Involvement of Tyrosyl-tRNA Synthetase in Splicing of Group I Introns in *Neurospora crassa* Mitochondria: Biochemical and Immunochemical Analyses of Splicing Activity

ARUN LAHIRI MAJUMDER,[†] ROBERT A. AKINS,[‡] JULIE G. WILKINSON, ROBERT L. KELLEY,[§]
AMY J. SNOOK, AND ALAN M. LAMBOWITZ*

*Departments of Molecular Genetics and Biochemistry and the Biotechnology Center,
The Ohio State University, 484 W. 12th Avenue, Columbus, Ohio 43210*

Received 23 November 1988/Accepted 6 February 1989

We reported previously that mitochondrial tyrosyl-tRNA synthetase, which is encoded by the nuclear gene *cyt-18* in *Neurospora crassa*, functions in splicing several group I introns in *N. crassa* mitochondria (R. A. Akins and A. M. Lambowitz, Cell 50:331–345, 1987). Two mutants in the *cyt-18* gene (*cyt-18-1* and *cyt-18-2*) are defective in both mitochondrial protein synthesis and splicing, and an activity that splices the mitochondrial large rRNA intron copurifies with a component of mitochondrial tyrosyl-tRNA synthetase. Here, we used antibodies against different trpE-*cyt-18* fusion proteins to identify the *cyt-18* gene product as a basic protein having an apparent molecular mass of 67 kilodaltons (kDa). Both the *cyt-18-1* and *cyt-18-2* mutants contain relatively high amounts of inactive *cyt-18* protein detected immunochemically. Biochemical experiments show that the 67-kDa *cyt-18* protein copurifies with splicing and synthetase activity through a number of different column chromatographic procedures. Some fractions having splicing activity contain only one or two prominent polypeptide bands, and the *cyt-18* protein is among the few, if not only, major bands in common between the different fractions that have splicing activity. Phosphocellulose columns resolve three different forms or complexes of the *cyt-18* protein that have splicing or synthetase activity or both. Gel filtration experiments show that splicing activity has a relatively small molecular mass (peak at 150 kDa with activity trailing to lower molecular masses) and could correspond simply to dimers or monomers, or both, of the *cyt-18* protein. Finally, antibodies against different segments of the *cyt-18* protein inhibit splicing of the large rRNA intron in vitro. Our results indicate that both splicing and tyrosyl-tRNA synthetase activity are associated with the same 67-kDa protein encoded by the *cyt-18* gene. This protein is a key constituent of splicing activity; it functions directly in splicing, and few, if any, additional components are required for splicing the large rRNA intron.

Group I introns include nuclear rRNA introns of *Tetrahymena* spp. and *Physarum polycephalum*, most fungal mitochondrial DNA introns, some chloroplast introns, and introns in T-even bacteriophages (5). All group I introns appear to use the same splicing mechanism first elucidated for the *Tetrahymena thermophila* nuclear rRNA intron by T. R. Cech and co-workers. This mechanism involves two sequential transesterification reactions: addition of guanosine to the 5' end of the intron coupled to cleavage at the 5' splice site and exon ligation coupled to cleavage at the 3' splice site (5). Some group I introns, including the *Tetrahymena* nuclear rRNA intron, some mitochondrial DNA introns in *Neurospora crassa* and *Saccharomyces cerevisiae*, and introns in T-even bacteriophage, are efficiently self-splicing in vitro (5). Self-splicing indicates that both the structural information and catalytic activities required for splicing are contained in the structure of the intron RNAs.

The basic outline of the catalytically active structure of group I introns has been elucidated by phylogenetic comparisons, analysis of in vivo and in vitro mutants, and direct RNA structure analysis (5). All group I introns can fold into

a core secondary structure that includes base pairing between short sequence elements P3[5'], P, Q, R, P3[3'], and S. Group I introns also contain an internal guide sequence that base pairs with flanking sequences in the 5' exon to position the 5' splice site for splicing and may also play a role in positioning the 3' splice site (4, 5). The internal region of the intron, including the core structure, possesses the catalytic activities that cleave at the 5' splice site and add guanosine to the 5' end of the intron (35). In those group I introns that are self-splicing in vitro, the catalytically active structure must be favored relative to alternative structures of deproteinized precursor RNAs.

Although some group I introns are self-splicing in vitro, genetic analysis has shown that most, if not all, group I introns are dependent upon proteins for splicing in vivo. The most likely hypothesis is that these proteins function to fold the RNA into the catalytically active conformation. We showed previously that *N. crassa cob* intron 1, which is efficiently self-splicing in vitro, is dependent on the protein encoded by the *cyt-18* gene for splicing in vivo (6, 12). The *N. crassa* mitochondrial large rRNA intron has been shown to require proteins for splicing both in vivo and in vitro (13).

The proteins required for splicing group I introns can be divided into three classes: (i) maturases, a family of structurally related proteins encoded within some, but not all, group I introns (26, 36); (ii) intron-specific proteins encoded by nuclear genes, e.g., the CBP2 and MRS1 proteins of *S. cerevisiae*, which are required for splicing *cob* introns 5 and 3, respectively (18, 21); and (iii) nuclear-encoded proteins

* Corresponding author.

[†] On leave from the Department of Botany, Visva Bharati University, Santiniketan 731235, West Bengal, India.

[‡] Present address: Department of Biochemistry, Wayne State University Medical School, Detroit, MI 48201.

[§] Present address: Institute of Gas Technology, Chicago, IL 60616.

required for splicing a number of different group I introns, e.g., the *cyt-18* and *cyt-19* gene products of *N. crassa* (25). For some introns, splicing may require both a maturase and a nuclear gene product (e.g., *Neurospora cob* intron 2 [6]).

Recently, we showed via cloning and sequencing that the *cyt-18* gene of *N. crassa* encodes mitochondrial tyrosyl-tRNA synthetase (1). Temperature-sensitive mutants in the *cyt-18* gene (*cyt-18-1* and *cyt-18-2*) had been shown previously to be defective in splicing the mitochondrial large rRNA intron and other group I introns in *N. crassa* mitochondria (6, 28). When grown at their nonpermissive temperature (37°C), these mutants are deficient in both mitochondrial tyrosyl-tRNA synthetase activity and a soluble activity that splices the mitochondrial large rRNA intron in vitro. Several lines of evidence, including biochemical experiments and analysis of intragenic partial revertants, indicated that the *cyt-18* gene product, mitochondrial tyrosyl-tRNA synthetase, or some derivative of this protein is required for splicing the large rRNA intron and other group I introns in *N. crassa* mitochondria (1).

The findings stated above raised a number of questions about the role of the synthetase in the splicing reaction. Does the synthetase itself function directly in the splicing reaction? Is it part of a complex with other synthetases or with maturases and other intron-specific factors? Is it regulated so that the synthetase and splicing activities are mutually exclusive? In our previous work, we showed that the activity that splices the mitochondrial large rRNA intron copurifies with a major component of mitochondrial tyrosyl-tRNA synthetase activity through several steps, including preparation of mitochondrial ribonucleoprotein particles (RNPs) and phosphocellulose chromatography (1). However, phosphocellulose column fractions containing both splicing and synthetase activity were relatively impure (20 to 30 polypeptides detected by silver staining). In addition, the phosphocellulose columns resolved mitochondrial tyrosyl-tRNA synthetase into two components, one that eluted at 0.5 M KCl and was associated with splicing activity and one that did not bind to the columns and was not associated with splicing activity (1).

Here, to gain further insight into how mitochondrial tyrosyl-tRNA synthetase functions in splicing, we carried out more detailed biochemical and immunochemical analyses of splicing activity. Our results indicate that splicing and synthetase activity are associated with the same 67-kilodalton (kDa) polypeptide encoded by the *cyt-18* gene. We find that this protein is a key constituent of splicing activity, that it functions directly in splicing, and that few, if any, additional components are required for splicing the mitochondrial large rRNA intron.

MATERIALS AND METHODS

Strains of *N. crassa* and growth conditions. *N. crassa* strains used in this study were wild-type 74-OR23-1A (designated 74A; FGSC 2489) and mutants *cyt-4-1* (AEG-193a), *cyt-18-1* RAC *al-2* (299-9 RAC *al-2 a*), and *cyt-18-2* (289-67 GG *al-2 A*) (2, 6, 11, 31). Procedures for maintaining strains, preparing conidia, and growing cells in liquid culture were as described previously (2, 7). Wild-type 74A was grown in liquid culture for 14 h at 25°C, *cyt-4-1* was grown in liquid culture for 24 h at 25°C, and *cyt-18-1* and *cyt-18-2* were grown in liquid culture for 24 h at 37°C. In specified instances, *cyt-18-1* and *cyt-18-2* were grown for 24 h at 39°C.

