catalyzed splicing reactions, many, if not all, require proteins for efficient splicing in vivo (for review, see Lambowitz and Perlman 1990). In the case of mitochondrial RNA splicing, these proteins have been shown to include maturases encoded within some group I introns, as well as a variety of splicing factors encoded by host chromosomal genes. The latter tend to differ between *Neurospora* and yeast, and they include aminoacyl-tRNA synthetases and other cellular RNA-binding proteins that have an additional function in their host cell. We suggested that host proteins that have already differentiated may have adapted to function in splicing relatively recently in evolution by taking advantage of their ability to recognize structural features of group I introns resembling those in their normal cellular RNA targets (Lambowitz and Perlman 1990). Most host-encoded splicing factors function in splicing one or a small number of related group I introns. However, the *Neurospora* CYT-18 protein, the mitochondrial tyrosyl-tRNA synthetase (mt TyrRS), functions in splicing a number of different group I introns in mitochondria and presumably recognizes conserved structural features of these introns (Collins and Lambowitz 1985). The fact that the CYT-18 protein is an aminoacyl-tRNA synthetase raises the possibility that

these structural features resemble those found in tRNAs (Akins and Lambowitz 1987).

To investigate how the *Neurospora* mt TyrRS functions in splicing, we developed an in vitro-splicing system based on the ability of the protein to splice the *Neurospora* mitochondrial large rRNA (mt large rRNA) intron, which is not self-splicing under any conditions examined (Garriga and Lambowitz 1986). Studies using this in vitro system showed that splicing occurs by the same guanosine-initiated transesterification reactions used by self-splicing group I introns and remains dependent on the integrity of the group I intron catalytic core (Guo et al. 1991). Furthermore, mutant CYT-18 proteins, with linker insertions near the predicted ATP-binding site, have little, if any, tyrosyl-adenylation activity but retain substantial splicing activity, indicating that the normal catalytic activity of the protein is not required for splicing (Kittle et al. 1991; G. Mohr, A. Cherniack, G. Guo, J. Gianelos, and A.M. Lambowitz, unpubl.). Together, these findings indicate that the splicing reaction remains essentially RNA catalyzed and that the ability of the protein to function in splicing requires only that it binds to the precursor RNA.

Regions of the CYT-18 protein required for splicing have been identified by analyzing mutant and partial revertant alleles of the *cyt-18* gene, as well as in vitro mutants of the CYT-18 protein synthesized in *Escherichia coli*. These regions include a small, idiosyncratic amino-

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terminal domain not found in other TyrRSs that do not function in splicing, parts of the nucleotide binding fold, and the putative tRNA-binding domain toward the carboxyl terminus of the protein (Cherniack et al. 1990; Kittle et al. 1991). A number of mutations in the carboxy-terminal domain result in parallel inhibition of splicing and TyrRS activity, suggesting that this region may be involved in binding both the intron and tRNA substrates. Deletions in the idiosyncratic amino-terminal domain inhibit splicing activity of the CYT-18 protein but leave substantial TyrRS activity assayed with *E. coli* tRNA^{Tyr}. However, recent experiments have shown that part of the idiosyncratic domain is required for efficient aminoacylation of the *Neurospora* mitochondrial tRNA^{Tyr} (mt tRNA^{Tyr}), suggesting that it interacts with some feature of this tRNA not found in the *E. coli* tRNA^{Tyr} (Cherniack 1991). The finding that different regions of the CYT-18 protein required for splicing may also be involved in binding the tRNA suggests that similar interactions may be used for the binding of both the intron and tRNA substrates.

To facilitate studies on the interaction between the CYT-18 protein and the intron RNA, we constructed a mini-intron of 388 nucleotides, compared with 2.3 kb for the original intron, that is still spliced in a protein-dependent manner (Guo et al. 1991). Experiments with mutant derivatives of this mini-intron suggested that the inability of the *Neurospora* intron to self-splice reflects instability of a structure in the intron core or in the interaction between the core and the P1 stem containing the 5' splice site (Guo et al. 1991). In reverse splicing catalyzed by the CYT-18 protein, the protein appears to bind to the free intron to form an active ribonucleoprotein (RNP) complex, which then binds ligated exon RNA (Mohr and Lambowitz 1991). Finally, recent experiments have shown that the CYT-18 protein can suppress structural defects in the catalytic core of mutant bacteriophage T4 *td* or yeast ω^+ introns (Mohr et al. 1992). Together, these findings suggest that the CYT-18 protein promotes splicing by binding to the intron and facilitating formation of the catalytically active structure of the intron core. Similarly, the yeast CBP2 protein, a group I intron splicing factor specific for *cob* intron 5, has been shown to require an intact catalytic core for binding, indicating either that the protein binds to the core or that the core is required for proper display of sequences that bind the protein (Gampel and Cech 1991).

The precise identification of the CYT-18 protein-binding site in the intron RNA is the key to understanding how the protein functions in splicing and its evolutionary significance. Here, we show that the CYT-18 protein binds strongly to a number of different group I introns, and we localized the CYT-18 binding site in the *Neurospora* mt large rRNA intron to a highly conserved region of the catalytic core. Considered together with recent functional analysis (Mohr et al. 1992), our results indicate that the CYT-18 protein promotes splicing by binding to the intron core and stabilizing it in the catalytically active conformation. Furthermore, our results suggest that the group I intron catalytic core, which is

specifically recognized by the synthetase, has structural features that resemble those in tRNAs.

Results

Binding of the CYT-18 protein to the Neurospora mt large rRNA intron

Table 1 summarizes intron constructs used in the present work, and Figure 1 shows the 388-nucleotide-derivative of the *Neurospora* mt large rRNA intron (pBD5A) used as the starting point for this study. This intron was constructed by deleting sequences corresponding to the open reading frame (ORF), which is ordinarily encoded in the intron, as well as nonessential parts of P2 and L8. We showed that splicing of this 388-nucleotide intron is completely dependent on the CYT-18 protein (Guo et al. 1991).

In initial experiments we used the nitrocellulose filter-binding method to assay the binding of purified CYT-18 protein to a 503-nucleotide in vitro transcript containing the 388-nucleotide intron. In these experiments, low concentrations of ³²P-labeled in vitro transcript were incubated with increasing concentrations of purified CYT-18 protein, and binding was assayed by retention of the ³²P-labeled RNA on a nitrocellulose filter. As shown in Figure 2A, the CYT-18 protein bound strongly to the transcript containing the intron (pBD5A/*Ban*I), whereas control transcripts (pHX11/*Pvu*II and pBD5A/*Nhe*I)

Table 1. Plasmids for synthesis of in vitro transcripts containing the *Neurospora* mt large rRNA intron and derivatives

