The *Neurospora* mitochondrial tyrosyl-tRNA synthetase is sufficient for group I intron splicing in vitro and uses the carboxy-terminal tRNA-binding domain along with other regions

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Neurospora mitochondrial tyrosyl-tRNA synthetase (mt tyrRS), which is encoded by nuclear gene *cyt-18*, functions in splicing of group I introns in mitochondria. Here, we overproduced functional *cyt-18* protein in *Escherichia coli* and purified it to near homogeneity. The purified protein has splicing and tyrRS activities similar to those of *cyt-18* protein isolated from mitochondria and is by itself sufficient to splice the mitochondrial large rRNA intron in vitro. Structure-function relationships in the *cyt-18* protein were analyzed by in vitro mutagenesis. We confirmed that a small amino-terminal domain not found in bacterial tyrRSs is required for splicing activity, but not tyrRS activity. Two linker insertion mutations, which disrupt the predicted ATP-binding site, completely inhibit tyrRS activity but leave substantial splicing activity. Finally, deletions or linker insertion mutations in the putative carboxy-terminal tRNA-binding domain inhibit both tyrRS and splicing activities, although some have differential effects on the two activities. Our results show that the normal catalytic activity of the *cyt-18* protein is not required for splicing and are consistent with the hypothesis that the protein functions by binding to the precursor RNA and facilitating formation of the correct RNA structure. Regions required for splicing are distributed throughout the *cyt-18* protein and overlap, but are not identical to, regions required for tyrRS activity. The finding that the putative carboxy-terminal tRNA-binding domain is required for both tyrRS and splicing activities suggests that the mechanism for binding the intron has similarities to the mechanism for binding tRNA^{Tyr}.

[Key Words: RNA splicing; group I intron; RNA catalysis; tyrosyl-tRNA synthetase; aminoacyl-tRNA synthetase; mitochondrial RNA processing]

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The *Neurospora* mitochondrial tyrosyl-tRNA synthetase (mt tyrRS), which is encoded by nuclear gene *cyt-18*, functions in splicing the large rRNA intron and other group I introns in *Neurospora* mitochondria (Akins and Lambowitz 1987; Majumder et al. 1989). As discussed elsewhere, the most likely hypothesis is that protein-assisted splicing of group I introns remains RNA catalyzed and proteins function primarily to facilitate correct folding of the intron RNA (Garriga and Lambowitz 1984; Lambowitz and Perlman 1990). Because the *cyt-18* protein is an aminoacyl-tRNA synthetase, we suggested that it might bind to the intron RNA via recognition of a sequence or structure that resembles the aminoacylation substrate tRNA^{Tyr} (Akins and Lambowitz 1987).

To investigate in more detail the involvement of tyrRS in splicing, we carried out biochemical analysis of the tyrRS protein isolated from mitochondria (Majumder et al. 1989). Using antibodies against *trpE*-*cyt-18* fusion proteins, we identified the *cyt-18* gene product as a basic protein having a relative molecular mass of ~70 kD. We then showed that this 70-kD protein copurified with both tyrRS activity and with a soluble activity that splices the mitochondrial large rRNA intron in vitro. The *cyt-18* protein was a major component of relatively pure fractions of splicing activity obtained by different chromatographic procedures, and these fractions had few, if any, other prominent polypeptide bands in common. Gel-filtration experiments also showed that splicing activity is relatively small (150 kD, with activity trailing to low molecular weights), raising the possibility that *cyt-18* protein dimers or monomers are sufficient to splice the mitochondrial large rRNA intron in vitro.

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Previously, we characterized two temperature-sensitive mutants in the *cyt-18* gene, *cyt-18-1* and *cyt-18-2* (Mannella et al. 1979). When grown at high temperature (37°C), these mutants are defective in splicing the mitochondrial large rRNA intron, as well as additional group I introns in mitochondrial mRNAs, and are also deficient in mt tyrRS activity (Mannella et al. 1979; Collins and Lambowitz 1985; Akins and Lambowitz 1987). Both *cyt-18* mutants have the same missense mutation leading to a single amino acid change in the *cyt-18* protein (Gly127 to Glu), which is responsible for both the splicing and aminoacylation defects (Cherniack et al. 1990). The mutant *cyt-18* protein was shown to be present at normal levels in mitochondria of cells grown at 37°C but is inactive in both splicing and aminoacylation *in vivo*. We also isolated intragenic partial revertants of the *cyt-18-2* mutant that specifically alleviate the splicing defect, but not the defect in aminoacylation (Cherniack et al. 1990). These partial revertants had secondary amino acid changes clustered at the amino terminus of the *cyt-18* protein (amino acids 50–81) in a region of ~60 amino acids that has no homolog in the *Escherichia coli* or yeast mt tyrRSs and may be a recent addition to the *cyt-18* protein. A single partial revertant had secondary changes in amino acids 189 and 190, which are located in the nucleotide-binding-fold region downstream of the *cyt-18-2* mutation.

In this study, we overproduced functional *cyt-18* protein in *E. coli* and used the *E. coli* expression system to analyze structure–function relationships in the *cyt-18* protein by *in vitro* mutagenesis. Our results show that the *cyt-18* protein is both necessary and sufficient to splice the mitochondrial large rRNA intron *in vitro*, and they are consistent with the hypothesis that the *cyt-18* protein functions by binding to the precursor RNA and facilitating formation of the correct RNA structure. Regions of the protein required for splicing include a small idiosyncratic domain at the amino terminus and the putative tRNA binding domain toward the carboxyl terminus, which may also function in binding the intron RNA.

Results

Expression of functional *cyt-18* protein in *E. coli*

Figure 1 (top) shows a map of the *Neurospora cyt-18* gene (Akins and Lambowitz 1987; Cherniack et al. 1990). The open reading frame (ORF) encoding the mt tyrRS has a length of 669 amino acids and is interrupted by a small intron near the amino terminus. The amino terminus of the ORF has the characteristics of a leader sequence required for targeting the protein to mitochondria. Amino-terminal sequencing of the mature *cyt-18* protein isolated from mitochondria showed that the amino terminus corresponds to Leu33 of the ORF. This indicates that the previously inferred initiation codon is correct (Akins and Lambowitz 1987) and that the leader is cleaved after the sequence ArgGly32, which fits the consensus sequence, Arg small amino acid, for a protein processing site in *Neurospora* mitochondria (von Heijne et al. 1989).

Figure 1. Map of *cyt-18* gene and plasmid constructs for expression of *cyt-18* protein in *E. coli*. The *cyt-18* gene (top) contains a single 64-bp intron (solid box) and encodes an amino-terminal presequence of 32 amino acids (striped box). The amino terminus of the processed intramitochondrial *cyt-18* protein corresponds to Leu33 of the *cyt-18* ORF. pEX550 expresses the *cyt-18* ORF beginning at Leu33 plus an additional amino-terminal methionine residue. pEX554 is the same as pEX550 but includes the amino-terminal presequence.

The *cyt-18* protein was expressed in *E. coli*, using a T7 expression plasmid pEX501, which was derived from pET3a (see Materials and methods). The primary construct, pEX550, consists of the *cyt-18* ORF, from which the intron was removed by site-directed mutagenesis and which has the amino terminus of the mature protein (Leu33) plus an additional amino-terminal Met residue. Other constructs tested in initial experiments were pEX554, which expresses the pre-*cyt-18* protein containing the amino-terminal mitochondrial targeting sequence, and pEX511, which has the mature amino terminus (Met-Leu33) but substitutes 18 amino acids encoded by vector sequence for the three carboxy-terminal amino acids. pEX553 has an in-frame deletion of 72 amino acids between the two *Xho*I sites in Figure 1 and was used as a negative control.