Isolation of mitochondria and mitochondrial RNPs. Mitochondria were isolated by the modified flotation gradient

method (24), and mitochondrial RNPs were isolated as described (1, 13). To make twice-washed RNPs, the initial RNP pellets were dissolved in 100 μ l of 10 mM Tris hydrochloride (pH 7.5), and insoluble material was removed by centrifugation in a Fisher microfuge for 1 min at 4°C. The supernatant was diluted to 4 ml with 500 mM KCl–25 mM CaCl₂–25 mM Tris hydrochloride (pH 7.5)–5 mM dithiothreitol (DTT) plus 0.2% Nonidet P-40, and RNPs were centrifuged through a 1.85 M sucrose cushion containing 500 mM KCl–25 mM CaCl₂–25 mM Tris hydrochloride (pH 7.5)–5 mM DTT, essentially as described for the first centrifugation step in RNP preparation (1, 13). RNP pellets after the first or second centrifugation were either used directly or stored frozen at –70°C.

Construction of recombinant plasmids and synthesis of fusion proteins. Subfragments of pBS337 (derived from mp *cyt-18-33* [1]), a plasmid containing the *cyt-18* gene, were cloned in pATH3 or pATH11 expression vectors obtained from A. Tzagoloff and T. J. Koerner (Columbia University, New York, N.Y.). The plasmids were constructed in *Escherichia coli* DH5 and expressed in *E. coli* RR1 (15, 17). The construction of each plasmid was verified by restriction mapping and sequencing across the anticipated *trpE*–*cyt-18* junction. *E. coli* RR1 containing the expression vectors was induced with indoleacrylic acid, and an insoluble protein fraction was prepared as described previously (20, 34). Large quantities of fusion proteins for immunizing rabbits were prepared from 1 liter of bacterial culture.

Production of antibodies and purification of IgG. *trpE*–*cyt-18* fusion proteins were purified from total insoluble *E. coli* protein by preparative sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (23) and used to raise antibodies in female New Zealand white rabbits, essentially as described (34). Two rabbits were injected with each fusion protein. In each case, both rabbits produced antibodies recognizing the same 67-kDa protein in *N. crassa* mitochondria, except for one preparation of C18-2, which also recognized a higher-molecular-weight polypeptide among total mitochondrial proteins.

All steps in the purification of immunoglobulin G (IgG) antibodies were at 4°C. First, serum (1 to 2 ml) was diluted with an equal volume of 0.2 M Tris hydrochloride (pH 7.4), and impurities were removed by batch adsorption to DEAE-Sephacel (Pharmacia, Inc., Piscataway, N.J.). The DEAE supernatants were then passed three times through a 1- to 2-ml affinity column containing *trpE* and insoluble proteins from induced *E. coli* RR1 containing the pATH vector linked to CNBr-activated Sepharose (5 mg of protein per ml of matrix; Sigma Chemical Co., St. Louis, Mo.) Finally, the eluate from the affinity column was applied to a 1- to 2-ml Protein A-Sepharose column (Sigma [10]). The protein A column was washed successively with 10-column volumes of the following: 0.1 M Tris hydrochloride (pH 7.4), 150 mM NaCl, 0.1 M Tris hydrochloride (pH 8.0), and 10 mM Tris hydrochloride (pH 7.4). IgG antibodies were eluted from the column with 0.1 M glycine hydrochloride (pH 3.0). Fractions containing IgG antibodies were pooled, dialyzed against 10 mM Tris hydrochloride buffer (pH 7.4), and brought to 0.4 mg of protein per ml. Aliquots (1 ml) of IgG fractions were stored at –70°C and thawed once as needed.

SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel analysis was carried out as described by Laemmli (23), with a 10% running gel and a 3.0% stacking gel. Electrophoresis was performed at 200 V for 45 min by using a Mini Protean II gel system (slab gels, 0.75 mm $\times$ 5 cm $\times$ 8 cm; Bio-Rad Laboratories, Richmond, Calif.). For analysis of column fractions, proteins were precipitated by adding 10

μg of cytochrome *c* carrier and an equal volume of 20% trichloroacetic acid (TCA)–10% acetone and incubating overnight on ice. The precipitated proteins were collected by centrifugation in a microfuge for 45 min at 4°C, washed several times with 100% acetone, and dried. The precipitates were suspended in 20 μl of SDS sample buffer. Gels were stained with Coomassie brilliant blue (38) or silver (29). In a few cases, gels were blotted directly onto nitrocellulose and proteins were stained with colloidal gold (AuroDye Forte; Janssen Life Sciences Products, Piscataway, N.J.). Molecular size estimates are based on migration relative to pre-stained molecular weight marker proteins (Rainbow markers; Amersham Corp., Arlington Heights, Ill.). Cytochrome *c*, which was used as a carrier for trichloroacetic acid precipitation of column fractions, runs anomalously in this gel system (M_r 25,000 to 30,000).

Two-dimensional gel electrophoresis. Nonequilibrium pH gradient electrophoresis (NEPHGE) was as described by O'Farrell et al. (30), with gels run in a Bio-Rad Mini Protean-II two-dimensional gel apparatus. Mitochondria were dissolved in NEPHGE sample buffer (0.5% SDS, 9.5 M urea, 5% β -mercaptoethanol, 0.2% ampholines [pH 3.5 to 10]) so the final protein concentration was 2 mg per ml. Aliquots (20 μl) were then mixed with an equal volume of NEPHGE lysis buffer (9.5 M urea, 2% [wt/vol] Nonidet P-40, 1.6% ampholines [pH 5 to 7], 0.4% ampholines [pH 3.5 to 10], 5% β -mercaptoethanol) and loaded immediately at the acidic end of the first-dimension tube gels. Dimension 1 was run at 750 V for 2 h, and dimension 2 was run at 200 V for 45 min.

Immunoblots. Proteins separated by SDS-polyacrylamide gel electrophoresis were transferred to nitrocellulose by the Polyblot system (American Bionetics, Emeryville, Calif.). For dot blots, 6- μl fractions were applied directly to nitrocellulose (3 μl at a time) and air dried. Nitrocellulose blots were blocked with 1% bovine serum albumin (BSA) in 10 mM Tris hydrochloride (pH 8.0)–150 mM NaCl–0.05% Tween 20 for 30 min at room temperature. The blocked membranes were incubated with appropriate dilutions of C18-1, C18-2, or C18-5 IgG antibodies (generally a 1:1,000 dilution of IgG antibodies adjusted to 0.4 mg of protein per ml) in 1% BSA–10 mM Tris hydrochloride (pH 8.0)–150 mM NaCl–0.05% Tween 20 for 30 min at room temperature with gentle shaking. Blots were processed further by using the alkaline phosphatase procedure as specified in the instructions for the ProtoBlot Western Blot AP System (Promega, Madison, Wis.), except that washes after incubation with primary and secondary antibody were with 1% BSA–10 mM Tris hydrochloride (pH 8.0)–150 mM NaCl–0.05% SDS for 15 min followed by 1% BSA–10 mM Tris hydrochloride (pH 8.0)–150 mM NaCl–0.05% Tween 20 for 15 min. After completion of immunoblot staining, the nitrocellulose membrane was washed thoroughly with double-distilled water to remove the color development solution and stained with a stabilized colloidal gold solution (AuroDye Forte) to reveal other proteins transferred to the blot.

Column chromatographic methods. The starting material for all chromatographic procedures was mitochondrial RNPs from nuclear mutant *cyt-4-1*, which contain elevated amounts of splicing activity (13). All operations were at 0 to 4°C. RNPs (150 to 400 μg of protein) from each of 4 liters of culture were suspended thoroughly in 100 μl of a solution containing 1 mM CaCl_2 –10 mM Tris hydrochloride (pH 8.2) and digested with 40 U of micrococcal nuclease (Pharmacia) for 30 min at 30°C. Digestion was stopped by addition of 10 μl of 50 mM ethylene glycol-bis (β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), and glycerol (ultra-

pure, autoclaved) and DTT were added to final concentrations of 10% and 1 mM, respectively, prior to loading onto the columns. Ionic strength of column fractions was monitored by using a YSI model 35 conductance meter (YSI Scientific, Yellow Springs, Ohio).

Phosphocellulose. Chromatography on phosphocellulose columns was essentially as described (1), with modifications in elution conditions indicated in the text.