Plasmid/enzyme	In vitro transcript ^a
pHX11/ <i>Ban</i> I	Nc mt large rRNA intron: 5'E/I/3'E, 844/2296/50 nt
pHX9422/ <i>Ban</i> I	Nc mt large rRNA intron: 5'E/I/3'E, 65/583/50 nt
pBD5A/ <i>Ban</i> I	Nc mt large rRNA intron: 5'E/I/3'E, 65/388/50 nt
pL95/ <i>Ban</i> I	pBD5A: 5' truncation to J2/3 (nt 95)
pL97/ <i>Ban</i> I	pBD5A: 5' truncation to P3 (nt 97)
pL107/ <i>Ban</i> I	pBD5A: 5' truncation to P4 (nt 107)
pL129/ <i>Ban</i> I	pBD5A: 5' truncation to P5 (nt 129)
pR367/ <i>Bam</i> HI	pBD5A: 3' truncation to P9.1 (nt 367)
pR359/ <i>Eco</i> RI	pBD5A: 3' truncation to P9 (nt 359)
pR342/ <i>Bam</i> HI	pBD5A: 3' truncation to J7/9 (nt 342)
pR312/ <i>Nco</i> I	pBD5A: 3' truncation to L8 (nt 312)
pR243/ <i>Bam</i> HI	pBD5A: 3' truncation to J6/7 (nt 243)
pR239/ <i>Bam</i> HI	pBD5A: 3' truncation to P6 (nt 239)
pR175/ <i>Nhe</i> I	pBD5A: 3' truncation to P6b (nt 175)
pΔ108–139/ <i>Ban</i> I	pBD5A: ΔP4–P5
pΔ115–133/ <i>Ban</i> I	pBD5A: ΔP5
pΔ149–233/ <i>Ban</i> I	pBD5A: ΔP6a–P6b
pΔ155–224/ <i>Ban</i> I	pBD5A: ΔL6a–P6b
pΔ252–282/ <i>Ban</i> I	pBD5A: ΔP7.1–P7.2
pΔ258–323/ <i>Bam</i> HI	pBD5A: 3' truncation to P7.1 (nt 258)
pΔ258–323/ <i>Ban</i> I	pBD5A: ΔP8
pI75/ <i>Bam</i> HI	RNA containing 75 nt of P4–P5–P6 with sequences distal to P6a deleted

^a[Nc] *Neurospora crassa*; [nt] nucleotide.

Figure 1. Predicted secondary structure of the 388-nucleotide derivative of the *Neurospora* mt large rRNA intron. The intron is cloned in pBD5A. Uppercase letters indicate intron sequences; lowercase letters indicate exon sequences. (5'SS, 3'SS) 5'- and 3'-splice sites, respectively.

showed only weaker binding at higher protein concentrations. Figure 2B shows that specific binding of the CYT-18 protein to the transcript containing the intron was competed efficiently by the same transcript (pBD5A/*Ban*I) but not by control transcripts (pHX11/*Pvu*II or pBTC20/*Kpn*I). Based on 11 nitrocellulose filter-binding experiments, like that in Figure 2A, we calculated that the CYT-18 protein binds to the intron with a K_d of 6 ± 2 nM. The K_d value was not affected by the presence of 0.2 or 0.5 mM GTP (not shown) and is essentially the same as that for the excised intron RNA (5.5 nM corrected for the proportion of active CYT-18 protein; Mohr and Lambowitz 1991). UV-cross-linking experiments confirmed that the CYT-18 protein is in direct contact with the intron RNA in the complex (Guo 1992).

The stoichiometry of the intron-protein complex was determined by using a protein concentration near the K_d and varying the RNA concentration by two orders of magnitude above and below the K_d . As shown in Figure 2C, a double-reciprocal plot of the binding data gave a straight line with a y-intercept close to one (1.16), indicating that there is one RNA-binding site per CYT-18 protein dimer. From the slope of the line in Figure 2C, we calculated a K_d of 7.8 ± 0.7 nM, in good agreement with the K_d measured by varying the protein concentration.

Binding of the CYT-18 protein to other group I introns

Additional nitrocellulose filter-binding experiments sum-

marized in Table 2 showed that the CYT-18 protein binds strongly to other group I introns, including mitochondrial introns from *Neurospora*, *Podospira*, and yeast, as well as the *td*, *sunY*, and *nrdB* introns of bacteriophage T4. The introns that bind CYT-18 strongly belong to different subclasses and include self-splicing, as well as non-self-splicing, introns. An apparent exception is the *Tetrahymena* nuclear rRNA intron, but recent studies have shown that CYT-18 binds strongly to a derivative of this intron that lacks the large peripheral structure P5a,b,c, which presumably obscures the CYT-18-binding site ($K_d = 9$ nM; Q. Guo, L. Zhao, and A. Lambowitz, unpubl.). The CYT-18 protein did not bind strongly to group II introns, as expected because it does not function in splicing group II introns (Collins and Lambowitz 1985). The finding that the CYT-18 protein binds strongly to a number of different group I introns suggests that it interacts with conserved structural features of these introns.

Competition between the intron RNA and *Neurospora* mt tRNA^{Tyr} for the CYT-18 protein

We wished to compare the ability of CYT-18 protein to bind group I introns with its ability to bind tRNA^{Tyr}. Because sufficient quantities of *Neurospora* mt tRNA^{Tyr} could not be isolated from mitochondria, we constructed a plasmid, pTYR, which enabled us to synthesize large amounts of *Neurospora* mt tRNA^{Tyr} in vitro from the

Figure 2. Nitrocellulose filter binding of the CYT-18 protein to the *Neurospora* mt large rRNA intron. (A) Binding of CYT-18 protein to the pBD5A/*Ban*I transcript containing the 388-nucleotide derivative of the *Neurospora* mt large rRNA intron and to nonspecific RNAs [pHX11/*Pvu*II, 171-nucleotide RNA containing 5' exon sequences; pBD5A/*Nhe*I, 239-nucleotide RNA containing 65 nucleotides of 5' exon plus 174 nucleotides of intron truncated in P6b]. The percentage of ³²P-labeled RNA retained on the filter is plotted as a function of concentration of the CYT-18 protein dimer (nM). (B) Binding of the CYT-18 protein to the intron RNA (pBD5A/*Ban*I) competed by the same RNA or nonspecific RNAs [pHX11/*Pvu*II (see above) and pBTC20/*Kpn*I (869-nucleotide transcript corresponding to the antisense strand of *Neurospora* nuclear gene *cyt-20*)]. The amount of RNA bound in the presence of the competitor divided by the amount bound in the absence of the competitor (θ) is plotted as the function of the competitor RNA concentration (nM). (C) Stoichiometry of the complex between the CYT-18 protein and pBD5A/*Ban*I intron RNA. The reciprocal of the fraction of the CYT-18 protein in the complex ($1/v$) is plotted against the reciprocal of the concentration of free RNA [$1/[RNA_{free}]$, nM⁻¹]. The amount of bound RNA was corrected for the efficiency of binding (0.64), which was determined from a plot of [RNA bound] (cpm) vs. [total RNA] (cpm) in separate experiments, where an excess of CYT-18 protein (377 nM) was bound to varying amounts of ³²P-labeled RNA from 0.4 to 400 pM (Yarus and Berg 1967). The concentration of free RNA [RNA_{free}] at each point was calculated by subtracting the concentration of bound RNA corrected for efficiency from the total RNA concentration (Yarus and Berg 1967). The data in C are averages for duplicate samples.

phage T3 promoter. The in vitro transcript lacks modified nucleotides but is still recognized efficiently by the CYT-18 protein (see Materials and methods).

To investigate whether the intron and tRNA bind to similar sites in the CYT-18 protein, we tested their ability to compete for synthetase and splicing activity, respectively. Figure 3 shows that the *Neurospora* mt tRNA^{Tyr} inhibited splicing of the mt large rRNA intron. Although inhibition required a relatively high concentration of the *Neurospora* mt tRNA^{Tyr} (>25 μ M at 0.11 μ M intron RNA; see legend to Fig. 3), this tRNA was nevertheless a better inhibitor of splicing than other tRNAs tested, including *E. coli* tRNA^{Tyr}, *E. coli* tRNA^{Val}, total *E. coli* or yeast tRNA, and in vitro-transcribed *E. coli* or yeast tRNA^{Phe}, all of which inhibited only at higher concentrations. Based on the amount of the *Neurospora* mt tRNA^{Tyr} required for half-maximal inhibition and the measured K_d for the complex between the CYT-18 protein and the intron RNA, we calculated that the K_d for the binding of *Neurospora* mt tRNA^{Tyr} is

~2 μ M, in reasonable agreement with the K_m value for the tRNA in the aminoacylation reaction (7 ± 3 μ M; Cherniack 1991). This K_m has been a reasonable approximation to K_d for other synthetases (Schimmel and Söll 1979; Schimmel 1989). The finding that *Neurospora* mt tRNA^{Tyr} is a better inhibitor of splicing than is *E. coli* tRNA^{Tyr} is consistent with their relative K_m values in the aminoacylation reaction (7 and 25 μ M, respectively; see Materials and methods) and suggests that the more efficient inhibition may depend on structural features that are present in the *Neurospora* mt tRNA^{Tyr} but not in *E. coli* tRNA^{Tyr}.