Figure 2A shows that *E. coli* containing the pEX550 plasmid synthesized the correct-sized protein (~70 kD compared with the predicted size of 72 kD), which was readily detected in the Coomassie blue-stained gels. The pEX550 protein was recognized by anti-*cyt-18* antibodies in immunoblots and comigrated with authentic *cyt-18* protein from mitochondrial ribonucleoprotein (mtRNP)

Figure 2. Expression of *cyt-18* proteins in *E. coli* and analysis of splicing and tyrRS activities. (A) Coomassie blue-stained SDS-polyacrylamide gel of proteins in lysates of *E. coli* strains expressing different forms of the *cyt-18* protein. Numbers at left show positions of molecular weight markers. (B) Immunoblot of SDS-polyacrylamide gel probed with anti-*cyt-18* antibody preparation C18-2. (C) Splicing activity assayed in lysates of *E. coli* strains containing expression plasmids. Lysates for pEX550, pEX554, and pEX553 are the same as those shown in A. Splicing was assayed by the [^{32}P]GTP addition method using in vitro transcript from pHX9422, as described in Materials and methods. Some *E. coli* lysates, including those of cells containing the vector pEX501, contain a putative end-labeling activity, which results in the appearance of a labeled band at the position of in vitro transcript precursor RNA (P). (D) TyrRS activity assayed in lysates of *E. coli* strain AL565 [DE3], which has a temperature-sensitive defect in tyrRS leading to grossly decreased levels of endogenous tyrRS activity assayed at 37°C. For the experiment shown, tyrRS activities were normalized for the amount of *cyt-18* protein estimated from SDS-polyacrylamide gels stained with Coomassie blue. The following samples are shown: (RNPs) Whole mt RNPs from *Neurospora* strain *cyt-4-1*; (mt *cyt-18*), *cyt-18* protein partially purified from mt RNPs by heparin-Sepharose chromatography; (pEX550) cognate of mature intramitochondrial form of *cyt-18* protein; (pEX554) *cyt-18* protein containing amino-terminal presequence; (pEX553), *cyt-18* protein containing an in-frame deletion of 72 amino acids between XhoI sites shown in Fig. 1; (pEX501) expression vector with no *cyt-18* insert; (pEX511) same as pEX550, except that the carboxy-terminal 18 amino acids are different from those of the authentic *cyt-18* protein.

preparations (Fig. 2B). Amino-terminal sequencing of the pEX550 protein confirmed that it has the expected amino-terminal sequence. After induction for 1 hr, the expressed pEX550 protein constituted from 5% to 15% of total *E. coli* protein and >50% of the protein was present in the soluble fraction, as determined by densitometry of Coomassie blue-stained SDS-polyacrylamide

gels. The other constructs also gave proteins of the expected size. In several experiments, pEX511, which has the altered carboxy-terminal sequence, gave about two-fold more soluble protein than pEX550 (not shown).

The immunoblots (Fig. 2B) show that strains expressing the *cyt-18* protein also contained smaller immunoreactive material, which may be due to proteolysis or incorrect translation. Immunoblots for the same *E. coli* strain containing the vector pEX501 and induced with isopropyl- β -D-thiogalactopyranoside (IPTG) showed no bands at the position of the *cyt-18* proteins nor the smaller immunoreactive material, but did show a band of M_r 60 kD, which corresponds to an abundant *E. coli* protein that cross-reacts with the secondary antibody used to develop the immunoblots (Fig. 2B).

In initial experiments, the splicing activity of the expressed *cyt-18* proteins was assayed directly in *E. coli* lysates, using an in vitro transcript that contains a 583-nucleotide derivative of the mitochondrial large rRNA intron and flanking exons. Incubation of this transcript with preparations containing splicing activity and [^{32}P]GTP results in production of [^{32}P]GTP-labeled bands of 584 nucleotides, corresponding to the excised intron, and 634 nucleotides, corresponding to the intron-3' exon intermediate resulting from GTP-dependent cleavage at the 5' splice site. Figure 2C shows that *E. coli* lysates expressing the pEX550 protein did have splicing activity equivalent to that in mitochondrial RNPs, as did lysates containing the pEX554 protein, which has the amino-terminal leader, and the pEX511 protein, which has the altered carboxyl terminus. In contrast, no splicing activity was detected in lysates of strains containing the vector pEX501 or the in-frame deletion pEX553. Some *E. coli* lysates, including those from cells transformed with the vector pEX501, had a putative end-labeling activity that resulted in ^{32}P -labeling of the in vitro transcript precursor (Fig. 2C). When present, most of this activity could be separated from the *cyt-18* protein by chromatography in DEAE-Sephacel columns (not shown), but residual activity was sometimes found in purified preparations of *cyt-18* protein and may be associated with the protein or the intron RNA.

To assay tyrRS activity of the expressed *cyt-18* proteins directly in lysates, we used *E. coli* K12 mutant 565cN. This mutant has a temperature-sensitive defect in its tyrRS so that endogenous tyrRS activity in crude extracts assayed at 37°C is decreased at least 50-fold compared with wild-type *E. coli* strains. The strain was lysogenized with bacteriophage DE3 to support T7 RNA polymerase-driven overexpression of *cyt-18* proteins from the constructs described above. As shown in Figure 2D, both the pEX550 and pEX554 proteins have tyrRS activity, which was readily detected in the mutant strain assayed at 37°C, whereas at least 10-fold less activity was detected in lysates of cells containing the vector pEX501 or the pEX553 protein with the internal deletion.

Purification of *cyt-18* protein from *E. coli*

The pEX550 protein synthesized in *E. coli* showed essentially the same chromatographic behavior in DEAE-

Sephacel and heparin-Sepharose columns as *cyt-18* protein isolated from mitochondria (see Majumder et al. 1989). On the basis of initial results, we devised a relatively simple procedure for obtaining substantially pure preparations of the *cyt-18* protein from *E. coli*. Lysates were first chromatographed on a medium-scale DEAE-Sepacel column, where the majority of active *cyt-18* protein eluted just ahead of the 0.25 M KCl peak containing the bulk of *E. coli* tyrRS and other proteins loaded on the column. DEAE-Sepacel fractions identified as containing *cyt-18* protein by dot blots were pooled, desalted by gel filtration through Sephadex G-75, and chromatographed on a heparin-Sepharose column to remove residual *E. coli* tyrRS activity.

Figure 3 shows results for two different purifications, one for the pEX550 protein and the other for the pEX511 protein, which has a substitution for the three carboxy-terminal amino acids. In the experiment shown for pEX550, the DEAE-Sepacel column provided the bulk of the purification, whereas in the experiment for pEX511, the pooled DEAE-Sepacel fractions contained additional proteins, which were removed by heparin-Sepharose chromatography. In both experiments, the final protein fractions were ~95% pure, with the major contaminants being two slightly smaller polypeptides that cross-react with anti-*cyt-18* antibody. For several different experiments, specific activity measurements

showed that the pEX550 protein was purified ~20-fold with a yield of up to 3 mg. The purified protein was highly active in charging tRNA^{Tyr} and in splicing the mitochondrial large rRNA intron. The specific activity of tyrRS varied from ~100–250 U/mg of protein, where a unit is 1 nmole of *E. coli* tRNA^{Tyr} aminoacylated per minute.

Characterization of splicing and tyrRS activity of *cyt-18* protein synthesized in *E. coli*

The splicing and tyrRS activities of the pEX550 protein synthesized in *E. coli* are similar in all respects to those of authentic *cyt-18* protein isolated from mt RNPs. In the aminoacylation reaction, the *E. coli*-synthesized and mitochondrial *cyt-18* proteins have K_m values for *E. coli* tRNA^{Tyr} of 17 ± 4 and 25 ± 6 μ M and K_m values for ATP of 0.34 ± 0.04 and 0.35 ± 0.05 mM, respectively. The *Neurospora* mt tyrRS can be distinguished from *E. coli* tyrRS by its sensitivity to GTP, which competitively inhibits the *Neurospora* enzyme but has no effect on the *E. coli* enzyme (J.D. Kittle Jr. and A.M. Lambowitz, unpubl.). We confirmed that the tyrRS activity of the pEX550 protein was inhibited by GTP, whereas *E. coli* tyrRS from the same heparin-Sepharose column (0.25 M KCl fractions) was not inhibited.

Splicing reactions with the pEX550 protein had temperature profiles and Mg²⁺ and KCl concentration dependencies similar to those of authentic *cyt-18* protein (see Garriga and Lambowitz 1986), except that splicing activity of the pEX550 protein appeared to fall off somewhat more rapidly at high KCl concentrations (>200 mM). Splicing reactions carried out with *E. coli*-synthesized or mitochondrial *cyt-18* proteins have K_m values for guanosine of 14 ± 4 and 17 ± 4 μ M, respectively, and K_m values for GTP of 91 ± 10 and 107 ± 3 μ M, respectively. The specific activity of the *E. coli*-synthesized pEX550 protein was similar to that of the *cyt-18* protein isolated from mitochondria (not shown).