Heparin-Sepharose. Heparin-Sepharose CL6B (Pharmacia) was equilibrated overnight in TEDG buffer (20 mM Tris hydrochloride [pH 7.5], 1 mM EDTA, 0.5 mM DTT, 10% glycerol) and packed into a glass column (0.7 $\times$ 3 cm) (bed volume, 1.5 ml). Nuclease-digested RNPs (approximately 100 μg of protein) were loaded onto the column, and the column was washed successively with (i) 5.25 ml of TEDG buffer, (ii) 4.55 ml of TEDG buffer containing 5 mM ATP and 0.1 mM tyrosine, (iii) 4.2 ml of TEDG buffer containing 0.05 M KCl, (iv) 4.2 ml of TEDG buffer containing 0.1 M KCl, (v) 4.2 ml of TEDG buffer containing 0.25 M KCl, and (vi) 4.2 ml of TEDG buffer containing 0.5 M KCl. Fractions of 350 μl were collected.

DEAE-Sephacel. DEAE-Sephacel was washed and equilibrated overnight in PEDG buffer (20 mM potassium phosphate [pH 7.7], 1 mM EDTA, 0.5 mM DTT, 10% glycerol) and packed in a glass column (0.7 $\times$ 3 cm) (bed volume, 1.5 ml). Nuclease-digested RNPs (approximately 96 μg of protein) were loaded onto the column, and the column was washed successively with (i) 5.25 ml of PEDG buffer, (ii) 4.2 ml of PEDG buffer containing 0.1 M KCl, (iii) 4.2 ml of PEDG containing 0.25 M KCl, and (iv) 4.2 ml of PEDG buffer containing 0.5 M KCl. Fractions of 350 μl were collected.

Blue Sepharose. Blue Sepharose CL-6B (Pharmacia) was washed according to the recommendations of the manufacturer and equilibrated with TMDG buffer (20 mM Tris hydrochloride [pH 7.5], 5 mM MgCl_2 , 0.5 mM DTT, 10% glycerol). Nuclease-digested RNPs (approximately 100 μg of protein) were added to the equilibrated Blue Sepharose and incubated overnight on ice. The material was then packed into a glass column (0.7 $\times$ 3 cm) (bed volume, 1.5 ml). The column was washed successively with (i) 5 ml of TMDG buffer, (ii) 4.2 ml of TMDG buffer containing 5 mM ATP and 1.0 mM tyrosine, (iii) 4.2 ml of TMDG buffer containing 0.25 M KCl, and (iv) 4.2 ml of TMDG buffer containing 0.5 M KCl. Fractions of 350 μl were collected.

Alternatively, Blue Sepharose CL-6B was washed according to the recommendations of the manufacturer, equilibrated in 10 mM Tris hydrochloride (pH 7.5) containing 1 mM DTT, and packed into a column (4 $\times$ 0.8 cm). Twice-washed RNPs were digested with micrococcal nuclease, and approximately 400 μg of protein was loaded onto the column. The column was washed successively with (i) 5 ml of 10 mM Tris hydrochloride (pH 7.5)–1 mM DTT, (ii) 5 ml of 10 mM Tris hydrochloride (pH 7.5)–1 mM DTT containing 5 mM ATP and 0.1 mM L-tyrosine, (iii) 5 ml of 10 mM Tris hydrochloride (pH 7.5)–1 mM DTT containing 50 mM KCl, and (iv) 5 ml of 10 mM Tris hydrochloride (pH 7.5)–1 mM DTT containing 500 mM KCl. Fractions of 250 μl were collected.

Gel filtration. Nuclease-digested RNPs (200 μl ; 400 to 500 μg of protein) containing 20% glycerol and 1 mM DTT were loaded onto a Sephacryl S-200 column (glass column, 41 $\times$ 0.8 cm; bed volume, 21 ml equilibrated with 250 or 50 mM KCl, 20 mM phosphate buffer [pH 7.5], 1 mM DTT, 20% glycerol). The column was calibrated with gel filtration marker proteins (MW-GF-200 Kit; Sigma), including β -amylase (200 kDa), alcohol dehydrogenase (150 kDa), BSA

(66 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.4 kDa). The void volume was determined by using blue dextran 2000. Gel filtration columns were particularly prone to contamination of splicing fractions by nonspecific nucleases. For that reason, it was essential to use twice-washed RNPs for these columns.

Splicing assays. Splicing was assayed by using *in vitro* transcripts containing the large rRNA intron and flanking exon sequences (1, 13). Reactions were carried out in 20 μ l of solution containing 4 μ l of column fraction, 0.5 to 1.0 μ g of *in vitro* transcript, 40 μ Ci of [α - ^{32} P]GTP (3,000 Ci/mmol; ICN Radiochemicals, Irvine, Calif.), 100 mM KCl, 5 mM MgCl₂, 20 mM Tris hydrochloride (pH 7.5), and 5 mM DTT. Reaction mixtures were incubated for 60 min at 37°C. At the beginning of the work, assays were carried out with an *in vitro* transcript synthesized from recombinant plasmid pHX-11 (1), which contains the large rRNA intron and flanking exons cloned in Bluescribe vector (pBS+; Stratagene, La Jolla, Calif.). Transcription of pHX-11 with bacteriophage T3 RNA polymerase gives a 3.7-kb transcript containing 818 nucleotides (nts) of 5' exon, the 2.3-kb intron, and 502 nts of 3' exon. Toward the end of the work, we switched to pHX9422, a smaller derivative of pHX-11 from which additional exon sequences and nonessential intron sequences had been deleted. This plasmid gives a much shorter (approximately 600 nts) *in vitro* transcript containing 30 nts of the 5' exon, a 582-nt intron, and 51 nts of 3' exon. The intron has deletions encompassing the open reading frame (positions 670 to 2184) and most of the central hairpin structure (positions 190 to 386) near the 5' splice site. Characterization of this transcript showed that it is not self splicing *in vitro* under any conditions examined (R. A. Akins and A. M. Lambowitz, unpublished data). Because of the smaller size of the excised intron, splicing products with this transcript were analyzed on a gel containing 5% polyacrylamide, 7 M urea, 89 mM boric acid, 89 mM Tris, and 2.5 mM EDTA. The salt concentration in the assays was adjusted to 100 or 200 mM KCl by adding the required volume of 2 M KCl; 200 mM KCl was used only for those column wash fractions which had to be assayed for splicing in the presence of antibody against *Neurospora* endo-exonuclease.

Assay of aminoacyl-tRNA synthetase activity. Tyrosyl-tRNA synthetase activity was assayed as described by Akins and Lambowitz (1), except that incubations were for 12 min at 30°C and reactions were terminated by addition of 0.5 ml of ice-cold 10% TCA-0.1% SDS. The salt concentration in all assays was adjusted to 100 mM KCl by adding the required volume of 2 M KCl.

Inhibition of splicing activity by anti-cyt-18 antibodies. Splicing activity was purified by phosphocellulose chromatography of nuclease-digested mitochondrial RNPs, as described above. A 4- μ l portion of phosphocellulose column fraction containing splicing activity was mixed with 2 μ l of antibody incubation buffer (500 mM KCl, 50 mM Tris hydrochloride [pH 7.5], 5 mM DTT, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1% Nonidet) and 4 μ l of the appropriate dilution of IgG antibody and was incubated on ice for 3 h. Then, the remaining components of the splicing reaction mixture were added, and splicing was assayed as described above. In boiled controls, antibodies were boiled for 15 min, prior to addition of incubation buffer and splicing activity.

Protein assay. The amount of protein in different samples was determined by the method of Lowry (27) or by the method of Bradford (3), with Bio-Rad protein assay reagent.

FIG. 1. Map of *cyt-18* gene and plasmids for synthesis of *trpE*-*cyt-18* fusion proteins in *E. coli*. Symbols: □, *cyt-18* open reading frame; ■, intron; ▨, *trpE*.

RESULTS

Preparation of anti-cyt-18 antibodies. Figure 1 shows a map of the *cyt-18* gene. The open reading frame encoding mitochondrial tyrosyl-tRNA synthetase has a length of 531 amino acids and is interrupted by a small intron near the N terminus (1). Homology between the mitochondrial and bacterial tyrosyl-tRNA synthetases begins at amino acid 28 of the *Neurospora* protein and extends throughout the protein but is weaker toward the C terminus.

We raised antibodies to different segments of the *cyt-18* protein expressed in *E. coli* as *trpE*-*cyt-18* fusion proteins. Plasmid constructions for expression of fusion proteins are shown in Fig. 1. Plasmid pC18-1 contains an N-terminal segment of the *cyt-18* open reading frame (amino acids 36 to 82) cloned in pATH-11, whereas pC18-2 and pC18-5 contain overlapping C-terminal segments (amino acids 103 to 531 and 280 to 531, respectively) cloned in pATH-3. The insert in pC18-1 extends into the intron in the *cyt-18* gene, but because a termination codon is located fortuitously at the beginning of the intron, the predicted fusion protein contains no additional amino acids beyond the first exon.