In reciprocal experiments, transcripts containing the *Neurospora* mt large rRNA intron (pHX9422/*Ban*I and pBD5A/*Ban*I) strongly inhibited TyrRS activity of the CYT-18 protein, with either *E. coli* or *Neurospora* mt tRNA^{Tyr}, whereas similar amounts of control RNAs (total yeast RNA and pHX11/*Pvu*II containing 5' exon) had a relatively small effect on synthetase activity (Table 3). To determine the nature of the inhibition, TyrRS activ-

Table 2. Binding of the CYT-18 protein to different introns

Intron	Plasmid	Group	K_d (nM)
Nc. mt LSU (mini-intron)	pBD5A/ <i>BanI</i> ^a	IA1	6 ± 2
Nc. mt LSU (full length)	pHX11/ <i>BanI</i> ^a	IA1	10 ± 2
Sc. mt LSU	pBSω/ <i>SalI</i> ^b	IA1	11 ± 2
Pa. mt LSU-II	pRI1/ <i>EcoRI</i> ^c	IA1	36 ± 2
T4 <i>td</i>	pBStd/ <i>EcoRV</i> ^d	IA2	16 ± 2
T4 <i>nrdB</i>	pJSE17/ <i>HpaI</i> ^c	IA2	13 ± 2
T4 <i>sunY</i>	pMAX1/ <i>XbaI</i> ^f	IA2	12 ± 2
Nc. <i>ND1</i>	pND1/ <i>PvuII</i> ^g	IB2	16 ± 3
Tt. LSU	pT7TT1A3/ <i>EcoRI</i> ^h	IC1	>188*
Nc. <i>ND4L</i>	pND4L/ <i>EcoRI</i> ^g	IC1	58 ± 9
Nc. <i>ND3</i>	pND3/ <i>XbaI</i> ⁱ	IC2	46 ± 8
Nc. <i>ND4</i>	pND4/ <i>PvuI</i> ^g	IC2	51 ± 1
Nc. <i>cob-11</i>	pSP64-H2a/ <i>XmnI</i> ^j	ID	38 ± 8
Sc. <i>cob-11</i>	pSDH1/ <i>BamHI</i> ^k	II	>188*
Sc. <i>cox1-15γ</i>	pJD20/ <i>HindIII</i> ^l	II	>188*

The binding of the CYT-18 protein to ³²P-labeled RNAs containing different introns was measured by the nitrocellulose filter method. Group I introns are classified according to Michel and Westhof (1990). References: ^aThis paper. ^bProvided by Dr. Peter Zassenhaus (St. Louis University, MO); the transcript was synthesized with T7 RNA polymerase and consists of the 483-nucleotide 5' exon, the 1143-nucleotide intron, and the 61-nucleotide 3' exon. ^cHeinen (1991). ^dBelfort et al. (1987). ^eGott et al. (1986). ^fShub et al. (1987). ^gG. Wallweber and A. Lambowitz (unpubl.). For pND1/*PvuII*, the transcript was synthesized with T3 RNA polymerase and consists of the 539-nucleotide 5' exon, the 1118-nucleotide intron, and the 738-nucleotide 3' exon. For pND4/*PvuI*, the transcript was synthesized with T3 RNA polymerase and consists of the 708-nucleotide 5' exon, the 1449-nucleotide intron, and the 984-nucleotide 3' exon. For pND4L/*EcoRI*, the transcript was synthesized with T3 RNA polymerase and consists of the 696-nucleotide 5' exon, the 1492-nucleotide intron, and the 178-nucleotide 3' exon. ^hZaug et al. (1986). ⁱQ. Guo and A. Lambowitz (unpubl.). The transcript was synthesized with T7 RNA polymerase and consists of the 130-nucleotide 5' exon, the 1449-nucleotide intron, and the 285-nucleotide 3' exon. ^jGarriga and Lambowitz (1984). ^kDib-Hajj (1990). ^lJarrell et al. (1988). (*) Binding was not saturated at the indicated concentration of the CYT-18 protein. Data are mean ± S.D. for at least two independent experiments for each intron. (For abbreviations, see legend to Fig. 8.)

ity was assayed with different amounts of *Neurospora* mt tRNA^{Tyr} substrate and intron inhibitor. A Hanes-Woolf plot of the data gave a series of parallel lines, indicating that the intron RNA is a competitive inhibitor (Fig. 4). The calculated K_i , ~39 nM, is in reasonable agreement with the K_d value for the intron at the pH of synthetase reaction medium (K_d = 26 nM at pH 8.8) and shows again that the intron binds more strongly than the tRNA (K_m = 5.7 μM in this experiment). The finding that the inhibition is competitive provides evidence that the binding site for the intron overlaps that for the tRNA.

The CYT-18 protein binds to the group I intron catalytic core

To localize the CYT-18 protein-binding site in the intron

RNA, we tested derivatives of the 388-nucleotide mt large rRNA intron containing truncations or internal deletions for their ability to bind the CYT-18 protein by the nitrocellulose filter-binding method. Binding curves for 5' and 3' truncations are shown in Figure 5, and K_d values for the mutant introns are summarized in Figure 6.

From the 5' end, introns having truncations that remove 97 nucleotides and extend up to the second nucleotide of P3[5'] retained essentially full binding activity

Figure 3. Splicing activity of the CYT-18 protein is inhibited by the *Neurospora* mt tRNA^{Tyr}. Different competitor RNAs in amounts (μg) indicated were premixed with 0.5 μg (0.11 μM) of pHX9422/*BanI* transcript, which contains a 583-nucleotide derivative of the *Neurospora* mt large rRNA intron. The molar concentrations of the tRNA species at 15 μg ranged from 25 to 29 μM. Splicing was initiated by the addition of the CYT-18 protein purified from wild-type 74A mitochondrial RNPs by heparin-Sepharose chromatography and assayed by the addition of [α -³²P]GTP to the 5' end of the excised intron. *Neurospora crassa* [N.c.] mt tRNA^{Tyr} was transcribed from plasmid pTYR/*BstNI*, and *E. coli* tRNA^{Phe} and yeast tRNA^{Phe} were transcribed from plasmids p67CF0/*BstNI* and p67YF0/*BstNI*, respectively. *E. coli* tRNA^{Tyr} was obtained from Subriden RNA (Rolling Bay, WA). *E. coli* tRNA^{Val} and mixed *E. coli* and yeast tRNAs were obtained from Sigma. The somewhat different concentrations of *Neurospora* mt tRNA^{Tyr} required for inhibition in the top and bottom panels reflect the use of different CYT-18 protein preparations. The experiments shown in both panels were repeated at least once with essentially the same results.