To confirm that the expressed protein carried out exon ligation, we used ³²P-labeled in vitro transcripts that differ in length of the 3' exon, so that the ligated exon product could be readily distinguished by gel electrophoresis. As shown in Figure 4, both the mitochondrial *cyt-18* protein (lanes 1) and the *E. coli* synthesized *cyt-18* protein (lanes 2) gave the same splicing products having sizes predicted for excised intron and ligated exons. The control (lanes 3) shows that these bands were not detected when ³²P-labeled precursor RNA was incubated under the same conditions in the absence of protein. Both the *E. coli*-synthesized and mitochondrial *cyt-18* proteins converted >50% of the precursor to spliced product in this experiment.

Analysis of the *cyt-18* protein by in vitro mutagenesis

To delineate regions of the *E. coli* protein required for splicing and tyrRS activity, we carried out in vitro mutagenesis, guided by the structural similarity between the *cyt-18* protein and the cognate *E. coli* and *Bacillus*

Figure 3. Purification of *E. coli*-synthesized *cyt-18* proteins. pEX550 and pEX511 proteins were synthesized in *E. coli* and subjected to medium-scale purification through DEAE-Sepacel, Sephadex, and heparin-Sepharose columns, as described in Materials and methods. Fractions at various stages of purification were analyzed by electrophoresis in 10% SDS-polyacrylamide gels. Shown are gels stained with Coomassie blue. The identity of the *cyt-18* protein bands was confirmed by the immunoblotting of parallel lanes (not shown). Proteins from a mt RNP preparation are shown for comparison.

Figure 4. Splicing of ^{32}P -labeled in vitro transcript by *cyt-18* protein synthesized in *E. coli* or isolated from mitochondria. *cyt-18* protein was synthesized in *E. coli* from plasmid pEX511, and splicing activity was purified as in Fig. 3. *Neurospora cyt-18* protein was isolated from mitochondrial RNP preparations by heparin–Sephadex chromatography. Splicing assays were carried out using ^{32}P -labeled in vitro transcripts from pHX9422 cut with *Ban*I or *Xba*I to vary the length of the 3' exon. To detect small bands corresponding to ligated exons, splicing products were analyzed by electrophoresis in 6% polyacrylamide/8 M urea gels. The intron and spliced exon products are indicated for each transcript length. (Lanes 1) *cyt-18* protein purified from mt RNPs; (lanes 2) pEX511 protein; (lanes 3) no protein added.

steatothermophilus tyrRSs, which are among the best-studied aminoacyl-tRNA synthetases (Schimmel 1987). As shown in Figure 5 (bottom), the *Neurospora* protein has an amino-terminal mitochondrial targeting sequence and additional domains at the amino and carboxyl termini, which are not present in the bacterial synthetases. The regions of strongest homology to the bacterial synthetases are in the amino-terminal half of the *cyt-18* protein and correspond to sequences in the nucleotide binding fold, including the ATP and tyrosine binding sites. In the bacterial tyrRSs, the carboxy-terminal domain downstream of the nucleotide binding fold contains amino acids involved in tRNA recognition (Waye et al. 1983;

Bedoulle and Winter 1986; Schimmel 1987). This region of the *cyt-18* protein, downstream of Gly402, is relatively long compared with that of bacterial tyrRSs and has little sequence similarity to the other tyrRSs, except for a stretch of 14 amino acids centered around Arg540, which is conserved at the carboxyl termini of the bacterial tyrRSs and the yeast mt tyrRS (Fig. 5).

Figure 5 (top) shows the structure of the amino-terminal 318 amino acids of *B. steatothermophilus* tyrRS determined by x-ray crystallography (Brick et al. 1989). Sequences that are highly conserved between the *Neurospora* mt tyrRS and the *B. steatothermophilus* tyrRS are spatially arranged near the tyrosyl-adenylation site, for example, the HVGH and QIGG sequences shown in Figure 5. More loosely conserved sequences can be identified in a patch extending across the dimer interface (small diagonal rod at the left in Fig. 5, top). The carboxy-terminal region of the *B. steatothermophilus* protein (~100 amino acids) was disordered in protein crystals, preventing determination of its structure.

In this study, mutant derivatives of the *cyt-18* protein were synthesized in *E. coli* and partially purified by small-scale heparin–Sephadex chromatography, which is sufficient to remove the endogenous bacterial tyrRS (see Materials and methods). Heparin–Sephadex column 0.5 M KCl fractions containing *cyt-18* protein were assayed for splicing and tyrRS activity, and *cyt-18* protein in the fractions was detected and quantitated by SDS-PAGE and immunoblotting. Representative data for mutant proteins are shown in Figure 6, and the data are summarized in Figure 7. Standardization of the assays and methods used for quantitation of splicing and tyrRS activities are described in the legend to Figure 6. We confirmed that fractions do not contain detectable *E. coli* tyrRS by immunoblotting with anti-*E. coli* tyrRS antibody (not shown).

The data show that both the pEX550 protein, which lacks the amino-terminal mitochondrial targeting sequence, and the pEX554 protein, which retains this sequence, have similar levels of splicing and tyrRS activity. The pEX550 protein begins with an ATG immediately upstream of Leu33 of the *cyt-18* ORF, which corresponds to the amino terminus of mature *cyt-18* protein in mitochondria. Immediately following Leu33 is the idiosyncratic domain, which extends from amino acids 34 to 95 and is the site of secondary mutations that restore splicing in partial revertants of the *cyt-18-2* mutant. pEX521 gives a mutant *cyt-18* protein with a deletion of amino acids 38–80 from this domain. As reported previously, this deletion abolished splicing activity of the *cyt-18* protein but had relatively little effect on tyrRS activity (Cherniack et al. 1990). Further characterization here showed that the pEX521 protein and the wild-type pEX550 protein have similar K_m values for ATP (0.30 ± 0.09 and 0.34 ± 0.04 mM, respectively) and *E. coli* tRNA^{Tyr} (21 ± 4 and 17 ± 4 μM , respectively), indicating that the deletion does not substantially affect the binding of ATP or *E. coli* tRNA^{Tyr} by other regions of the *cyt-18* protein. In addition to the pEX521 deletion, we analyzed two linker insertion mutations in this region

Figure 5. X-ray crystal structure of the *B. stearo-*
thermophilus tyrRS and regions of homology be-
tween the *N. crassa* mt tyrRS and other tyrRSs.
(*Top*) The structure of the amino-terminal 318
amino acids of the *B. stearo-*
thermophilus tyrRS protein determined by Brick et al. (1989). Numbers
in the structure indicate amino acid positions in
the *B. stearo-*
thermophilus tyrRS. The protein
dimer interface is adjacent to the thin diagonal rod
at the left. Numbers highlighted outside of the
structure refer to mutant *cyt-18* proteins and are
shown close to the inferred position of the muta-
tions, on the basis of amino acid sequence align-
ment with the *B. stearo-*
thermophilus tyrRS. (*Bot-*
tom) Comparison of sequences of the *N. crassa* mt
tyrRS (*N.c.*; *cyt-18* protein), *Saccharomyces cere-*
visiae mt tyrRS (*S.c.*; G. Hill and A. Tzagoloff, un-
publ.), and *B. stearo-*
thermophilus tyrRS (*B.s.*; Win-
ter et al. 1983). The mt tyrRS proteins from *S. cere-*
visiae and *N. crassa* begin with an amino-terminal
mitochondrial targeting sequence (striped box).
Highly conserved regions are shown in black.
Short conserved sequences within highly con-
served regions are identified and numbered below.
The conserved residue Gly402 marks the end of
the last highly conserved region of the *cyt-18* pro-
tein that has a counterpart in the crystal structure.
This residue corresponds to *B. stearo-*
thermophilus Gly308.

that introduce four extra amino acids after codons 37 and 81 (pEX515 and pEX523). Neither insertion has any detectable effect on splicing activity. The pEX515 insertion retained wild-type levels of tyrRS activity, whereas the pEX523 insertion retained $\geq 50\%$ wild-type tyrRS activity. These two linker insertions are located near either end of the idiosyncratic amino-terminal domain and may demarcate the region required for splicing.