Identification of *cyt-18* gene product in mitochondrial preparations. The antibodies were used first to identify the *cyt-18* gene product in mitochondrial lysates and RNP preparations. We showed previously that mitochondrial RNP preparations contain both mitochondrial tyrosyl-tRNA synthetase and splicing activity and that nuclear mutant *cyt-4-1*, in which splicing of the large rRNA intron may be inhibited indirectly, accumulates high amounts of splicing activity in RNPs, possibly associated with unspliced precursor RNAs that accumulate in the mutant (1, 13). Figure 2 shows SDS-polyacrylamide gels and immunoblots of proteins present in mitochondrial lysates and RNP preparations from wild-type 74A and mutant *cyt-4-1*. For each antibody preparation, the left lanes show immunoblots and the right lanes show the same blots restained with AuroDye to reveal other proteins. All three antibody preparations bind to the same polypeptide, which is detected in both mitochondrial lysates and RNP preparations. This protein has an *M_r* of 67,000 determined by Ferguson plots, but in the 10% SDS-polyacrylamide gels used routinely, the protein runs anomalously slowly relative to the molecular weight markers. Control experiments showed that binding of each antibody prepara-

FIG. 2. SDS-polyacrylamide gel electrophoresis and immunoblot analyses of proteins in mitochondrial lysates and RNP preparations. Proteins in mitochondrial lysates (A) and RNP preparations (B) from wild-type 74A and mutant *cyt-4-1* were separated by electrophoresis in 10% SDS-polyacrylamide gels and immunoblotted with C18-1, C18-2, and C18-5 antibodies. Left lanes show immunoblots and right lanes show the same blots stained with AuroDye to reveal other proteins. Positions of molecular mass marker proteins are shown on the left. The molecular mass of the *cyt-18* protein (67 kDa) was determined by Ferguson plots. (C) Binding of antibodies against *trpE-cyt-18* fusion proteins to the 67-kDa polypeptide was competed with the appropriate *trpE-cyt-18* fusion protein but not by other *trpE-cyt-18* fusion proteins or by BSA. In each case, competition was carried out by incubating the antibody preparation with 30 μ g of insoluble protein fraction containing the indicated fusion protein or with BSA (fraction 5), prior to probing the immunoblot. The mixtures were incubated in 50 μ l of 10 mM Tris hydrochloride (pH 7.5) at 37°C for 30 min, followed by 4°C for 90 min. Strips are parallel lanes from the same gel.

tion to the 67-kDa polypeptide is competed with the appropriate fusion protein but not by other fusion proteins or BSA at the same concentrations (Fig. 2C). The finding that antibodies to different regions of the *cyt-18* open reading

FIG. 3. NEPHGE and immunoblot analysis of proteins in whole mitochondria and RNP preparations. Proteins in a mitochondrial preparation (40 μ g of protein) (A and B) and in an RNP preparation (20 μ g of protein) (C) from the *cyt-4-1* mutant were separated by NEPHGE and immunoblotted with C18-2 antibodies. The RNP preparations had been digested with 9 U of micrococcal nuclease for 30 min at 30°C prior to electrophoresis. (B) Immunoblot of proteins in mitochondrial lysates. (C) Immunoblot of proteins in RNP preparations. Figure 3A shows the same blot as in Fig. 3B stained with AuroDye to reveal other proteins. Positions of molecular weight marker proteins in the SDS-polyacrylamide gel are indicated on the right.

frame bind to the same 67-kDa polypeptide, together with the specificity of binding demonstrated by the competition experiments, identify the 67-kDa polypeptide as the *cyt-18* gene product.

Mitochondrial lysates and RNP preparations from the *cyt-4-1* mutant contain 1.5- to 10-fold more 67-kDa polypeptide per microgram of protein than do those from wild-type 74A, consistent with the higher amounts of splicing and mitochondrial tyrosyl-tRNA synthetase found previously in *cyt-4-1* RNP preparations (1, 13). From Western blots (immunoblots), we calculated that 6 to 19% of the 67-kDa polypeptide present in mitochondrial lysates is recovered in mitochondrial RNP preparations in both wild-type 74A and *cyt-4-1*. The observed M_r of the *cyt-18* protein, 67,000, is higher than the calculated molecular weight of 60,000 which includes a potential N-terminal mitochondrial targeting sequence of 20 to 30 amino acids. This higher apparent molecular weight could reflect anomalously slow migration in SDS-polyacrylamide gels, as is sometimes the case for basic proteins, or cleavage of little or none of the N-terminal targeting peptide after transport into mitochondria.

Two-dimensional gel electrophoretic analysis. Two-dimensional gel electrophoretic analysis of the *cyt-18* protein was carried out by using NEPHGE (30). Figure 3 shows Western

FIG. 4. SDS-polyacrylamide gel electrophoresis and immunoblot analysis of proteins in whole mitochondria and RNP preparations. Shown are proteins in mitochondrial lysates (15 μ g) (A) and in RNP preparations (7.5 μ g) (B) from wild-type 74A and nuclear mutants *cyt-4-1*, *cyt-18-1*, and *cyt-18-2*. Wild-type 74A and mutant *cyt-4-1* were grown at 25°C. Mutants *cyt-18-1* and *cyt-18-2* were grown at 37°C. Other experiments showed no substantial difference in the amount of *cyt-18* protein in mitochondria of wild-type 74A grown at 25 or 37°C (Cherniack and Lambowitz, unpublished data.) Proteins were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting with C18-1 (A) and C18-5 (B) antibodies. Immunoblots are shown on the left, and the same blots stained with AuroDye to reveal other proteins are shown on the right. Positions of the molecular weight marker proteins are indicated on the left.

blots of total mitochondrial proteins and mitochondrial RNP proteins from mutant *cyt-4-1* probed with the C18-2 antibody preparation. The *cyt-18* protein migrates as a single spot and is one of the most basic proteins in the gel. The basicity of the protein is in agreement with a pI of 9.6 calculated from amino acid composition of the 531-amino-acid open reading frame. The two-dimensional gels provide no indication that the *cyt-18* protein is subject to covalent modifications resulting in charge changes (e.g., phosphorylation).

Analysis of *cyt-18* mutants. Figure 4 shows immunoblot and SDS-polyacrylamide gel analyses of proteins in mitochondrial preparations from wild-type 74A and mutant *cyt-4-1* and 37°C-grown mutants *cyt-18-1* and *cyt-18-2*. The amount of *cyt-18* protein in mitochondrial lysates and RNP preparations from the *cyt-18* mutants is as high as or higher than that in wild-type 74A. We showed previously that mitochondrial lysates and RNP preparations from the mutant have less than 10% wild-type splicing and synthetase activities (1, 13). Consequently, the mutations must result primarily in inactivation of the protein rather than inhibition of its synthesis or transport into mitochondria. When grown at a more stringent temperature, 39°C, the *cyt-18* mutants also show somewhat decreased amounts of *cyt-18* protein (10 to 40% less than wild-type 74A [data not shown]).

Both *cyt-18* mutants have a single base change that is responsible for the mutant phenotype and results in the change of amino acid 127 from glycine to glutamic acid (A.

Cherniack and A. M. Lambowitz, unpublished data). In two-dimensional gels containing mixtures of wild-type and mutant proteins, the mutant *cyt-18* proteins appear to be shifted in the direction expected for the predicted charge change of -1 (data not shown).

Copurification of *cyt-18* gene product with splicing activity. The antibodies were used to investigate whether the *cyt-18* gene product copurifies with splicing activity through different chromatographic procedures. The starting material in these experiments was mitochondrial RNP preparations from mutant *cyt-4-1*, which contain elevated amounts of *cyt-18* protein and splicing activity. The RNPs were digested with micrococcal nuclease and fractionated on different types of columns. Column fractions were assayed for splicing and synthetase activity, and proteins in the column fractions were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting to detect the *cyt-18* protein. Tyrosyl-tRNA synthetase activity was unstable in dilute solutions and after repeated freezing and thawing. In addition, only limited amounts of splicing and tyrosyl-tRNA synthetase activity could be obtained from mitochondrial preparations. For these reasons, column volumes were kept as small as possible, and step gradients were preferred over continuous gradients. All columns were run at 4°C, and glycerol (up to 20%) was added to fractions to stabilize activities. Splicing activity was generally more stable than synthetase activity.

Phosphocellulose. We reported previously that phosphocellulose columns eluted with a KCl step gradient resolve mitochondrial tyrosyl-tRNA synthetase into two components, one that elutes at a high salt concentration (0.5 M KCl) and is associated with splicing activity and one that does not bind to the column in 0 M KCl buffer and is not associated with splicing activity (1). In many repeats of this experiment, the ratio of the two peaks of tyrosyl-tRNA synthetase activity varied in different RNP preparations. Mixing experiments indicated that the 0 M KCl fraction did not contain a diffusible inhibitor of splicing activity (1).