Table 3. The *Neurospora* mt large rRNA intron inhibits TyrRS activity of the CYT-18 protein

Substrate	Competitor	Amount (μ g)	cpm	Activity (%)
<i>E. coli</i> tRNA ^{Tyr}	none	0	17,841	100
	Nc mt large rRNA intron: pHX9422/ <i>Ban</i> I	5	50	0.3
		10	74	0.4
		20	132	0.7
		5	0	0
	Nc mt large rRNA intron: pBD5A/ <i>Ban</i> I	10	6	0
		20	1,070	3
		5	15,712	88
		10	14,992	84
	5' exon: pHX11/ <i>Pvu</i> II	20	11,196	63
		5	25,427	142
		10	25,357	142
		20	22,759	127
Nc mt tRNA ^{Tyr}	none	0	16,561	100
	Nc mt large rRNA intron: pHX9422/ <i>Ban</i> I	5	407	2.5
		10	381	2.3
		20	401	2.4
	Nc mt large rRNA intron: pBD5A/ <i>Ban</i> I	5	479	2.9
		10	451	2.7
		20	494	3.0
	5' exon: pHX11/ <i>Pvu</i> II	5	16,566	100
		10	15,869	96
		20	13,632	82

tRNA^{Tyr} substrate (1 μ g) was premixed with competitor RNA at the concentrations indicated, and TyrRS reactions were initiated by adding CYT-18 protein preparation (28 μ g of micrococcal nuclease-digested mitochondrial RNP preparation from *N. crassa* wild-type 74A). The experiment was repeated three times using different CYT-18 protein preparations with similar results. (Nc) *Neurospora crassa*.

for the CYT-18 protein (1 and 2, L95 and L97; $K_d = 7$ and 6 nM, respectively), indicating that the 5' splice site, P1, P2, and part of P3 are not required for binding. However, truncations that extend as few as 10 nucleotides farther to the second nucleotide of P4[5'] resulted in sharply decreased binding (3 and 4, L107 and L129). This sharp decrease was confirmed by additional experiments testing the ability of the mutant introns to compete with the wild-type intron for splicing activity of the CYT-18 protein (not shown).

From the 3' end, the largest truncation that retained full binding activity was R367, which removes 22 nucleotides from the 3' end of the intron (5, $K_d = 5$ nM). More extensive 3' truncations that delete sequences between P9 and P6 resulted in a progressive increase in K_d up to 13 nM for a truncation in J6/7 (9, R243). Although this corresponds to a relatively small decrease in binding affinity, it is easily detectable in the binding assays and appears to be significant. The same effect was observed in two independent repeats of the experiment in which transcripts were assayed in parallel and was confirmed by additional experiments in which the 3' truncations were tested for ability to compete binding of the wild-type intron (pBD5A/*Ban*I; Fig. 5C) and by experiments testing the ability of mutant introns to compete with wild-type intron for splicing activity (not shown). Truncations that extend beyond J6/7 into P6 resulted in strongly decreased binding, as judged by loss of ability to

Figure 4. Competitive inhibition of TyrRS activity of the CYT-18 protein by the *Neurospora* mt large rRNA intron. Different amounts of *Neurospora* mt tRNA^{Tyr} substrate were premixed with intron RNA (pBD5A/*Ban*I) at the concentrations indicated. TyrRS reactions were initiated by the addition of the CYT-18 protein preparation (28 μ g of micrococcal nuclease-digested mitochondrial RNPs from *N. crassa* wild-type 74A). The substrate concentration $[S]$ (μ M) *Neurospora* mt tRNA^{Tyr} divided by the rate of product formation (v , μ M/min) is plotted as a function of the substrate concentration $[S]$ (μ M) (Dixon and Webb 1979). $[I]$ The concentration of the inhibitor intron RNA (pBD5A/*Ban*I). The data are averages for duplicate samples.

Figure 5. Nitrocellulose filter assay of binding of the CYT-18 protein to derivatives of the *Neurospora* mt large rRNA intron. (A,B) Binding of the CYT-18 protein to derivatives of the mt large rRNA intron having 5'- or 3'-truncations. The percentage of 32 P-labeled RNA retained on the filter is plotted as a function of concentration of the CYT-18 protein dimer (nM). (C) Binding of the *Neurospora* mt large rRNA intron competed by a series of RNAs having 3' truncations of intron sequences. 32 P-labeled RNA containing the intron (pBD5A/*Ban*I) was mixed with different concentrations of unlabeled competitor RNAs, as indicated. The CYT-18 protein was added, and nitrocellulose filter-binding assays were carried out under standard conditions. The amount of RNA bound in the presence of the competitor divided by the amount bound in the absence of the competitor (θ) is plotted as the function of the competitor RNA concentration. All experiments were repeated at least once with essentially the same results. Circled numbers identify RNAs depicted schematically in Fig. 6.

compete with the wild-type intron for splicing activity (not shown) and by nitrocellulose filter-binding assays (e.g., R175, 10 in Fig. 5B, or R239, which deletes only 1 nucleotide into P6, in Fig. 5C).

In addition to the regions identified by 5' and 3' truncation, we found that several internal regions of the intron could be removed without affecting binding (Fig. 6B). These include P6b and part of L6a (14, $K_d = 6$ nM), P7.1 and P7.2 (15, $K_d = 6$ nM) and part of P8 (16, $K_d = 7$ nM). Deletion of P5 or P6a resulted in moderate decreases in binding (12, $K_d = 43$ nM; 13, $K_d = 52$ nM), whereas deletion of P4 and P5 near the base of P4 resulted in strongly decreased binding (11; Fig. 6B).

Construction of small RNAs containing the CYT-18 protein-binding site

Together, the above results indicate that the intron contains a high-affinity binding site for the CYT-18 protein in the P4-P5-P6-P6a region, and there may be an additional contribution from the P7-P9 region. To confirm the location of the high-affinity binding site, we synthesized an RNA that contains a 75-nucleotide region of the intron encompassing P4[5'] to P6[3'], excluding se-

quences distal to L6a (RNA 1; Fig. 7). The CYT-18 protein bound this RNA with a K_d of 13 nM, in good agreement with the results expected from 5' and 3' truncations. Starting with this small RNA, we tested derivatives that add additional 3' sequences. Addition of P7[5'] had no effect on K_d (RNA 2; Fig. 7), whereas addition of the 3' end of the intron, including P7 and P9, gave a K_d of 6 nM, similar to that for the full-length intron (RNA 3; Fig. 7). Because the experiments in Figure 6 show that sequences distal to P9 have no effect on binding, we infer that the tighter binding of the latter RNA is the result of sequences in P7-P9. In control experiments, addition of similar-length sequences that did not restore P7 and P9 either had no effect on binding or resulted in an increased K_d , presumably owing to nonspecific interference with the binding of CYT-18 (not shown).

The contribution of P7-P9 could indicate that P7-P9 is required for correct folding of P4-P5-P6-P6a or that the intron contains at least two binding sites for CYT-18, one in P4-P5-P6-P6a and the other in P7-P9. Based on the measured K_d values, we calculate that binding of CYT-18 to P4-P5-P6-P6a contributes -10.7 kcal/mole to ΔG° , whereas its potential binding to P7-P9 would contribute only an additional -0.5 kcal/mole. By itself, P7-P9 is

A. 5'- and 3'- Truncations

B. Internal Deletions

Figure 6. Summary of the binding of the CYT-18 protein to derivatives of the *Neurospora* mt large rRNA intron. (A) 5' or 3' truncations; (B) internal deletions. RNAs were [1] L95, $K_d = 7$ nM; [2] L97, $K_d = 6$ nM; [3,4] L107 and L129, respectively, binding not saturated at 168 nM CYT-18 protein; [5] R367, $K_d = 5$ nM; [6] R359, $K_d = 9$ nM; [7] R342, $K_d = 11$ nM; [8] R312, $K_d = 11$ nM; [9] R243, $K_d = 13$ nM; [10] R175, binding not saturated at 168 nM CYT-18 protein; [11] Δ 108-139, binding not saturated at the 188 nM CYT-18 protein; [12] Δ 115-133, $K_d = 43$ nM; [13] Δ 149-233, $K_d = 52$ nM; [14] Δ 155-224, $K_d = 6$ nM; [15] Δ 252-282, $K_d = 6$ nM; [16] Δ 258-323, $K_d = 7$ nM. K_d values are averages from two independent nitrocellulose filter-binding experiments, except for 14, 15, and 16, which are from one experiment. The intact 388-nucleotide mt large rRNA intron (pBD5A/BamI) was run in parallel with mutant RNAs in all experiments.