Four mutations (pEX551, pEX558, pEX571, and pEX573) were made in different regions of the nucleotide-binding fold. Assuming that the three-dimensional structure of this conserved region is similar to that of *B. stearo-*
thermophilus tyrRS, the pEX551 and pEX558 proteins would have four extra amino acids inserted in the adenylate-binding cleft in a position that should interfere directly with binding of tyrosyl adenylate (see Fig. 5). As expected from this model, both linker insertions completely inhibited tyrRS activity. However, both retained splicing activity, with the pEX558 protein about as active as wild-type and the pEX551 protein $\sim 25\%$ as active (corrected for amount of *cyt-18* protein; see legend to Fig. 6). These findings suggest that the functional integrity of the ATP-binding site is not required for splicing activity. In contrast, the pEX571 and pEX573 proteins, which have mutations in two other regions of the

nucleotide-binding fold, retained essentially wild-type levels of splicing and tyrRS activity. The pEX573 mutation is a 4-amino-acid insertion after Leu281, whereas the pEX571 mutation is a 3-nucleotide deletion, which replaces 2 amino acids, Asp189 and Trp190, with a single Gly residue. We were somewhat surprised to find this latter replacement had no effect on splicing activity, because the 2 amino acids affected in pEX571 are the site of secondary mutations that partially suppressed the splicing defect in the *cyt-18-2* mutant (Asp189 and Trp190 changed to Val and Arg, respectively; Cherniack et al. 1990).

Analysis of mutant *cyt-18* proteins having carboxy-terminal truncations showed that the 39 carboxy-terminal amino acids could be removed without inhibiting splicing or tyrRS activity in vitro (pEX533, pEX534). In addition, in the pEX511 protein, replacement of the last 3 amino acids of the authentic *cyt-18* protein with 18 amino acids encoded by vector sequence had little, if any, effect on splicing or tyrRS activity. More extensive carboxy-terminal truncations (pEX531, pEX532, pEX602, and pEX603) that extend toward and into the region that would correspond to the tRNA-binding domain of bacterial tyrRSs inhibited both tyrRS and splicing activities. The pEX531 protein, which deletes 116

Figure 6. Assays of splicing and tyrRS activities of mutant *cyt-18* proteins. *cyt-18* proteins were synthesized in *E. coli* and partially purified on small-scale heparin–Sephadex columns. (A) Splicing assayed by the [³²P]GTP addition reaction, using in vitro transcript from pHX9422. Some fractions show labeling of in vitro transcript precursor due to putative end-labeling activity discussed in the text. (B) Immunoblot analysis of fractions used for splicing assays. Immunoblots were probed with anti-*cyt-18* antibodies C18-1 or C18-2, as described in Materials and methods. Asterisks denote *cyt-18* band. (C) TyrRS activities of the mutant *cyt-18* proteins. TyrRS activities are expressed as percent of wild-type protein (pEX550 or pEX550A), adjusted for *cyt-18* protein concentration estimated by immunoblotting with anti-*cyt-18* antibody. Measured counts per minute for active proteins ranged from 6×10^4 to 12×10^4 . Background in the tyrRS assays was ~5% of the counts per minute measured for the wild-type protein and was not subtracted in calculating final percent values. Both splicing and tyrRS reactions were carried out for

times established in separate time course experiments as being prior to the plateau in amount of product with the highest concentrations of wild-type protein (pEX550) used in the assays. For reactions carried out for these times, we verified that both splicing and tyrRS activities increase linearly from 0.05 to 0.4 μ g of pEX550 protein, encompassing all *cyt-18* protein concentrations in the assays. In all cases, splicing and tyrRS activities were assayed across the 0.5 M KCl peak, which involves a range of protein concentrations. For quantitation, splicing and tyrRS activities of mutant proteins were compared with those of wild-type protein over similar concentration ranges, determined by immunoblots across the 0.5 M KCl peak and a separate dilution series for each sample. The splicing assays, which are meant to show presence or absence of splicing activity, are those carried out with the highest amounts of mutant *cyt-18* proteins; these assays were generally within a factor of two of wild-type protein (pEX550 or pEX550A). Percent values for splicing activity in the text are based on the normalization procedure described above. The data are representative of at least two independent experiments (separate inductions) for each mutant protein.

amino acids, had no detectable tyrRS activity, even at elevated levels of *E. coli* tRNA^{Tyr} substrate (30 μ M) but did retain very low, but detectable, splicing activity (<5% of wild-type activity; splicing product bands are barely visible in original autoradiogram but too light to be seen in Fig. 6). The pEX532 truncation, which removes an additional 9 amino acids, as well as pEX602, which removes 246 amino acids, had no detectable tyrRS or splicing activities, even with elevated levels of *E. coli* tRNA^{Tyr} or in vitro transcript substrates (30 μ M and 250 μ g/ml, respectively). In the case of the largest carboxy-terminal truncations, pEX602 and pEX603, we confirmed that the proteins partially purified by heparin–Sephadex chromatography had high tyrosyl–adenylation activity (Table 1), suggesting that even extensive carboxy-terminal truncations did not substantially alter the conformation of the nucleotide-binding fold in the amino-terminal half of the protein. Some of the carboxy-terminal truncations (e.g., 531 and 532) strongly reduced the proportion of expressed protein recovered in the soluble fraction, but the solubilities of the proteins from pEX602 and pEX603, the most radical carboxy-terminal truncations, returned to a high level.

To address the possibility that inhibition of splicing or tyrRS activity is due to gross conformational changes in

the carboxy-terminal region, we also analyzed two linker insertion mutations and one single codon deletion in this region (pEX561, pEX562, and pEX575). All of these constructs gave high yields of soluble protein. In pEX562, a 4-amino-acid insertion in an unconserved region of the protein (Tyr417) had no detectable effect on either splicing or tyrRS activity, whereas in pEX561, a 4-amino-acid insertion in another part of the carboxy-terminal region (Gly470) resulted in complete loss of both activities, even with high concentrations of RNA substrates described above. Finally, in pEX575, a single amino acid deletion (Asn545) within the short sequence conserved in bacterial and yeast mt tyrRS, gave a protein that lacked tyrRS activity, even at high concentrations of *E. coli* tRNA^{Tyr}, but had detectable, albeit reduced, splicing activity (5–10% wild type; Fig. 6).

Discussion

Here, we show that functional *cyt-18* protein can be expressed in large quantities in *E. coli*. The expressed protein was purified to near homogeneity and shown to have tyrRS and splicing activities similar to those of authentic *cyt-18* protein isolated from mitochondria. These findings indicate that the *cyt-18* protein is both

necessary and sufficient to splice the *Neurospora* mitochondrial large rRNA intron in vitro. The ability of purified *cyt-18* protein to splice the mitochondrial large rRNA intron in vitro implies that it binds directly to intron RNA.

Although the *cyt-18* protein is, by itself, sufficient to splice the mitochondrial large rRNA intron in vitro, additional components are required for efficient splicing in vivo. Mutations in two other nuclear genes, *cyt-4* and *cyt-19*, inhibit splicing of the mitochondrial large rRNA intron and other group I introns but do not result in deficiency of *cyt-18* protein or splicing activity assayed in vitro (Bertrand et al. 1982; Garriga and Lambowitz 1986; Q. Guo and A. Lambowitz, unpubl.) The *cyt-4* mutation may inhibit splicing indirectly by affecting 3'-end synthesis of the mitochondrial large rRNA (Garriga et al. 1984). The *cyt-19* component, which has not yet been identified, may function as part of a complex with the *cyt-18* protein or it may act independently on the intron

RNA to stabilize structures that form spontaneously in vitro, so that only the final addition of the protein is required for splicing to occur.