Figure 5 shows RNP proteins fractionated on a phosphocellulose column by using a similar step gradient elution, except that splicing activity was eluted with 0.45 M KCl, which gives better purification of the activity. Splicing was assayed by incubating an in vitro transcript containing the large rRNA intron and flanking exons with the column fraction and [^{32} P]GTP, as described in Materials and Methods. Products were analyzed by gel electrophoresis and autoradiography to detect excised intron labeled by addition of [^{32} P]GTP to its 5' end during splicing. The resulting autoradiograms generally show a major band corresponding to the excised intron and a slightly larger band corresponding to the splicing intermediate resulting from addition of [^{32} P]GTP to the 5' splice site.

As in the previous work, the phosphocellulose column resolves mitochondrial tyrosyl-tRNA synthetase into two major components, one that elutes at 0.45 M KCl (Fig. 5A, peak fraction 32) and is associated with splicing activity and one that does not bind to the column (Fig. 5A, peak fractions 5 and 6) and is not associated with splicing activity. Immunoblots show that both peaks of synthetase activity contain the 67-kDa *cyt-18* protein detected by anti-*cyt-18* antibodies and that there is no difference in electrophoretic mobility of *cyt-18* protein associated with each of the peaks (Fig. 5C).

In the previous work using 0.5 M KCl, splicing activity was eluted from phosphocellulose columns as a sharp peak at the salt front, and all fractions containing splicing activity also contained tyrosyl-tRNA synthetase activity (1). Here, by using 0.45 M KCl, the bound peak was resolved into two

FIG. 5. Phosphocellulose column chromatography of tyrosyl-tRNA synthetase and splicing activities associated with mitochondrial RNPs. Mitochondrial RNP particles (300 μl ; 800 μg of protein), which had been digested with micrococcal nuclease, were loaded on a 1-ml phosphocellulose column as described previously (13). The column was washed with 5 ml of 0, 0.3, and 0.45 M KCl in 20 mM phosphate buffer (pH 6.5), 1 mM EDTA, 0.5 mM DTT. Fractions (250 μl) were collected. (A) Chart of tyrosyl-tRNA synthetase activity in 4- μl portions of fractions. (B) Gel showing splicing activity in 4- μl aliquots of selected fractions assayed by using the in vitro transcript from pHX-11. Lengths of bands are given on the right. (C) SDS-polyacrylamide gel and immunoblot analyses of proteins in different fractions. Panels on the left show immunoblots (Western blots) probed with C18-2 antibody; similar results were obtained with C18-1 antibody (data not shown). Panels on the right show immunoblots after staining with AuroDye to reveal other proteins. Cytochrome *c* was added as a carrier during TCA precipitation. Positions of molecular mass markers are indicated on the left.

components, one that contains both splicing and synthetase activity (Fig. 5, fractions 32 to 33) and one that contains only splicing activity (Fig. 5, fractions 34 to 36). Immunoblots of SDS-polyacrylamide gels showed that all fractions that have splicing activity contain the 67-kDa *cyt-18* protein.

We attempted to rerun the 0 and 0.5 M peaks of synthetase activity from phosphocellulose columns on a second phosphocellulose column but encountered difficulty due to sub-

stantial loss of synthetase activity after the first column or during dialysis of the 0.5 M KCl fraction. In one experiment, both the 0 and 0.5 M KCl peaks were eluted at the same position as they were in the original column. However, this was not reproducible, and in other experiments small peaks of activity were scattered throughout the rerun column, possibly reflecting partial denaturation of the protein.

We also attempted to specifically elute tyrosyl-tRNA

FIG. 6. Heparin-Sepharose column chromatography of tyrosyl-tRNA synthetase and splicing activities associated with mitochondrial RNPs. The column was eluted with buffers containing increasing concentrations of KCl, as described in Materials and Methods. (A) Portions (4 μl) of paired fractions (2 μl of each fraction) were assayed for tyrosyl-tRNA synthetase activity. The datum points are plotted between the fractions that were paired for assay. (B) Splicing activity in 4- μl portions of indicated fractions assayed with in vitro transcript from plasmid pHX9422. The smear in lane 3 is due to unincorporated nucleotides that were not removed by ethanol precipitation. Reassay of this fraction showed roughly the same amount of splicing activity as lane 4. (C) Dot blot analysis of 6- μl portions of indicated fractions. (D) SDS-polyacrylamide gel and immunoblot analyses of proteins in column fractions. Immunoblots were probed with C18-2 antibody and then stained with AuroDye to reveal other proteins. Only the final AuroDye-stained blots are shown. Positions of molecular weight markers are indicated on the left of each gel. Lines to the right show positions of *cyt-18* protein in immunoblots and cytochrome *c* added as carrier prior to TCA precipitation.

FIG. 7. DEAE-Sephacel column chromatography of tyrosyl-tRNA synthetase and splicing activity associated with mitochondrial RNPs. The column was eluted with buffers containing increasing concentrations of KCl, as described in Materials and Methods, and 350- μl fractions were collected. (A) Portions (4 μl) of paired fractions (2 μl of each fraction) were assayed for tyrosyl-tRNA synthetase activity. The datum points are plotted between the fractions that were paired for assay. (B) Splicing activity in 4- μl portions of indicated fractions assayed with in vitro transcript from plasmid pHX9422. The upper panel shows initial assay of paired fractions. The lower panels show reassay of individual fractions 1 to 8 and 27 to 32. Splicing activity in flowthrough fractions 1 and 2 was lost after the initial assay, possibly reflecting protease activity present in these complex fractions. (C) SDS-polyacrylamide gel and immunoblot analyses of proteins in column fractions. Immunoblots were probed with C18-2 antibody and then stained with AuroDye to reveal other proteins. Only the final AuroDye-stained blots are shown. Fractions 40 and 41, which correspond to a small peak of splicing and synthetase activity, show a light band of 67-kDa *cyt-18* protein, which is detectable in the immunoblot prior to the AuroDye staining. Positions of molecular weight markers are indicated on the left. Lines on the right of each gel indicate positions of *cyt-18* protein in immunoblots and cytochrome *c* added as carrier prior to TCA precipitation.

FIG. 8. Blue-Sepharose chromatography of tyrosyl-tRNA synthetase and splicing activities associated with mitochondrial RNPs. The column was eluted with buffers containing increasing concentrations of KCl, as described in Materials and Methods, and 350- μl fractions were collected. (A) Portions (4- μl) of paired fractions (2 μl of each fraction) were assayed for tyrosyl-tRNA synthetase activity. The datum points are plotted between the fractions that were paired for assay. (B) Splicing activity in 4- μl portions of indicated fractions assayed with in vitro transcript from plasmid pHX9422. The smear in lane 17/18 is due to unincorporated nucleotides that were not removed by ethanol precipitation. (C) Dot blot analysis of 6- μl portions of indicated fractions. (D) SDS-polyacrylamide gel and immunoblot analyses of proteins in column fractions. Immunoblots were probed with C18-2 antibody (dilution 1:1,000), and the blots were then stained with AuroDye to reveal other proteins. Only the final AuroDye-stained blots are shown. Flowthrough fraction 1, which has a small amount of synthetase activity, contains a small amount of 67-kDa *cyt-18* protein, which is detectable in the dot blot and in some immunoblots of this fraction prior to AuroDye staining (data not shown). Positions of molecular weight markers are indicated on the left. Lines to the right indicate positions of *cyt-18* protein in immunoblots and cytochrome *c* added as carrier prior to TCA precipitation.

synthetase activity bound to a phosphocellulose column with 0.3 M KCl plus *E. coli* tRNA^{Tyr} (20 OD₂₆₀ [optical density at 260 nm] units), a procedure that has been used for purification of some *E. coli* synthetases (37). In this case, only a small proportion of the mitochondrial tyrosyl-tRNA synthetase activity was eluted with *E. coli* tRNA^{Tyr} (approximately 25% of the synthetase activity bound to the column), and it contained no detectable splicing activity. Splicing activity was eluted subsequently at 0.5 M KCl along with the major peak of synthetase activity (data not shown).

Considered together, the results of phosphocellulose chromatography show that the *cyt-18* protein can exist in at least three different forms or complexes, one that does not bind to phosphocellulose and two eluted at 0.45 M KCl. The different chromatographically resolved components have either splicing or tyrosyl-tRNA synthetase activity or both.