Figure 7. Binding of the CYT-18 protein to small RNAs containing different regions of the *Neurospora* mt large rRNA intron catalytic core. (1) p175/*Bam*HI, RNA containing 75 nucleotides of the intron encompassing J3/4 to J6/7 with sequences distal to P6a deleted. (2) pR258/*Bam*HI, RNA containing the same 75-nucleotide intron sequences as RNA 1 plus an additional 15 nucleotide adding P7[5']. (3) pΔ258-323/*Ban*I, RNA containing the same intron sequences as RNA 2 plus an additional segment restoring P7 and P9 and extending into the 3' exon. In addition to intron sequences, all three RNAs have the vector sequence between the T3 promoter and the *Hind*III site (GGGAACAAAAGCTTGG) attached to the 5' end of the RNA, and RNAs 1 and 2 have part of a *Bam*HI linker (CCGGATC) attached to the 3' end. RNA 3 has a *Bam*HI linker sequence (CCGGATCCGG) between P7[5'] and P7[3']. K_d values for 1 and 3 are averages from two separate experiments, whereas the K_d value for 2 is from one experiment.

expected to have a relatively high K_d , above the range readily measured by nitrocellulose filter-binding assay. We attempted to measure the binding of the P7–P9 region to CYT-18 by inhibition of TyrRS activity, but in this experiment an RNA beginning in P6b (*Nhe*I site) and extending through the 3' end of the intron had a K_i of 2.4 μ M, only slightly better than inhibition by nonspecific RNAs (K_i 's = 4–12 μ M; not shown).

Comparison of group I introns that bind and are spliced by the CYT-18 protein

In general, different group I introns have minimal sequence homology (<10%), but all share conserved secondary and tertiary structures that are required for catalytic activity (Cech 1990; Michel and Westhof 1990; Heuer et al. 1991). Figure 8 is a schematic comparing six different introns (Nc LSU, Nc ND1, Sc LSU, Pa LSU1, T4 *td*, and Tt LSU with P5abc deleted) that both bind and can be spliced in vitro by the CYT-18 protein. The region containing the high-affinity binding site, P4–P5–P6–P6a, has little sequence similarity in these introns, except for

the A residues in J3/4, L4/5, and L6/6a, which are conserved in most group I introns. Outside of this region, the only extensive conserved sequence is P7, which is highly conserved in all group I introns.

Discussion

We show here that the CYT-18 protein, the *Neurospora* mt TyrRS, binds strongly to a number of different group I introns, and we localized the CYT-18 protein-binding site in the *Neurospora* mt large rRNA intron to a highly conserved region of the group I intron catalytic core. Previous work showed that the CYT-18 protein functions in splicing a number of different group I introns in *Neurospora* mitochondria (Collins and Lambowitz 1985) and could also promote splicing of mutants of the phage T4 *td* or yeast ω^+ introns that have structural defects in different regions of the catalytic core (e.g., P4, P5, P6, J6/7, P7, P8, and P9; Mohr et al. 1992). Together, these results indicate that the CYT-18 protein functions in splicing by binding to highly conserved structural features of the group I intron core and stabilizing the core in a conformation required for catalytic activity.

Figure 8. Comparison of the different introns that bind and are spliced by the CYT-18 protein. The introns compared are the *N. crassa* (Nc) mt large rRNA intron, the *Saccharomyces cerevisiae* (Sc) mt large rRNA intron [(Dujon 1980); in vitro splicing (G. Mohr, G. Wallweber, and A. Lambowitz, unpubl.)], the *Po-dospira anserina* (Pa) mt large rRNA intron 1 (Heinen 1991), the bacteriophage T4 *td* intron (Chu et al. 1984; Mohr et al. 1992), the *N. crassa* ND1 intron [(Burger and Werner 1985); in vitro splicing (G. Wallweber and A. Lambowitz, unpubl.)], and the *Tetrahymena* (Tt) large rRNA intron derivative lacking P5a,b,c [(Joyce et al. 1989); in vitro splicing (Q. Guo and A. Lambowitz, unpubl.)]. Nucleotides conserved in all introns are indicated in the structure. (R,Y) Conserved purine or pyrimidine, respectively. (N) Any nucleotide found at a position. For some introns, the published structures of the P6/P6a junction region have been modified to maximize similarity.

The findings that the *Neurospora* mt large rRNA intron is a competitive inhibitor of aminoacylation and that the *Neurospora* mt tRNA^{Tyr} inhibits splicing indicate that the intron-binding site in the CYT-18 protein overlaps the binding site for the tRNA. This conclusion is consistent with findings that regions of the CYT-18 protein required for splicing, including part of the idiosyncratic amino-terminal domain, may also be involved in binding tRNA^{Tyr} (see introductory section) and that small linker insertions in the putative carboxy-terminal tRNA-binding domain have parallel effects on splicing and synthetase activity (Kittle et al. 1991; G. Mohr, A. Chermiack, G. Guo, J. Gianelos, and A.M. Lambowitz, unpubl.).

The binding of CYT-18 protein to the intron RNA ($K_d = 6$ nM) appears to be substantially stronger than is its binding to tRNA^{Tyr} (estimated $K_d = 2\text{--}7$ μ M; see Results). A caveat here is that for experimental reasons the tRNAs used for assays were either *E. coli* tRNA^{Tyr} or an in vitro transcript of *Neurospora crassa* mt tRNA^{Tyr}, which lacks modified nucleotides and, for that reason, may not be optimally recognized by the synthetase. Nevertheless, the observed K_d in the micromolar range is similar to that for other tRNA/synthetase complexes, which generally have relatively high K_d values to facilitate rapid turnover for protein synthesis (Schimmel 1987, 1989). The tighter binding of the intron indicates either that additional interactions are involved and/or that binding of the intron requires less distortion of RNA structure than does binding of the tRNA (cf. Rould et al. 1989). Because the difference in K_d values for the intron and tRNA corresponds to a relatively small difference in ΔG° (-11.2 kcal/mole for the intron compared with -8.5 kcal/mole for the tRNA), a relatively small number of additional interactions may be involved.

Although different group I introns have only minimal sequence homology and can be divided into subclasses based on ancillary structures, all have similar secondary and tertiary structures that include a highly conserved core region required for catalytic activity (Cech 1990; Michel and Westhof 1990; Heuer et al. 1991). The CYT-18 protein binds directly to this catalytic core and presumably recognizes structural features that are conserved in different group I introns. Because most group I introns have not coevolved with the CYT-18 protein and are not normally dependent on it for splicing, we infer that these structural features are conserved for some other reason, presumably because they are required for catalytic activity.

Our results show that the CYT-18 protein binds strongly to the P4-P5-P6-P6a region of the intron core. By itself, an RNA containing 75 nucleotides from this region binds the CYT-18 protein with a K_d of 13 nM (-10.7 kcal/mole). Intron mutants lacking P5 and P6a still bind CYT-18 relatively strongly ($K_d = 43$ and 52 nM, respectively; Fig. 6B), suggesting that key features are probably in P4–P6. Comparison of six different group I introns that both bind and can be spliced in vitro by the CYT-18 protein showed relatively little sequence similarity in P4–P6, raising the possibility that the protein recognizes

conserved secondary and/or tertiary structure features of this region.

The previous finding that regions required for splicing are distributed throughout the CYT-18 protein and include both amino- and carboxy-terminal domains suggests that the protein contacts the intron at more than one site (see introductory section). In addition to P4-P5-P6-P6a, we find that the P7–P9 region is required for maximal binding of the CYT-18 protein, either because it is necessary for optimal folding of P4-P5-P6-P6a or because it contains a second binding site for the protein. The possibility that CYT-18 binds to these two regions is consistent with findings with the phage *td* intron that CYT-18 exerts its effect over a wide area of the catalytic core and can efficiently suppress mutations in P7 and P9, as well as P4 and P6 (Mohr et al. 1992). If there is a second binding site in the P7/P9 region, the higher K_d for this site could reflect either weaker interaction with the protein or the coupling of binding to an energetically unfavorable conformational change.