We used in vitro mutagenesis of the *E. coli* synthesized *cyt-18* protein to delineate regions that are required for splicing or tyrRS activity. The structural organization of the related bacterial tyrRSs and the x-ray crystal structure of the *B. stearotherophilus* tyrRS (Fig. 5) appear to be useful guides in interpreting the in vitro mutants, as well as the original *cyt-18-1* and *cyt-18-2* mutants and the previously described revertants (Cherniack et al. 1990). Compared with the cognate bacterial tyrRSs, the *cyt-18* protein has an amino-terminal extension, which presumably targets the protein to mitochondria, and a carboxy-terminal extension of 122 amino acids (downstream of amino acid 547; Fig. 5). The presence of the amino-terminal leader in the pEX554 protein does not strongly affect either splicing or mt tyrRS activities, raising the possibility that these "foreign" activities may be

Table 1. Tyrosyl adenylation activity of *cyt-18* proteins synthesized in *E. coli*

Construct	Tyrosyl adenylation (cpm)	TyrRS (cpm)
pEX501	3,000 ± 2,100	763
pEX511 (wt)	14,196 ± 86	247,590
pEX602	27,360 ± 110	10,043
pEX603	24,000 ± 5,500	2,025
No protein	570 ± 130	—

Derivatives of the *cyt-18* protein synthesized in *E. coli* and partially purified by heparin-Sepharose chromatography (0.5 M KCl fractions) were assayed for tyrosyl-adenylation and tyrRS activity. Synthesis of tyrosyl-adenylate was assayed by the binding of ³H-labeled tyrosyl-adenylate/protein complex to nitrocellulose filters, as described in Materials and methods. Portions of column fractions used for assays contained 2.7 µg of total protein and approximately equal amounts of *cyt-18* proteins (pEX511, pEX602, and pEX603), as judged by immunoblots. Data shown are the average of two assays for each sample and are similar to results in repeated experiments.

associated with pre-*cyt-18* protein in the *Neurospora* cytosol. The carboxy-terminal 39 amino acids of the *cyt-18* protein can be removed (pEX533) or the carboxyl terminus can be replaced with vector sequences (pEX511) without substantially affecting either splicing or tyrRS activity in vitro. However, it is possible that these carboxy-terminal amino acids have some function in vivo, such as interaction with another protein or larger complex. Deletion of 116 amino acids in pEX531 inhibits both tyrRS and splicing activity.

Mutations in the nucleotide binding fold region have different effects depending on their location. The original *cyt-18-1* and *cyt-18-2* mutations, which result in temperature-sensitive defects in both splicing and aminoacylation in vivo, affect a conserved Gly residue, which in the *B. stearrowthermophilus* tyrRS is a turn residue on the exterior of the nucleotide binding fold (Fig. 5). The *cyt-18* mutations may identify a region of the nucleotide binding fold that functions directly in splicing or they may inhibit splicing and tyrRS activities by affecting the conformation of other regions of the protein. In this study, we find that two linker insertion mutations elsewhere in the nucleotide binding fold (pEX571 and pEX573) have no discernible effect on either splicing or tyrRS activity. However, two other mutations (pEX551 and pEX558), which are within the predicted adenylate-binding cleft in a position to interfere directly with binding of tyrosyl adenylate (Fig. 5), inhibit tyrRS activity, but leave some splicing activity. These findings indicate that neither the functional integrity of the ATP-binding site nor the normal catalytic activity of the *cyt-18* protein is required for splicing activity.

Our results confirm that a small idiosyncratic domain at the amino terminus of the *cyt-18* protein is required for splicing, but not tyrRS activity. This domain was identified initially by the clustering of suppressor mutations in partial revertants of the *cyt-18-2* mutation that

restore splicing, but not tyrRS activity (Cherniack et al. 1990). The suppressor mutations in this region are readily accounted for by the three-dimensional model of Figure 5, which suggests that this amino-terminal domain may adjoin and interact with the region containing the *cyt-18-2* mutation, Gly127. In this study we confirmed that a large deletion in this domain (43 amino acids; pEX521) inhibits splicing completely while having relatively little effect on tyrRS activity, including little or no effect on the K_m values for *E. coli* tRNA^{Tyr} or ATP. These findings are also rationalized by the model, which suggests that the idiosyncratic domain could be deleted without significantly affecting the structure of the nucleotide binding fold or carboxy-terminal domain, which are required for tyrRS activity. The idiosyncratic amino-terminal domain may function directly in binding the intron RNA or it may interact with other regions of the protein that bind the intron RNA. If this region functions in binding the intron, it presumably interacts with RNA sequences or structures that are present in the intron, but not in *E. coli* tRNA^{Tyr}.

In the previous study, we identified a single partial revertant, R67-10, which has secondary amino acid changes at Asp189 and Thr190 (Cherniack et al. 1990). The three-dimensional model shows that these amino acids are also in proximity to the *cyt-18-2* mutation (positions marked by 571 in Fig. 5). However, replacing these amino acids with a single Gly in pEX571 did not substantially affect splicing or tyrRS activity. The second-site suppression in R67-10 could be explained in either of two ways: The entire surface of the *cyt-18* protein identified in Figure 5 by pEX521, *cyt-18-2*, and pEX571 may be required for splicing, with the change in pEX571 being one that is permissible to retain function; or, alternatively, the suppressor mutation in R67-10 may act indirectly by affecting the abnormal structure caused by the *cyt-18-2* mutation.

Finally, we find that a number of deletions or linker insertion mutations in the carboxy-terminal region corresponding to the tRNA-binding domain of bacterial tyrRSs inhibit both splicing and tyrRS activity, consistent with the idea that some sequences that function in binding *E. coli* tRNA^{Tyr} also function in binding the intron RNA. This finding is also consistent with the hypothesis that the protein recognizes a structure or sequence within the intron that resembles the tRNA^{Tyr} substrate. Interestingly, the pEX575 protein, which has a single amino acid deletion (Asn545) within the short stretch conserved in bacterial and yeast mt tyrRS, lacks tyrRS activity but has detectable, albeit reduced, splicing activity. The differential effects observed with pEX575 indicate that some amino acids do not function identically in both processes.

Considered together, our results show that regions required for splicing are distributed throughout the *cyt-18* protein and overlap but are not identical to regions required for tyrRS activity. The finding that the normal catalytic activity of the protein is not required for splicing is consistent with the hypothesis that the protein functions primarily by facilitating formation of the cor-

rect RNA structure. As discussed previously, the involvement of a small amino-terminal idiosyncratic domain, which is not found in *E. coli* or yeast mt tyrRSs, suggests that splicing activity of the *Neurospora* protein was acquired recently in evolution after the divergence of *Neurospora* and yeast (Cherniack et al. 1990). However, our results also show that this region must act in concert with other regions of the *cyt-18* protein, particularly the putative tRNA-binding domain toward the carboxyl terminus of the protein. By using the *E. coli* expression system, it should be possible to investigate in detail the interaction of *cyt-18* protein with the intron RNA and the role of *cyt-18* protein in splicing.

Materials and methods

N. crassa strains

The *N. crassa* strain used in this study was mutant *cyt-4-1* (AEG-193a; Bertrand et al. 1982). Procedures for maintaining the strain, preparing conidia, and growing mycelia in liquid culture were described previously (Davis and de Serres 1970; Bertrand et al. 1982).

E. coli strains

E. coli strain BL21 [DE3][plysS], which was used for expression of *cyt-18* proteins, contains an IPTG-inducible T7 RNA polymerase gene (Rosenberg et al. 1987). *E. coli* K12 565cN has a temperature-sensitive mutation in the tyrRS (Bedouille and Winter 1986; obtained from the University Chemical Laboratory Cambridge, England). AL565(DE3) is 565cN lysogenized with T7 bacteriophage DE3.

Recombinant plasmids and phage

Plasmid p337 contains the *cyt-18* gene (3.3-kb *Bam*HI fragment) cloned in Bluescribe vector [pBS(+); Stratagene] (Cherniack et al. 1990). To prepare the *cyt-18* gene for expression in *E. coli*, the 3.3-kb *Bam*HI fragment containing the *cyt-18* gene from p337 was inserted into the *Bam*HI site of M13mp18 and the intron was deleted by oligonucleotide-directed mutagenesis (Kunkel et al. 1987), using the oligonucleotide 5'-ATGTGCTCTTTGGT-GCCAGCTGTATCCTTG-3'. The resulting M13 phage, which contains a continuous *cyt-18* ORF, is designated mpALM1. This phage was constructed by A. Majumder.