Heparin-Sepharose. Heparin-Sepharose also resolved mitochondrial tyrosyl-tRNA synthetase activity into two major components, one that does not bind to the column in 0 M KCl (Fig. 6, fractions 2 to 8) and the other eluted by 0.5 M KCl (Fig. 6, fractions 65 to 69). The recovery of synthetase activity was substantially greater than that from phosphocellulose columns. As in the phosphocellulose columns, the ratio of splicing to synthetase activity was much higher in the 0.5 M KCl fractions, but in this case, a small amount of splicing activity could also be detected in the 0 M KCl fractions, possibly reflecting incomplete adsorption to Heparin-Sepharose. Fractions trailing the 0.5 M KCl peak of synthetase activity have relatively high ratios of splicing to synthetase activity (e.g., fractions 70 and 71) and may reflect the third component resolved on phosphocellulose columns. As before, all fractions that have either splicing or synthetase activity contain *cyt-18* protein identified immunochemically. Figure 6 also shows that neither synthetase nor splicing activity could be eluted from Heparin-Sepharose by washing the column with equilibration buffer containing the ATP and tyrosine substrates of the aminoacylation reaction.

DEAE-Sephacel. Figure 7 shows fractionation of RNP proteins on a DEAE-Sephacel column. In this experiment, large amounts of synthetase and splicing activity were present in the flowthrough (fractions 1 to 6). A major peak of synthetase activity was eluted from the column at 0.25 M KCl (Fig. 7, fractions 27 to 30) and minor peaks at 0.1 M KCl (Fig. 7, fractions 16 to 19) and 0.5 M KCl (Fig. 7, fractions 41 and 42). All three peaks contained *cyt-18* protein detected immunochemically, and all three peaks had splicing activity roughly proportional to the amount of *cyt-18* protein detected immunochemically.

Blue-Sepharose. In initial experiments (data not shown), micrococcal nuclease-digested RNPs were chromatographed on a Blue-Sepharose column equilibrated with 10 mM Tris hydrochloride (pH 7.5)–1 mM DTT–20% glycerol. Under these conditions, most of the splicing and synthetase activity were not adsorbed, but a small peak of splicing and synthetase activity (less than 10%) was eluted with equilibration buffer plus 5 mM ATP and 0.1 mM tyrosine or with the same buffer containing 50 mM KCl. This minor peak was relatively pure and contained *cyt-18* protein detected immunochemically. (See Fig. 11 for SDS-polyacrylamide gel and immunoblot analyses of a fraction from this peak.)

Subsequently, we used different conditions in which nuclease-digested RNPs were adsorbed to the column matrix by incubation overnight on ice in buffer containing 20 mM Tris hydrochloride (pH 7.5)–5 mM MgCl₂–0.5 mM DTT–10% glycerol (Fig. 8). Under these conditions, most of the synthetase activity was bound to the column and was eluted at 0.5 M KCl (Fig. 8A, fractions 39 to 44). A smaller amount of

FIG. 9. Gel filtration analysis of tyrosyl-tRNA synthetase and splicing activities associated with mitochondrial RNPs. Fractions (90 μ l/3 min) were collected after the void volume (up to fraction 30) had passed. Numbers at the top indicate positions of molecular mass standards (kDa) in the same column. Top panel shows tyrosyl-tRNA synthetase activity in 4- μ l portions. Middle panel shows splicing activity assayed with 4- μ l portions of indicated paired fractions. Splicing was assayed with in vitro transcript from pHX-11. Lower panel shows SDS-polyacrylamide gel and immunoblot analyses of proteins in indicated fractions. Immunoblots were probed with C18-2 antibody.

synthetase activity was present in the flowthrough, and a minor peak of synthetase activity was eluted at 0.25 M KCl (fractions 27 to 30, plus trailing fractions). No synthetase activity was eluted by equilibration buffer containing 5 mM ATP plus 1.0 mM tyrosine. The majority of splicing activity was associated with the major synthetase peak eluted at 0.5 M KCl (Fig. 8, fractions 39 to 48), but a small amount of splicing activity was also detected in the small peak of synthetase activity eluted at 0.25 M KCl (Fig. 8, fractions 31 and 32). All fractions having synthetase or splicing activity contained the *cyt-18* protein detected immunochemically.

Gel filtration. To determine the molecular mass of tyrosyl-tRNA synthetase and splicing activity, RNP proteins were separated by gel filtration through Sephacryl S-200 (Fig. 9). Tyrosyl-tRNA synthetase activity eluted as a sharp peak corresponding to a molecular mass of approximately 150 kDa (Fig. 9, fraction 37), and the major peak of splicing activity was detected in fractions 36 to 41, coinciding roughly with the major peak of tyrosyl-tRNA synthetase activity. However, relatively high levels of splicing activity were also detected trailing the main peak up to at least fraction 54. Immunoblots showed that the *cyt-18* protein peaks at fractions 36 to 38 but is detectable throughout the column. The presence of inactive *cyt-18* protein in higher-molecular-weight fractions may reflect aggregation, whereas its presence in trailing fractions may reflect dissociation of dimers to monomers, interaction of the *cyt-18* protein with

FIG. 10. Gel filtration on Sephacryl S-200, followed by phosphocellulose column chromatography of tyrosyl-tRNA synthetase and splicing activity. (A) Gel filtration of nuclease-digested RNPs through Sephacryl S-200 was carried out as in Fig. 9, except that the column buffer contained 50 mM KCl. Numbers at the top indicate positions of molecular mass standards (kDa). Top panel shows tyrosyl-tRNA synthetase activity in 4- μl portions of fractions. Middle panel shows splicing activity in 4- μl portions of the indicated paired fractions, assayed by using the in vitro transcript from pHX-11. Lower panel shows dot blot analysis of 6- μl portions of the fractions. (B) Phosphocellulose column chromatography of pooled Sephacryl S-200 fractions (fractions 35 to 40, indicated with a bar in A). A 300- μl portion of the pooled Sephacryl S-200 fractions was loaded on a 1.5-ml phosphocellulose column equilibrated with 0.05 M KCl–20 mM sodium phosphate buffer (pH 6.5)–1 mM EDTA–0.5 mM DTT. The column was washed with five column volumes of 0.05, 0.3, and 0.5 M KCl in 20 mM phosphate buffer (pH 6.5)–1 mM EDTA–0.5 mM DTT. Fractions (250 μl) were collected. Panel B shows tyrosyl-tRNA synthetase activity in 4- μl portions of indicated fractions. (C) Splicing assay with 4- μl portions of selected fractions. Splicing was assayed by using in vitro transcript from pHX-11. (D) SDS-polyacrylamide gel and immunoblot analyses of proteins in indicated fractions. Left lanes show immunoblot probed with antibody C18-2. Right lanes show immunoblot after staining with AuroDye to reveal other proteins. Positions of molecular mass markers are indicated on the left.

the column, or both. Neither tyrosyl-tRNA synthetase activity nor splicing activity was detected in the void volume.

The column profile and the molecular weight of the major peak of tyrosyl-tRNA synthetase activity were essentially the same with column buffers containing either 50 or 250 mM KCl (cf., Fig. 9 and 10A). However, loading of larger amounts of protein (more than 700 μg) caused the major peak of synthetase and splicing activity to shift to higher molecular masses (approximately 200 kDa or higher), presumably reflecting aggregation. When peak fractions of synthetase activity from these more heavily loaded columns were rerun on the same column under identical conditions, synthetase activity reemerged as a sharp peak at 150 kDa (data not shown). The finding that the major peak of synthetase activity has a molecular mass of 150 kDa suggests that the active form of mitochondrial tyrosyl-tRNA synthetase is a homodimer, as are bacterial tyrosyl-tRNA syn-

thetases (33). The trailing of splicing activity to lower molecular weights suggests, but does not prove, that monomers of the *cyt-18* protein may be sufficient for splicing activity. Substantial splicing activity is present in fractions 44 to 46, the position expected for monomers (Fig. 9).

Sequential gel filtration and phosphocellulose columns. Figure 10 shows an experiment in which fractions from a gel filtration column, corresponding to the major peak of tyrosyl-tRNA synthetase activity at 150 kDa, were pooled as shown and then chromatographed on a phosphocellulose column. The phosphocellulose column (Fig. 10B) gave two peaks of tyrosyl-tRNA synthetase activity, one in the column flowthrough (0.05 M KCl; fractions 26 to 28) and one at 0.5 M KCl (peak fractions 38 to 44). Thus, the putative dimer peak from the gel filtration columns contains the two major components of synthetase activity resolved on phosphocellulose columns, and these components are stable through an

intermediate gel filtration step. These results show that the *cyt-18* protein in the 0 M KCl peak is not stably associated with a large complex that prevents its binding to phosphocellulose. As before, splicing activity was associated with the peak of synthetase activity eluted at 0.5 M KCl but not with synthetase activity in the flowthrough fraction (Fig. 10C). Immunoblots of SDS-polyacrylamide gels showed that phosphocellulose column fractions having splicing activity contain the 67-kDa *cyt-18* protein as the major constituent and relatively few other proteins (Fig. 10D).