Figure 9A shows a three-dimensional model for the *Tetrahymena* rRNA intron catalytic core interacting with P1 and P10 (Michel and Westhof 1990). Although the *Tetrahymena* intron belongs to a different structural class than the *Neurospora* mt large rRNA intron, it nevertheless binds CYT-18 strongly ($K_d = 9$ nM; Q. Guo, L. Zhao, and A.M. Lambowitz, unpubl.) and must therefore contain all structural features essential for tight binding. According to the structural model, the catalytic core of the intron consists of two extended helices, one formed by the stacking of P6-P4-P5 and the other, P8-P3-P7, with the active site formed by a cleft between the two helices. The high-affinity binding site for CYT-18 is located on one helix, and the possible low affinity site is on the other helix. If there are binding sites on both helices, a major function of the CYT-18 protein may be to stabilize the two helices in the correct relative orientation to form the active site. This possibility is consistent with the finding that the CYT-18 protein suppresses mutations of the J6/7 region in the phage *td* or yeast ω^+ introns, which is believed to be involved in tertiary contacts that play a key role in establishing the correct orientation of the two major helices (Michel and Westhof 1990; Mohr et al. 1992). Furthermore, it may also explain why the CYT-18 protein can suppress mutations in many different regions of the intron core, all of which potentially interfere with establishing the correct orientation of the two helices.

The findings that the CYT-18 protein is a synthetase and uses the same or overlapping regions to bind both the intron and tRNA have three explanations, which are not mutually exclusive: (1) The protein may bind to different RNA motifs in the intron core and the tRNA; (2) the protein may bind to small sequences or structural motifs that are present in both the intron core and the tRNA; or (3) the protein may recognize similar overall three-dimensional structures of the intron core and tRNA.

Figure 9, B and C, compares the model for the part of the group I intron core involved in binding CYT-18 pro-

Figure 9. Comparison of the three-dimensional structures of group I introns and tRNA. (A) Three-dimensional model of the *Tetrahymena* large rRNA intron from Michel and Westhof (1990). [G1, G414] The 5' and 3' nucleotides of the intron. (B) Same as A, except that regions not required for binding the CYT-18 protein have been deleted and the segment corresponding to P6a has been closed by a loop. (C). Three-dimensional structure of yeast tRNA^{Phe} adapted from Freifelder (1987).

tein with the three-dimensional structure of yeast tRNA^{Phe}. Michel and Westhof (1990) noted that the stacked helix formed by P6 and P4 is structurally analogous to the tRNA helix formed by the stacking of the anticodon and D-arms in being stabilized by base triples with both the incoming and outgoing single-stranded regions (J3/4 and J6/7, in the case of the intron). The potential correspondence between the P4/P6 helix and the D-arm/anticodon arm helix is supported by the finding that the carboxy-terminal domain of the protein, which in bacterial TyrRSs is believed to interact with the anticodon arm of tRNA^{Tyr} (Bedouelle 1990), is required for high-affinity binding of the intron and may therefore interact with the P4/P6 region (G. Mohr, A. Cherniack, G. Guo, J. Gianelos, and A.M. Lambowitz, unpubl.).

Figure 10 compares the sequences of the *Neurospora* mt tRNA^{Tyr} and the *Neurospora* mt large rRNA intron. For the purpose of comparison, the intron is drawn unconventionally in the form of a cloverleaf, using the potential correspondence between the D-arm/anticodon arm and the P4/P6 region of the intron for orientation. The degree of sequence similarity is surprisingly high (circled nucleotides), but only a subset of the matching nucleotides correspond to those conserved in other group I introns shown to bind CYT-18 functionally (shaded nucleotides). Of the features that are conserved, the most striking is the similarity in both sequence and secondary structure between the P7 stem and the variable arm of the tRNA. In addition, P9 has a length of 7 bp, the same as that of an acceptor stem in five of the six introns to which CYT-18 binds functionally (Fig. 8), the exception being the *Tetrahymena* intron where P9 consists of 5 bp with a bulged nucleotide and there is one extra nucleotide in L9. The relatively high degree of sequence similarities between the *Neurospora* mt large rRNA and mt tRNA^{Tyr} may reflect coevolution with the CYT-18 pro-

tein and could contribute to the somewhat tighter binding of the *Neurospora* mt large rRNA intron relative to other group I introns (Table 2).

The potential correspondence between P7 and the variable arm is intriguing, because the *Neurospora* mt tRNA^{Tyr} belongs to a subclass of tRNAs (type II) characterized by long variable arms (Rich and RajBhandary 1976). The long variable arm has been implicated in the recognition of *E. coli* tRNA^{Tyr} (Himeno et al. 1990) and is an idiosyncratic feature that could interact with the idiosyncratic amino-terminal domain of the CYT-18 protein and contribute to the *Neurospora* mt tRNA^{Tyr} being a better inhibitor of splicing than is *E. coli* tRNA^{Tyr}. In the case of yeast tRNA^{Ser}, which also has a long variable arm, footprinting experiments have shown that SerRS binds to both the variable arm and the base of the anticodon stem (Dock-Bregeon et al. 1990). If the situation is similar for the *Neurospora* mt TyrRS, a key structure involved in recognition may be the cognate of the variable arm, P7, hinged off the cognate of the anticodon arm, P4/P6 (cf. Dock-Bregeon et al. 1989, 1990).

The finding that splicing activity of the *Neurospora* mt TyrRS requires an idiosyncratic amino-terminal domain not found in bacterial or yeast mt TyrRSs suggests that the *Neurospora* mt TyrRS may have adapted to function in splicing relatively recently in evolution, after the divergence of *Neurospora* and yeast (Cherniack et al. 1990; Kämper et al. 1992). However, the present results raise the possibility that the TyrRS may have been predisposed to function in splicing by virtue of its ability to recognize conserved structural features of group I introns that resemble those in tRNAs. The possible structural similarities between group I introns and tRNAs could reflect convergent evolution leading to the presence of similar RNA sequence or structural motifs. The alternate possibility is that group I introns and tRNAs are

Figure 10. Comparison of the *Neurospora* mt tRNA^{Tyr} and the *Neurospora* mt large rRNA intron core. The structure of *Neurospora* mt tRNA^{Tyr} is from Heckman et al. (1979). The intron structure is drawn as a cloverleaf to facilitate comparison to the tRNA^{Tyr}. Circled nucleotides are identical in the two structures. Shaded nucleotides are those conserved in the six group I introns that bind and are spliced by CYT-18 [see Fig. 8].

evolutionarily related. In principle, either RNA species could have evolved into the other. However, if we assume that group I introns are ancient, we can speculate that catalytically active RNAs, which were the ancestors of group I introns, also gave rise to tRNAs.

The possibility that group I RNAs evolved into tRNAs is consistent with the hypothesis that life began in an "RNA world," in which case all the machinery for protein synthesis, including tRNAs, would have had to evolve from catalytic RNAs. The possible involvement of catalytic RNAs in the evolution of protein synthesis was suggested early on (for review, see Darnell and Doolittle 1986) and has been reinforced by recent findings that the *Tetrahymena* ribozyme binds arginine (Yarus 1988) and that antibiotics that interact with the decoding region of *E. coli* 16S rRNA specifically inhibit splicing of group I introns (von Ahsen et al. 1991). Weiner and Maizels (1987) proposed that tRNAs originated as "genomic tags" at the 3' ends of self-replicating, catalytically active RNAs and that such RNAs gave rise to both tRNAs and synthetases, which functioned in their own aminoacylation by RNA catalysis. Recently, Piccirilli et al. (1992) provided evidence for the feasibility of such a reaction by demonstrating that the *Tetrahymena* ribozyme could catalyze the reverse of an aminoacylation reaction, hydrolysis of an aminoacyl-ester bond attached to an oligonucleotide ending in CCA. Although present-day group I introns do not contain a CCA terminus, the Mauriceville plasmid, an autonomous element, which may be related to group I introns, has a CCA ter-

minus and a 3'-terminal tRNA-like structure that may be a genomic tag for a reverse transcriptase encoded by the element (Kuiper and Lambowitz 1988). The objective now will be to solve the structure of the intron and its complex with the TyrRS to determine the extent of the structural similarities.