Plasmids *plysS* and pET3a and bacteriophage DE3, which were used in the bacterial expression strains, were constructed by Rosenberg et al. (1987). pEX501 is a simple derivative of pET3a in which the *Eco*RI site was disrupted by filling in with Klenow fragment of DNA polymerase I. pEX503 is a derivative of pEX501, which has the palindromic oligonucleotide 5'-TATGCTGCGCAGAATTCTGCGCAGCA-3' inserted in the unique *Nde*I cloning site. This insert places the first 5 codons of the mature *cyt-18* protein (Leu33 to Phe37) in the correct reading frame downstream of the ATG start codon of the vector and introduces a unique *Eco*RI site, which was used later to insert the remainder of the *cyt-18* ORF. pEX503 also lacks the *Cla*I site of pEX501, which was destroyed by filling in with Klenow fragment.

pEX511 expresses a cognate of the mature form of the *cyt-18* protein beginning with Leu33 inserted immediately downstream of the ATG start codon in the expression vector pEX503. To construct this plasmid, pEX503, which already contains the vector ATG codon in-frame with the first 5 amino acids of the

cyt-18 ORF (see above), was digested with *Eco*RI and *Bam*HI. The resulting 4.4-kb *Eco*RI-*Bam*HI fragment was ligated with two different segment of the *cyt-18* gene: a sequenced 200-bp *Eco*RI-*Cla*I fragment encompassing the site of the intron deletion from mpALM1, and the 1.7-kb *Cla*I-*Bam*HI fragment containing the main body of the *cyt-18* gene from p337. pEX511 still lacks the final 3 codons of the *cyt-18* ORF and instead substitutes 18 codons from vector sequence at the carboxyl terminus.

pEX550 expresses a cognate of the mature form of the *cyt-18* protein, including the correct carboxyl terminus. This plasmid was derived from pEX511 by inserting a double-stranded oligonucleotide (top, 5'-GATCCGTATAAGTGGTGACGCGT-3'; bottom, 5'-GATCACGCGTCACCACTTATACG-3') into the *Bam*HI site of pEX511. The correct orientation restores the final 3 codons of the *cyt-18* gene and creates an *Mlu*I site at the stop codon. pEX550A is the same as pEX550, except that to facilitate in vitro mutagenesis most of the residual *tet*^R gene in the vector was deleted by digestion with *Nar*I and religation, leaving a single *Nar*I site.

pEX554 expresses the full-length *cyt-18* ORF, including the amino-terminal mitochondrial targeting sequence. The construction of this plasmid began with M13 phage mpALM1, which contains the full-length *cyt-18* ORF. An *Nde*I site was introduced at the ATG start codon of the *cyt-18* ORF by oligonucleotide-directed mutagenesis, using the oligonucleotide 5'-TGTGCGAAGCAGCATATGTGATGTTTGGCT-3' to create mpALM2. The mutagenized region was sequenced, and the 107-bp *Nde*I-*Eco*RI fragment was used to replace the 14-bp *Nde*I-*Eco*RI fragment of pEX550, thereby restoring the amino-terminal targeting sequence.

pEX553 is a derivative of pEX550 that has a deletion of 216 bp (72 amino acids) between the two *Xho*I sites in the *cyt-18* ORF.

pEX521 was derived from pEX511 by deleting sequences between the *Eco*RI site (filled in with Klenow fragment) and the *Pvu*II site of the *cyt-18* gene, with the insertion of a *Nco*I linker (CCCATGGG) to maintain the reading frame. The correct carboxyl terminus of the ORF was restored by inserting a double-stranded oligonucleotide, as described above for pEX550. The pEX521 construction deletes 45 amino acids, but 2 of these are restored in the correct position by the linker. This protein was referred to previously as Δ45 (Cherniack et al. 1990).

pEX531, pEX532, pEX533, and pEX534 are a series of plasmids that produce *cyt-18* proteins having carboxy-terminal truncations. These plasmids were created by inserting a palindromic linker 5'-TGAGTGACTGAATTCAGTCACTCA-3', which contains stop codons in all three reading frames, into pEX511 at the *Aat*II, *Dra*III, *Hind*III, and *Bam*HI sites, respectively.

pEX515 and pEX523 are derivatives of pEX511 in which *Nco*I linkers were inserted into the *cyt-18* ORF at the *Eco*RI and *Pvu*II sites, respectively. In the case of pEX523, it was necessary to restore the reading frame by filling in the *Nco*I site with Klenow fragment of DNA polymerase I.

pEX551 is a derivative of pEX550 in which a *Bam*HI linker (5'-GCGGATCCGC-3') was inserted into the *cyt-18* ORF at the *Cla*I site.

pEX558, pEX561, and pEX573 are derivatives of pEX550A in which an *Nco*I linker was inserted into the *cyt-18* ORF at the *Bcl*I, *Xma*III, or downstream *Xho*I site, respectively. pEX562 has a *Bam*HI linker (10-mer) inserted in the *Acc*I site of the *cyt-18* ORF in pEX550A.

pEX571 and pEX575 have 3-bp deletions in the *cyt-18* ORF. These plasmids were constructed by digestion of pEX550A with *Bgl*II or *Dra*III, respectively, removal of the 3' overhangs using the 3' → 5' exonuclease activity of Klenow fragment (Maniatis et al. 1982), and religation.

pEX602 and pEX603 are derivatives of pEX511, which have extensive carboxy-terminal truncations. To create these plasmids, the 2.2-kb *SalI*–*HindIII* fragment, containing the *cyt-18* gene from p337, was recloned in M13mp19. The resulting M13 clone, designated mpWLI, was subjected to oligonucleotide-directed mutagenesis to juxtapose a TAA stop codon, normally out-of-frame with the *cyt-18* ORF, to the truncation end points. In the case of pEX603, the oligonucleotide 5'-AGCAGGTTGCCGTTAGTCTTTGATGTGCTC-3' was used to delete 449 nucleotides from the *cyt-18* gene, placing the TAA codon just after Asp382; for pEX602, the oligonucleotide 5'-AGCAGGTTGCCGTTAAATGGTCATCTGGCC-3' was used to delete 326 nucleotides of the *cyt-18* gene, placing a stop codon just after Ile423. The deletion end points were sequenced, and the plasmids were assembled by replacing the resident 1.6-kb *Clal*–*HindIII* fragment from the *cyt-18* portion of pEX511 with the corresponding *Clal*–*HindIII* fragments containing the deletion end points from the double-stranded M13 phage DNA.

Expression of *cyt-18* protein in *E. coli*

Fresh Amp^R Cam^R colonies of BL21(DE3)[pLysS], transformed with the desired pEX plasmid, were grown overnight at 37°C in 3-ml cultures containing LB plus ampicillin (50 µg/ml) and chloramphenicol (15 µg/ml). The overnight cultures were inoculated (1 : 100) into 30 ml of N-Z/M9 media in 125-ml Erlenmeyer flasks and incubated with aeration at 37°C until OD₅₉₅ was 0.6–0.8. The synthesis of *cyt-18* protein was then induced by the addition of 3 mM IPTG in 15 ml of prewarmed N-Z/M9 media and continued incubation for 1 hr at 37°C. For the temperature-sensitive strain AL565(DE3), bacteria were cultured and induced at 30°C.

Prior to harvesting, each induced culture was diluted with 120 ml of ice-cold TES buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10 mM NaCl]. The cells were then pelleted by centrifugation in a Beckman JA-14 rotor at 2500g for 10 min. Pelleted cells were resuspended by brief vortexing with 400 µl of TES buffer containing lysozyme (10–12 mg/ml) and incubated on ice for 30 min, followed by addition of 1.2 ml of lysis II buffer [20 mM Tris HCl (pH 8.0), 1.5 mM 2-mercaptoethanol, 1 mM PMSF, 1% Triton X-100] with gentle swirling. For small-scale purifications, the cell suspension was then aliquoted into five Eppendorf tubes, frozen immediately on dry ice, and stored at –70°C until used. For medium-scale purifications, cells were transferred to a single 40-ml Oak Ridge tube prior to freezing.

To prepare soluble fractions for chromatography in small-scale DEAE–Sephacel or heparin–Sephacel columns, cells frozen in lysis II buffer were thawed by gentle swirling at 0°C to 4°C for 5–10 min until lysis was evident. Following addition of 150 µl of TC buffer [50 mM Tris-HCl (pH 8.3), 3 mM CaCl₂] plus 100 units of micrococcal nuclease, the solution was incubated on ice for 10 min, until it was no longer highly viscous. It was then spun in an Eppendorf microcentrifuge at 14,000g for 5 min to pellet cellular debris and insoluble protein.