For unknown reasons, the column in Fig. 10A and some other Sephacryl columns (not shown) gave an additional peak of apparent synthetase activity at low molecular weight (Fig. 10A, fraction 62). This peak contains little, if any, *cyt-18* protein.

Copurification of *cyt-18* protein with splicing activity. The results given above show that *cyt-18* protein copurifies with splicing activity through several different types of chromatographic procedures. Figure 11 shows SDS-polyacrylamide gel and immunoblot analyses of selected fractions having splicing activity from the different columns. The combined Western blot plus AuroDye staining may overestimate the amount of *cyt-18* protein relative to other proteins. However, selected fractions were also analyzed by silver staining (Fig. 11C). Fractions having splicing activity contained as few as one or two prominent bands and several lighter bands detected either by colloidal gold or silver staining. All fractions that had splicing activity contained the *cyt-18* protein identified immunochemically, and there were no other prominent bands in common between the different fractions. To avoid missing small polypeptides, tracking dye was run to the bottom of the gel (M_r , 13,000 to 18,000) and

transferred to the Western blots. However, we cannot exclude the possibility that some small proteins were missed.

Inhibition of splicing activity by anti-*cyt-18* antibodies. We also investigated whether the antibody preparations could inhibit splicing activity in vitro. Splicing activity was inhibited by IgG antibody preparations C18-1, C18-2, and C18-5 but not by boiled antibody preparations or the same amount of IgG from preimmune serum (repeated three times) (Fig. 12) or IgG antibodies to an unrelated protein encoded by the Mauriceville mitochondrial plasmid (not shown). Control experiments using whole mitochondrial RNA from nuclear mutant *cyt-18-1*, which accumulates unspliced precursor RNAs, showed that the C18-2 antibody preparation completely inhibited the protein-dependent splicing of the large rRNA intron but did not inhibit self splicing of *cob* intron 1 or the 1.4-kb ND5 intron occurring in the same reaction mixture (data not shown).

We also attempted to carry out antibody inhibition experiments for mitochondrial tyrosyl-tRNA synthetase but could not find inhibitory conditions under which incubation with preimmune IgG antibodies inhibited less than 50% of the synthetase activity in phosphocellulose column fractions. Although the anti-*cyt-18* antibodies gave greater inhibition than preimmune serum, the results were compromised by the high level of inhibition in the preimmune control.

DISCUSSION

Here, we show that a 67-kDa polypeptide, identified immunochemically as the *cyt-18* gene product, copurifies with both mitochondrial tyrosyl-tRNA synthetase and splic-

FIG. 11. SDS-polyacrylamide gel and immunoblot analyses of fractions having splicing activity from different columns. (A) SDS-polyacrylamide gel and immunoblot analyses of fractions from indicated columns. Immunoblots were probed with antibody C18-2 and stained with AuroDye to reveal other proteins. The DEAE-Sephacel column was run differently from that shown in Fig. 7 but gave the same order of elution of small and large synthetase peaks. DEAE-Sephacel fractions 45 and 46 are from the major peak of tyrosyl-tRNA synthetase activity, and fractions 33 and 34 are from the smaller, earlier-eluting peak of tyrosyl-tRNA synthetase activity. The Blue-Sepharose column was run under the alternative conditions described in Materials and Methods, with column buffer containing 10 mM Tris-hydrochloride (pH 7.5)–1 mM DTT. The fraction shown was eluted with 5 mM ATP–0.1 mM tyrosine–50 mM KCl. The phosphocellulose column fractions (PC) are from the column shown in Fig. 5. The fraction from sequential Sephacryl S-200 plus phosphocellulose columns is from the column shown in Fig. 10. Positions of *cyt-18* protein and cytochrome *c* are indicated on each gel. Numbers on the left indicate positions of molecular mass markers. (B) Splicing assays with 4 μ l of DEAE-Sephacel and Blue-Sepharose column fractions (from panel A) with in vitro transcript from pHX-11. The position of the excised intron is indicated on each gel. (C) SDS-polyacrylamide gel electrophoresis and silver staining of fractions having splicing activity. Fractions were eluted with 0.45 M KCl from a phosphocellulose (PC) column or from sequential Sephacryl S-400 plus phosphocellulose columns. For the PC column fraction, the left lane is a silver-stained SDS polyacrylamide gel and the right lane is an immunoblot of a parallel lane probed with C18-2 antibody. The position of *cyt-18* protein is indicated on the left. Numbers on the right indicate positions of molecular weight mass markers.

ing activity through different column chromatographic procedures, including phosphocellulose, Heparin-Sepharose, DEAE-Sephacel, Blue-Sepharose, and gel filtration through Sephacryl S-200. Some fractions having splicing activity contain only one or two prominent polypeptides and several lighter bands, and the 67-kDa *cyt-18* protein is among the few, if not only, major bands in common between different fractions that have splicing activity. Gel filtration experiments show that splicing activity has a relatively small molecular mass (peak at molecular mass, 150 kDa) and does not require association of the *cyt-18* protein with a large protein or RNP complex. Finally, antibodies against different segments of the *cyt-18* protein inhibit splicing of the large rRNA intron in vitro. Considered together, our results

FIG. 12. Inhibition of splicing activity by anti-*cyt-18* antibodies. Splicing activity was purified by phosphocellulose chromatography and incubated with antibodies as described in Materials and Methods. Splicing assays were performed under standard conditions, except that the KCl concentration was 140 mM. (A) Inhibition of splicing by 4 μ l of C18-1 antibody. Lanes labeled control, preimmune, and C18-1, Boiled show incubations with 4 μ l of either distilled H₂O, preimmune IgG antibodies, or boiled antibodies. Splicing was assayed using in vitro transcript synthesized from pHX-11. Sizes in kilobases are indicated at the left. (B) Inhibition of splicing by antibody preparations C18-1 (1 μ l), C18-2 (2 μ l), and C18-5 (4 μ l). Controls show incubations with 4 μ l of distilled H₂O or the same amount of boiled antibody. In other experiments, 4 μ l of C18-5 antibody inhibited splicing completely. Splicing in part B was assayed with in vitro transcript synthesized from pHX9422. The data shown are representative of four experiments with C18-1 and C18-2 and two with C18-5. The amount of C18-1 and C18-2 antibodies required for complete inhibition of splicing ranged from 1 to 4 μ l. A 4- μ l volume of C18-5 was required for inhibition of splicing. The position of the excised intron is indicated at the right.

indicate that the *cyt-18* gene product is a key constituent of splicing activity, that it functions directly in the splicing reaction, and that few, if any, additional components are required for splicing the large rRNA intron.

Our results also show that both splicing and synthetase activity are associated with the same 67-kDa polypeptide encoded by the *cyt-18* gene. However, this polypeptide appears to exist in at least three different forms or complexes that have splicing or synthetase activity or both (Fig. 5). The different chromatographically resolved forms of the *cyt-18* protein could reflect covalent modifications, differences in quaternary structure, or additional tightly bound components. Furthermore, it is not yet clear whether the different forms are related to regulation of the protein in vivo or are generated in vitro during isolation of the protein. Since immunoblots of SDS-polyacrylamide gels showed no difference in the mobility of the *cyt-18* protein associated with the different chromatographically resolved activities (Fig. 5), and since the *cyt-18* protein runs as a single spot in two-dimensional gels (Fig. 3), we infer that any covalent modification affecting activity of the protein would have to be small and not result in a charge change or else it would have to affect only a small proportion of the protein not detectable in two-dimensional gels. Tightly bound components that might affect activity could include a small number of additional polypeptides, cofactors, or substrates of the synthetase or splicing reaction. In addition, the form of the *cyt-18* protein

that lacks splicing activity and does not bind to phosphocellulose columns could be generated in vitro by denaturation of a specific region of the protein or by tight binding of small RNA fragments resulting from micrococcal nuclease digestion.