Materials and methods

Strains of *Neurospora* and growth conditions

The *N. crassa* wild-type 74-OR23-1A (designated 74A) was used in this study. Procedures for maintaining the strain, preparing conidia, and growing cells in liquid culture were as described (Davis and de Serres 1970; Lambowitz et al. 1979).

Recombinant plasmids and construction of mutant introns

Constructs containing the *Neurospora* mt large rRNA intron and its derivatives are summarized in Table 1. Plasmids pHX11, pHX9422, and pBD5A contain different forms of the *Neurospora* mt large rRNA intron cloned behind the phage T3 promoter in the pBS(+) vector (Guo et al. 1991). All three plasmids were linearized with *Ban*I for in vitro transcription. The transcript from pHX11 consists of an 844-nucleotide 5' exon, the full-length 2296-nucleotide intron, and a 50-nucleotide 3' exon. The transcripts from pHX9422 and pBD5A consist of a 65-nucleotide 5' exon, 583- or 388-nucleotide derivatives of the intron, respectively, and a 50-nucleotide 3' exon.

The mutant introns used in this study are related to pBD5A. To construct plasmids having 5' truncations of intron sequences, plasmid pBD5, which is the same as pBD5A except for

the polylinker region (Guo et al. 1991), was linearized at the *SphI* site in the P2 stem and digested with BAL31 (Slow) nuclease (International Biotechnologies, Inc., New Haven, CT) at 30°C for 10–60 min. The BAL31-digested plasmids were ligated with T4 DNA ligase in the presence of a *HindIII* linker (CCAAGCTTGG). Plasmids containing both the *HindIII* linker and appropriate inserts were isolated, and the deletion end points were sequenced (Sanger et al. 1977), by using the M13 reverse primer. *HindIII*–*EcoRI* fragments containing selected truncated introns were recloned between the *HindIII* and *EcoRI* sites of pBS(–) to give plasmids pL95, pL97, pL107, and pL129, with the number in the plasmid name indicating the last nucleotide of the truncation. These plasmids were linearized with *BanI* and transcribed with phage T3 RNA polymerase (Stratagene, La Jolla, CA.).

Plasmids pBD5910, pBD591, and pBD5908, used to obtain RNAs having 3' truncations of intron sequences, were described (Guo et al. 1991) but have been renamed pR342, pR359, and pR367 to indicate the last nucleotide of the truncation. Plasmids with 3' truncations upstream of L8 were constructed by BAL31 digestion of pBD5A linearized at the *NcoI* site at the end of P8. The BAL31-treated plasmids were ligated with T4 DNA ligase in the presence of a *BamHI* linker (CCGGATCCGG). Plasmids containing both the *BamHI* linker and inserts of appropriate size were isolated, and the deletion end points were sequenced (Sanger et al. 1977), by use of a primer complementary to the 3' exon (CCGCGCACTCATTTTGTACAC). The resulting plasmids are named pR239, pR243, pR244, and pR258. These plasmids were linearized with *BamHI* and transcribed with T3 RNA polymerase. RNAs R175 and R312 were obtained from pBD5A linearized with *NheI* or *NcoI*, respectively.

Plasmid pBD541, which has a deletion of P4 and P5, was described (Guo et al. 1991) but has been renamed pΔ108–139 to indicate the extent of the deletion. pΔ258–323 was constructed by combining pR258 and pR244 at the *BamHI* sites in the linkers. Other plasmids having internal deletions of intron sequences were constructed from pBD5A by site-directed mutagenesis (Kunkel et al. 1987), by use of the following synthetic oligonucleotides: pΔ115–133: 5'-GATAAATTTTCGCTTCAAA-TTATTTTGAATTAACCC-3'; pΔ149–233: 5'-CCTTATAGTCGTTGAACGATTATTTTCGCTTCAAATT-3'; pΔ155–224: 5'-GAACGATTTTATCTTNTAGATAAAATTTTCGCTTCAAA-TT-3'; pΔ252–282: 5'-AAAAAGAAAGTTGGGTGTTTATAGTCGTTGAACG-3'.

Plasmids having internal deletions were linearized with *BanI* and transcribed with T3 RNA polymerase.

pRL96–242 and pRL98–242 were constructed by combining 5' truncations pL95 and pL97, respectively, with 3' truncation pR243 via the *NheI* site in P6b. Starting from these plasmids, pI75 and pI77 were constructed by deleting sequences distal to P6a by site-directed mutagenesis, by using the oligonucleotide for pΔ155–224 (see above). These plasmids were linearized with *BamHI* and transcribed with T3 RNA polymerase.

Nonspecific transcripts used as controls were synthesized from pHXII/*PvuII* (*Neurospora* mt large rRNA gene, 171-nucleotide RNA containing sequences in the 5' exon) or pBTC20/*KpnI* (*Neurospora* *cyt*-20 gene, 869-nucleotide RNA corresponding to antisense strand; Kubelik et al. 1991), with T3 RNA polymerase.

In vitro synthesis of *Neurospora* mt tRNA^{Tyr} and other tRNAs

Plasmid pTYR, which was used to synthesize the *Neurospora* mt tRNA^{Tyr}, was constructed by polymerase chain reaction (PCR) cloning the *Neurospora* mt tRNA^{Tyr} gene from plasmid

pHP2, which contains the *PstI*-5a fragment of wild-type 74A mitochondrial DNA in pBR322 (Heckman and RajBhandary 1979). The primers for PCR cloning were 5'-AGCTAGCAAT-TAACCTCACTAAA[A/G]GGAGGGTCCGTTTGTGG-3' and 5'-CGGTACCTGGTAGGAGAGAAAGGAA-3'. The 5' primer contains a terminal *NheI* site for cloning and introduces a phage T3 promoter at the 5' end of the tRNA^{Tyr} gene. The 3' primer contains a *BstNI* site, which is used to linearize the plasmid for synthesis of an RNA ending in CCA, and a terminal *KpnI* site for cloning. After PCR amplification, the 121-nucleotide PCR product was digested with *NheI* and *KpnI* and inserted into the *XbaI*–*KpnI* site of pUC19.

To synthesize the *Neurospora* mt tRNA^{Tyr}, pTYR was linearized with *BstNI* and transcribed with phage T3 RNA polymerase. Following transcription, the DNA template was digested with DNase I (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ; 5 units for 5 min at 37°C) and subjected to two cycles of extraction with phenol–CIA (phenol/chloroform/isoamyl alcohol, 25 : 24 : 1) and ethanol precipitation. The RNA pellet was then dissolved in 300 μl of 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA, and ~1 mg of dissolved RNA was loaded on a DEAE–Sephacel column (0.7 × 4-cm column; 0.6-ml bed volume), which had been equilibrated with 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), and 1 mM EDTA (room temperature). The column was washed with 250 mM NaCl, 10 mM Tris-HCl (pH 7.5), and 1 mM EDTA, and the tRNA was eluted with 800 mM NaCl, 10 mM Tris-HCl (pH 7.5), and 1 mM EDTA. The eluted tRNA was precipitated with ethanol and redissolved in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA.