Preparation of soluble fractions for medium-scale purification of the *cyt-18* protein was essentially the same as that described above, except the larger volume of cells required the addition of 1.2 ml of TC buffer along with 500 units of micrococcal nuclease. Insoluble material was removed by centrifugation in a Beckman JA-17 rotor at 14,000g for 10 min at 0–4°C.

Small-scale heparin–Sephacel chromatography

Chromatography of *E. coli*-synthesized *cyt-18* proteins in small-scale heparin–Sephacel columns was essentially as described

for *cyt-18* protein from mitochondria (Majumder et al. 1989), except that 1 mM PMSF was included in all column buffers. Chromatography was at 4°C in 0.7 × 4-cm glass columns with bed volumes of 1.5 ml. The columns were loaded with 400–600 µl of *E. coli*-soluble protein fraction containing 1–3 mg of protein and were eluted with a step gradient of 0, 0.25, and 0.5 M KCl in TEDG buffer [20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.5 mM DTT, 1 mM PMSF, 10% glycerol]. As found previously for the mitochondrial *cyt-18* protein (Majumder et al. 1989), the *E. coli*-synthesized *cyt-18* protein was present in both 0 and 0.5 M KCl fractions. Endogenous *E. coli* tyrRS was found only in the 0.25 M KCl fractions, as judged by assays of tyrRS activity and by immunoblots probed with anti-*E. coli* tyrRS antibody. The 0.5 M KCl fractions, which contained ≥50% *cyt-18* protein, were used for analysis of splicing and tyrRS activities of mutant *cyt-18* proteins.

Medium-scale purification of *cyt-18* protein from *E. coli*

For medium-scale purification, *E. coli* soluble protein fractions from eight pooled lysates, prepared as described above, were loaded onto a 1.5 × 20-cm glass column containing 30 ml of DEAE–Sephacel equilibrated with PEDG buffer [20 mM KPO₄ (pH 7.6), 1 mM EDTA, 0.5 mM DTT, 1 mM PMSF, 10% glycerol]. After loading, the column was washed successively with 75 ml of PEDG, 30 ml of 0.1 M KCl in PEDG, and 30 ml of 0.25 M KCl in PEDG. The flow rate was ~300 µl/min. Fractions (5 ml) were collected for the 0 and 0.1 M KCl washes, and 3-ml fractions were collected thereafter. The major peak of *cyt-18* protein, identified by dot blots, eluted just before the large change in conductivity at the 0.25 M KCl salt front.

In the next step, the pooled peak fractions containing *cyt-18* protein from the DEAE–Sephacel column (6–11 ml, containing up to 3 mg of protein) were desalted by gel filtration through Sephadex G-75–120 (Pharmacia). Gel filtration was in a 1.5 × 20-cm glass column containing 28 ml of Sephadex G-75–120 equilibrated with TEDG buffer. The column was eluted with 30 ml of TEDG, 1-ml fractions were collected, and the fractions containing most of the excluded peak (~18 ml) were pooled for heparin–Sephacel chromatography.

Finally, heparin–Sephacel chromatography was carried out in a 1.5 × 5-cm glass column containing 5 ml of heparin–Sephacel CL6B (Pharmacia) equilibrated with TEDG buffer (see above). After loading, the column was washed successively with 20 ml of TEDG, 0.25 M KCl in TEDG, and 0.5 M KCl in TEDG, and 1-ml fractions were collected. At this stage of purification, active *cyt-18* protein eluted from the heparin–Sephacel column predominantly at 0.5 M KCl, with only trace amounts in flowthrough fractions. Residual *E. coli* tyrRS was in the 0.25 M KCl fraction.

Preparation of *cyt-18* protein from *N. crassa* mitochondria

cyt-18 protein was partially purified from mt RNP preparations of *Neurospora* nuclear mutant *cyt-4-1*, which has elevated levels of *cyt-18* protein, by heparin–Sephacel chromatography (Majumder et al. 1989). The columns were eluted with a step gradient, and 0.5 M KCl fractions, which contain *cyt-18* protein, were used for assays of splicing and tyrRS activities.

Splicing assays

Splicing was assayed as described previously, using a 698-nucleotide in vitro transcript containing a 583-nucleotide derivative of the mitochondrial large rRNA intron that is not self-splicing (Guo et al. 1991). This transcript was synthesized from

plasmid pHX9422 linearized with *Ban*I, using bacteriophage T3 RNA polymerase (GIBCO, BRL). Splicing reactions were carried out in 20 μ l of solution containing 1 μ g of in vitro transcript, 4 μ l of protein preparation, 100 mM KCl, 5 mM MgCl₂, 20 mM Tris-HCl (pH 7.5), 5 mM DTT, 40 μ Ci of [α -³²P]GTP (0.6 μ M; 3000 Ci/mmol; NEN), and 40 units of RNasin (Promega) or 0.25 units of Inhibit-ACE (5 Prime $\rightarrow$ 3 Prime, Inc.). Reactions were initiated by the addition of the protein preparation, incubated for 15 min or 30 min at 37°C, and terminated by the addition of 6.5 volumes of 20 mM Tris-HCl (pH 7.5) and 50 mM EDTA, followed by phenol extraction and ethanol precipitation in the presence of 2 M ammonium acetate. Splicing products were analyzed by electrophoresis in 4% polyacrylamide/8 M urea gels (unless otherwise specified), followed by autoradiography. In some experiments, splicing was assayed by using ³²P-labeled in vitro transcripts, which were labeled internally by adding 50 μ Ci of [α -³²P]GTP to 200 μ l of the standard transcription mixture containing 500 μ M of each unlabeled NTP. ³²P-labeled in vitro transcripts were phenol extracted and purified by using a Sephadex G-50 spun column (Maniatis et al. 1982). Splicing assays were performed as described above, using 5 nCi of the ³²P-labeled in vitro transcript plus 500 μ M GTP or guanosine (see below). Reactions were terminated by the addition of 4 volumes of 80% formamide/1% xylene cyanol, and products were incubated at 85°C for 5 min, prior to loading onto a gel. To determine K_m values for guanosine or GTP, guanosine concentrations were varied from 1 to 100 μ M and GTP concentrations were varied from 10 to 400 μ M. K_m values were determined by Eadie-Hofstee plots; values indicated in Results are the average of at least two determinations.

TyrRS assays

TyrRS activity in lysates or column fractions was assayed by using *E. coli* tRNA_{Tyr} substrate (Subriden RNA), as described by Majumder et al. (1989). For both *E. coli*-synthesized and mitochondrial *cyt-18* protein, tyrRS activity is less stable than splicing activity, so that tyrRS assays were carried out with freshly isolated protein. To determine the K_m values for the tRNA, 0.1–20 μ g of tRNA was used per 20- μ l reaction. The K_m values for ATP were determined by varying ATP concentrations from 0.1 to 2 mM, with K_m values obtained from Eadie-Hofstee plots.

Tyrosyl-adenylation assay

Tyrosyl-adenylation was assayed by incorporation of [³H]-tyrosine into protein-bound tyrosyl-adenylate (Leatherbarrow and Fersht 1987). Assays were carried out in 50 μ l of reaction mixture containing 10 μ l of *cyt-18* protein preparation. The protein preparations were adjusted so that all reactions used 2.7 μ g of protein and the final KCl concentration in reaction medium was 100 mM. The reaction medium also contained 12.5 μ Ci of L-[3-, ³H]tyrosine (54 Ci/mmol; Amersham) diluted to a final concentration of 4.6 μ M L-tyrosine, 5 mM ATP, 10 mM MgCl₂, 144 mM Tris-HCl (pH 7.8), 14 mM 2-mercaptoethanol, 0.1 mM PMSF, and 0.1 unit of pyrophosphatase (Boehringer Mannheim). Reactions were incubated for 40 min at 25°C, after which protein-bound tyrosyl-adenylate was collected on a nitrocellulose filter. The filter was washed three times with 6 ml of the ice-cold reaction buffer, and radioactivity was determined by liquid scintillation counting, using Ready Protein⁺ liquid scintillation cocktail (Beckman Instruments, Inc.). The amount of *cyt-18* protein was determined by probing immunoblots with anti-*cyt-18* antibody (see below).

SDS-PAGE and immunoblotting

Procedures for SDS-PAGE and immunochemical analysis were described in detail previously (Majumder et al. 1989; Cherniack et al. 1990). SDS-PAGE was carried out by the method of Laemmli (1970), using a 3% stacking gel and a 10% separating gel. Gels were run in a mini-PROTEAN II apparatus (0.75-mm $\times$ 5-cm $\times$ 8-cm slab gels; Bio-Rad Laboratories) at 100–150 V, until the bromophenol blue dye marker reached the bottom of the gel. For immunoblot analysis, proteins separated by SDS-PAGE were electroblotted to nitrocellulose, using a polyblot apparatus (American Bionetics), and probed with a 1 : 1000 dilution of anti-*cyt-18* antibody preparation C18-2, which is directed against a *trpE*-*cyt-18* fusion protein containing amino acids 103–563 of the *cyt-18* protein. For proteins having carboxy-terminal truncations (i.e., 602, 532, and 531), immunoblots were probed with antibody preparation C18-1, which is directed against amino acids 36–82 at the amino terminus of the *cyt-18* protein. To detect endogenous *E. coli* tyrRS in column fractions, immunoblots were probed with 1 : 1000 dilutions of antibody against *E. coli* tyrRS obtained from Edwards and Schimmel (1987). For dot blots, 1.5 μ l of column fraction was dotted onto nitrocellulose, dried, and developed with anti-*cyt-18* antibody (Majumder et al. 1989).

Protein sequencing

To determine amino-terminal amino acid sequence, *cyt-18* protein was partially purified from mitochondria of *N. crassa cyt-4-1* by heparin-Sepharose chromatography. The final 0.5 M KCl heparin-Sepharose fraction, containing ~35 μ g of protein, was precipitated with 4 volumes of acetone. After washing with acetone, the pellet was resuspended in 50 μ l of SDS-gel sample buffer, and SDS-PAGE was carried out as described above, except that the gel thickness was 1.5 mm and the gel was run until a 30-kD prestained protein marker reached the bottom. Following electrophoresis, the gel was electroblotted onto a polyvinylidene difluoride (PVDF) membrane, as described (Matsudaira 1987). The membrane was stained with Coomassie brilliant blue, and the band containing *cyt-18* protein was identified by the immunoblotting of a parallel lane. Ten amino acids of amino-terminal sequence were determined, by an Applied Biosystems model 470A gas-phase sequencer interfaced to a model 120A PTA amino acid analyzer.

The amino-terminal sequence of *cyt-18* protein synthesized in *E. coli* was determined by the same procedure, by using pEX550 protein purified as in Figure 3, with 20 μ g of pEX550 protein preparation loaded onto the SDS-polyacrylamide gel.

Protein determination

Protein concentrations were determined by the method of Bradford (1976), using a Bio-Rad assay kit.

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References

- Akins, R.A. and A.M. Lambowitz. 1987. A protein required for splicing group I introns in *Neurospora* mitochondria is mitochondrial tyrosyl-tRNA synthetase or a derivative thereof. *Cell* **50**: 331–345.
- Bedoulle, H. and G. Winter. 1986. A model of synthetase/transfer RNA interaction as deduced by protein engineering. *Nature* **320**: 371–373.
- Bertrand, H., P. Bridge, R.A. Collins, G. Garriga, and A.M. Lambowitz. 1982. RNA splicing in *Neurospora* mitochondria. Characterization of new nuclear mutants with defects in splicing the mitochondrial large rRNA. *Cell* **29**: 517–526.
- Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**: 248–254.
- Brick, P., T.N. Bhat, and D.M. Blow. 1989. Structure of tyrosyl-tRNA synthetase refined at 2.3 Å resolution. Interaction of the enzyme with tyrosyl adenylate intermediate. *J. Mol. Biol.* **208**: 83–98.
- Cherniack, A.D., G. Garriga, J.D. Kittle, R.A. Akins, and A.M. Lambowitz. 1990. Function of *Neurospora* mitochondrial tyrosyl-tRNA synthetase in RNA splicing requires an idiosyncratic domain not found in other synthetases. *Cell* **62**: 745–755.
- Collins, R.A. and A.M. Lambowitz. 1985. RNA splicing in *Neurospora* mitochondria. Defective splicing of mitochondrial mRNA precursors in the nuclear mutant *cyt-18-1*. *J. Mol. Biol.* **184**: 413–428.
- Davis, R.H. and F.J. deSerres. 1970. Genetic and microbiological research techniques for *Neurospora crassa*. *Methods Enzymol.* **17A**: 79–143.
- Edwards, H. and P. Schimmel. 1987. An *E. coli* aminoacyl synthetase can substitute for yeast mitochondrial enzyme function in vivo. *Cell* **51**: 643–649.
- Garriga, G. and A.M. Lambowitz. 1984. RNA splicing in *Neurospora* mitochondria. Self-splicing of a mitochondrial intron in vitro. *Cell* **39**: 631–641.
- . 1986. Protein-dependent splicing of a group I intron in ribonucleoprotein particles and soluble fractions. *Cell* **46**: 669–680.
- Garriga, G., H. Bertrand, and A.M. Lambowitz. 1984. RNA splicing in *Neurospora* mitochondria: Nuclear mutants defective in both splicing and 3' end synthesis of the large rRNA. *Cell* **36**: 623–634.
- Guo, Q., R.A. Akins, G. Garriga, and A.M. Lambowitz. 1991. Structural analysis of the *Neurospora* mitochondrial large rRNA intron and construction of a mini-intron that shows protein dependent splicing. *J. Biol. Chem.* **266**: 1809–1819.
- Kunkel, T.A., J.D. Roberts, and R.A. Zakour. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* **154**: 367–382.
- Laemmli, U.K. 1970. Cleavage of structural proteins during assembly of the head of the bacteriophage T4. *Nature* **227**: 680–685.
- Lambowitz, A.M. and P.S. Perlman. 1990. Involvement of aminoacyl-tRNA synthetases and other proteins in splicing of group I and II introns. *Trends Biochem. Sci.* **15**: 440–444.
- Leatherbarrow, R.J. and A.R. Fersht. 1987. Investigation of transition-state stabilization by residues histidine-45 and threonine-40 in the tyrosyl-tRNA synthetase. *Biochemistry* **26**: 8524–8528.
- Majumder, A.L., R.A. Akins, J.G. Wilkinson, R.L. Kelley, A.J. Snook, and A.M. Lambowitz. 1989. Involvement of tyrosyl-tRNA synthetase in splicing of group I introns in *Neurospora crassa* mitochondria. Biochemical and immunochemical analysis of splicing activity. *Mol. Cell Biol.* **9**: 2089–2104.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular cloning: A laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
- Mannella, C.A., R.A. Collins, M.R. Green, and A.M. Lambowitz. 1979. Defective splicing of mitochondrial rRNA in cytochrome-deficient nuclear mutants of *Neurospora crassa*. *Proc. Natl. Acad. Sci.* **76**: 2635–2639.
- Matsudaira, P. 1987. Sequence from picomolar quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J. Biol. Chem.* **262**: 10035–10038.
- Rosenberg, A.H., B.N. Laden, C. Dao-shan, L. Shu-Wha, J.J. Dunn, and F.W. Studier. 1987. Vectors for selective expression of cloned DNAs by T7 RNA polymerase. *Gene* **56**: 125–135.
- Schimmel, P. 1987. Aminoacyl tRNA synthetases: General scheme of structure-function relationships in the polypeptides and recognition of transfer RNAs. *Annu. Rev. Biochem.* **56**: 125–158.
- von Heijne, G., J. Steppuhn, and R.G. Herrmann. 1989. Domain structure of mitochondrial and chloroplast targeting peptides. *Eur. J. Biochem.* **180**: 535–545.
- Waye, M.M.Y., G. Winter, A.J. Wilkinson, and A.R. Fersht. 1983. Deletion mutagenesis using an "M13 splint": The N-terminal structural domain of tyrosyl-tRNA synthetase (*B. stearothermophilus*) catalyses the formation of tyrosyl adenylate. *EMBO J.* **10**: 1827–1829.
- Winter, G., G.L.E. Koch, B.S. Hartley, and D.G. Barker. 1983. The amino acid sequence of the tyrosyl-tRNA synthetase from *Bacillus stearothermophilus*. *Eur. J. Biochem.* **132**: 383–387.

The Neurospora mitochondrial tyrosyl-tRNA synthetase is sufficient for group I intron splicing in vitro and uses the carboxy-terminal tRNA-binding domain along with other regions.

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