The *in vitro* tRNA^{Tyr} transcript is recognized efficiently by CYT-18 protein. In parallel experiments with the same CYT-18 protein preparation, the K_m value for the *in vitro*-synthesized *Neurospora* mt tRNA^{Tyr} was 7 ± 3 μM compared with 25 ± 6 μM for *E. coli* tRNA^{Tyr}, and the V_{max} was about half that for the *E. coli* tRNA^{Tyr} (Cherniack et al. 1990; Cherniack 1991; Kittle et al. 1991).

E. coli tRNA^{Phe} and yeast tRNA^{Phe} were transcribed from plasmids p67CF0 and p67YF0 (provided by Dr. Olke Uhlenbeck, University of Colorado, Boulder). The plasmids were digested with *BstNI* and transcribed with phage T7 RNA polymerase (Sampson and Uhlenbeck 1988). The resulting tRNA transcripts were purified as described above for the *Neurospora* mt tRNA^{Tyr} transcript.

Isolation of mitochondrial RNA and mt RNP particles

Mitochondria were purified by the flotation gradient method (Lambowitz 1979). Mitochondrial RNA was extracted by the SDS–urea plus phenol–CIA procedure (Garriga et al. 1984). RNP particles were isolated by lysing mitochondria with 1% Nonidet P-40, followed by centrifugation of the lysate through a 1.85 M sucrose cushion containing 0.5 M KCl buffer (Akina and Lambowitz 1987).

Preparation of CYT-18 protein from *Neurospora* and *E. coli*

CYT-18 protein was obtained from mitochondrial RNPs of *Neurospora* wild-type 74A by heparin–Sephacel chromatography (Majumder et al. 1989) or synthesized in *E. coli* from the expression plasmid pEX560 (Kittle et al. 1991). The *E. coli*-synthesized CYT-18 protein used for binding assays was purified extensively by using a recently developed procedure involving polyethyleneimine precipitation of nucleic acids, ammonium sulfate fractionation, and chromatography in a CM–Sephacel column (R. Saldanha and A.M. Lambowitz, unpubl.). The final protein fractions were ~95% pure, with the major impurities being slightly

smaller proteins that cross-reacted with anti-CYT-18 antibodies.

Synthesis of in vitro transcripts

In vitro transcripts were synthesized from recombinant plasmids linearized with restriction enzymes, by use of phage T3 or T7 RNA polymerase (Stratagene), essentially as described (Yisraeli and Melton 1989). Following transcription, the DNA template was digested with DNase I (Pharmacia; 5 units for 5 min at 37°C), and the transcript was phenol/CIA-extracted and ethanol-precipitated twice. Standard transcription reactions were carried out in a volume of 200 μ l with 0.5 mM rNTPs for 60 min at 37°C. To synthesize large amounts of RNAs for competition experiments, transcription was carried out in a volume of 400 μ l with 2 mM rNTPs. To synthesize high-specific-activity 32 P-labeled transcripts for nitrocellulose filter-binding assays, transcription was carried out in a volume of 50 μ l with 0.4 mM ATP, GTP, and CTP, 0.04 mM UTP, and 150 μ Ci of [α - 32 P]UTP (3000 Ci/mmol; NEN-DuPont, Boston, MA). To synthesize 32 P-labeled transcripts for splicing assays, transcription was carried out in a volume of 50 μ l with 0.4 mM ATP, GTP, CTP, and UTP plus 150 μ Ci [α - 32 P]UTP (3,000 Ci/mmol; NEN-DuPont).

Nitrocellulose filter-binding assay

Approximately 5 pM 32 P-labeled RNA (18 μ Ci/pmol) was mixed with different amounts of CYT-18 protein in 500 μ l of TMK buffer [10 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 100 mM KCl] containing 50 μ g acetylated bovine serum albumin (New England Biolabs, Inc., Beverly, MA) plus nonspecific competitors 40 μ g of poly(rC) (Pharmacia) and 10 μ g of yeast total RNA (Sigma Chemical Co., St. Louis, MO). The samples were incubated for 2 min at 37°C, followed by 15 min at room temperature and placed on ice to await filtration. Control experiments with pBD5A/*Ban*I showed that K_d values at 0°C and 25°C are essentially the same (6–7 nM) and that the K_d at 37°C is 12 nM (not shown). The values indicated in the text correspond to the K_d at 25°C. Samples were filtered under gentle vacuum through a nitrocellulose filter (HAWP02500; Millipore Co., Bedford, MA), which had been prewetted with the TMK buffer plus 40 μ g/ml of denatured salmon sperm DNA (Sigma). After filtration, the filters were washed three times with 0.9 ml of TMK buffer plus 40 μ g/ml of denatured salmon sperm DNA and dried at 80°C. Radioactivity retained on the filter was measured by liquid scintillation counting, by use of Ready Protein Liquid Scintillation Cocktail (Beckman Instruments, Inc., Fullerton, CA). Binding data were analyzed as described by Yarus and Berg (1967), with the proportion of active CYT-18 protein (~60%) determined by the method of Riggs et al. (1970). The binding of the CYT-18 protein to the intron-containing transcript was saturated after 1 min at 37°C and was stable for at least 60 min at 0°C or 25°C.

Assay of tyrosyl-tRNA synthetase activity

TyrRS activity of the CYT-18 protein was assayed essentially as described [Akina and Lambowitz 1987]. For experiments involving large numbers of samples, TyrRS assays were carried out with CYT-18 protein in micrococcal nuclease-digested mitochondrial RNP preparations from wild-type 74A [Akina and Lambowitz 1987]. The use of these preparations circumvents problems owing to instability of TyrRS activity associated with purified mitochondrial CYT-18 protein [Majumder et al. 1989]. TyrRS assays were carried out in 20 μ l of reaction medium containing 50 mM Tris-HCl (pH 8.8), 15 mM MgCl₂, 5 mM ATP,

1 μ Ci of L-[3,5- 3 H]-tyrosine (50 Ci/mmol; Amersham Co., Arlington Heights, IL) and 1 μ g of tRNA substrate (1.63 μ M for *Neurospora* mt tRNA^{Tyr}; 1.73 μ M for *E. coli* tRNA^{Tyr}). Reactions were initiated by the addition of enzyme preparation, incubated at 30°C for 10 min, and terminated by the addition of 0.5 ml of 10% TCA–0.1% SDS. TCA-precipitable radioactivity was collected on Whatman GF/C filters and counted as described previously [Akina and Lambowitz 1987].

Splicing assays

Splicing reactions with unlabeled precursor RNA were carried out as described [Majumder et al. 1989] in 20 μ l of reaction medium containing 0.5 μ g of in vitro transcript substrate, 4 μ l of CYT-18 protein preparation (0.8 μ g of CYT-18 protein preparation from *Neurospora* 74A RNPs or 6–30 nM purified *E. coli*-synthesized CYT-18 protein), 100 mM KCl, 5 mM MgCl₂, 20 mM Tris-HCl (pH 7.5), 5 mM DTT, and 40 μ Ci of [α - 32 P]GTP (3000 Ci/mmol; NEN-DuPont). Reactions were initiated by the addition of the CYT-18 protein, incubated at 37°C for 10–30 min, and stopped by the addition of 180 μ l of 10 mM Tris-HCl (pH 7.5), 5 mM EDTA, 10 μ g of total *E. coli* tRNA (Sigma), and 200 μ l of phenol–CIA. Splicing assays with 32 P-labeled in vitro transcripts (~20,000 cpm; 2 ng) were the same, except that the GTP concentration was 250 μ M and reactions were incubated for 7 min. After phenol–CIA extraction and ethanol precipitation, samples were analyzed by electrophoresis in a 5% polyacrylamide gel (20 : 1 acrylamide/bisacrylamide) containing 8 mM urea, 90 mM Tris, 90 mM boric acid, and 2.5 mM EDTA.

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