There is some indication that the form of the *cyt-18* protein that has splicing but not synthetase activity may correspond to monomers of the synthetase polypeptide. In gel filtration experiments, the major peak of splicing activity has a molecular mass of 150 kDa and is coincident with the major peak of tyrosyl-tRNA synthetase activity. Synthetase activity elutes as a sharp peak at 150 kDa and presumably corresponds to homodimers of the synthetase polypeptide, which are the active form of the synthetase in *E. coli* and *Bacillus stearothermophilus* (33). By contrast, splicing activity and the 67-kDa *cyt-18* protein trail to lower molecular weights, most likely reflecting dissociation of dimers to monomers. If so, the results suggest that dimers of the *cyt-18* protein are required for synthetase activity, whereas monomers may be sufficient for splicing activity. The component resolved on phosphocellulose columns, which has splicing activity but not synthetase activity (fractions at the end of the 0.45 M KCl peak; Fig. 5), may also consist of *cyt-18* monomers which could conceivably have higher affinity for phosphocellulose columns due to exposure of positively charged residues. In addition, dissociation of subunits could account for the finding that synthetase activity is less stable than splicing activity. In the case of bacterial tyrosyl-tRNA synthetases, monomers are capable of binding tRNA^{Tyr}, even though they have no synthetase activity (19). Thus, synthetase monomers may still be capable of binding intron RNA to facilitate splicing even though they are not active in aminoacylation. The association and dissociation of synthetase monomers may be one means by which the two activities of the *cyt-18* protein are regulated in vivo.

The *cyt-18-1* and *cyt-18-2* mutants grown at 37°C are grossly deficient in both splicing and mitochondrial tyrosyl-tRNA synthetase activity (1, 13) but contain relatively high amounts of *cyt-18* protein detected immunochemically in mitochondrial lysates and RNP preparations. Thus, the mutations must affect primarily the activity of the protein rather than its synthesis or transport into mitochondria.

Surprisingly, the present work shows that the activity required for splicing the large rRNA intron is relatively small (150 kDa by gel filtration) and consists of the *cyt-18* protein plus few, if any, other constituents. We reported previously that the *cyt-18* mutants are defective in splicing not only the large rRNA intron but also a number of additional group I introns including both introns in the *cob* gene, the 1.4-kb ATPase 6 intron, and optional introns in the *col* gene (6, 25). Recent experiments have shown that the 0.45 M KCl-phosphocellulose column fractions that splice the large rRNA intron are capable of splicing at least some additional group I introns (e.g., ND5 intron 2 and probably the intron in ND1). However, these fractions gave no detectable splicing of other introns, including *cob* intron 2 or the ATPase 6 intron, both of which contain open reading frames that potentially encode maturases (Akins and Lambowitz, unpublished data). A likely interpretation is that splicing of some group I introns, including the large rRNA intron, can be carried out by the relatively simple activity described here, whereas splicing of other group I introns requires both the *cyt-18* protein and one or more additional polypeptides. The different polypeptides may bind separately to the intron or as a pre-formed complex.

In *Saccharomyces cerevisiae*, recent studies have implicated mitochondrial leucyl-tRNA synthetase in splicing of

cob intron 4 and *oxi3* intron 4, two closely related introns that encode maturases. Initially, P. P. Slonimski and co-workers showed that dominant mutations in nuclear gene NAM2 could suppress the splicing defect in mitochondrial DNA mutants defective in *cob* intron 4 maturase. Cloning and sequencing of the NAM2 gene showed that it encodes a protein with synthetase-like characteristics (22), and this protein was subsequently identified as mitochondrial leucyl-tRNA synthetase (16). The situation is complicated, however, because the ability of NAM2 mutations to restore splicing requires the presence of the latent maturase encoded by *oxi3* intron 4, which is ordinarily inactive in splicing but can be activated by certain missense mutations (9). Thus, based on results published thus far, the mitochondrial leucyl-tRNA synthetase could activate the *oxi3* intron 4 maturase either directly by protein-protein interaction or indirectly by increasing the frequency of misacylation, leading to synthesis of a small amount of active maturases.

Assuming that the *S. cerevisiae* mitochondrial leucyl-tRNA synthetase functions directly in splicing, the results for *S. cerevisiae* and *N. crassa* can be reconciled in at least two ways. First, the different aminoacyl-tRNA synthetases may interact with each other in a complex analogous to the synthetase complex that exists in mammalian cells (8). The gel filtration experiments presented here (Fig. 9 and 10) show that *N. crassa* splicing activity, solubilized by digestion of RNPs with micrococcal nuclease, is relatively small (molecular mass, 150 kDa). However, it remains possible that splicing activity in vivo is part of a larger protein or RNP complex and that this association is required for or enhances splicing activity. In that case, tyrosyl-tRNA synthetase by itself or in association with a few other proteins may be sufficient for splicing in vitro, but its activity or stability in vivo may be modulated by association with mitochondrial leucyl-tRNA synthetase or other mitochondrial synthetases.

Alternatively, different aminoacyl-tRNA synthetases may function in splicing different group I introns in *N. crassa*, *S. cerevisiae*, and other organisms. A possible analogy would be different plant RNA viruses, which have related tRNA-like structures at their 3' ends but are aminoacylated by different synthetases (14, 32). Despite having a common conserved secondary structure, group I introns have diverged considerably in primary structure and also have optional stems and loops that could permit interaction with different proteins. Base-pairing interactions involving the IGS and elements P3 [5'] and P3 [3'], which are not conserved in sequence in different group I introns, could also provide binding sites for intron-specific proteins. The finding that different aminoacyl-tRNA synthetases may be adapted for splicing different introns in *N. crassa* and *S. cerevisiae* could indicate that these interactions evolved relatively late in evolution after the divergence of *Saccharomyces* and *Neurospora* spp.

ACKNOWLEDGMENTS

We thank Richard Swenson from the Ohio State University and Gianni Piperno from Rockefeller University for their comments on the manuscript and Murray Fraser from McGill University for antisera against *Neurospora* endo-exonuclease.

This work was supported by Public Health Service grant GM37951 from the National Institutes of Health.

LITERATURE CITED

1. 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.

2. 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.
3. 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.
4. Burke, J. M., M. Belfort, T. R. Cech, R. W. Davies, R. J. Schweyen, D. A. Shub, J. W. Szostak, and H. F. Tabak. 1987. Structural conventions for group I introns. *Nucleic Acids Res.* 15:7217-7221.
5. Cech, T. R., and B. L. Bass. 1986. Biological catalysis by RNA. *Annu. Rev. Biochem.* 55:599-629.
6. Collins, R. A., and A. M. Lambowitz. 1985. RNA splicing in *Neurospora* mitochondria. Defective splicing of mitochondrial mRNA precursors in the nuclear mutant *cyt18-1*. *J. Mol. Biol.* 184:413-428.
7. Davis, R. H., and F. J. de Serres. 1970. Genetic and microbiological research techniques for *Neurospora crassa*. *Methods Enzymol.* 17A:79-143.
8. Deutscher, M. P. 1984. The eukaryotic aminoacyl-tRNA synthetase complex: suggestions for its structure and function. *J. Cell Biol.* 99:373-377.
9. Dujardin, G., C. Jacq, and P. P. Slonimski. 1982. Single base substitution in an intron of oxidase gene compensates splicing defects of the cytochrome *b* gene. *Nature (London)* 298:628-632.
10. Ey, P. L., S. J. Prowse, and C. R. Jenkin. 1978. Isolation of pure IgG1, IgG2a and IgG2b immunoglobulins from mouse serum using protein A-sepharose. *Immunochemistry* 15:429-436.
11. 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.
12. Garriga, G., and A. M. Lambowitz. 1984. RNA splicing in *Neurospora* mitochondria: self-splicing of a mitochondrial intron in vitro. *Cell* 39:631-641.
13. Garriga, G., and A. M. Lambowitz. 1986. Protein-dependent splicing of a group I intron in ribonucleoprotein particles and soluble fractions. *Cell* 46:669-680.
14. Haenni, A.-L., S. Joshi, and F. Chapeville. 1982. tRNA-like structures in the genomes of RNA viruses. *Prog. Nucleic Acid Res. Mol. Biol.* 27:85-104.
15. Hanahan, D. 1985. Techniques for transformation of *E. coli*, p. 109-135. In D. M. Glover (ed.), *DNA cloning: a practical approach*, vol. 1. IRL Press Limited, Oxford.
16. Herbert, C. J., M. Labouesse, G. Dujardin, and P. P. Slonimski. 1988. The NAM2 proteins from *S. cerevisiae* and *S. douglasii* are mitochondrial leucyl-tRNA synthetases, and are involved in mRNA splicing. *EMBO J.* 7:473-483.
17. Herzog, N. K., and H. R. Bose, Jr. 1986. Expression of the oncogene of avian reticuloendotheliosis virus in *Escherichia coli* and identification of the transforming protein in reticuloendotheliosis virus T-transformed cells. *Proc. Natl. Acad. Sci. USA* 83:812-816.
18. Hill, J., P. McGraw, and A. Tzagoloff. 1985. A mutation in yeast mitochondrial DNA results in a precise excision of the terminal intron of the cytochrome *b* gene. *J. Biol. Chem.* 260:3235-3238.
19. Jones, D. H., A. J. McMillan, A. R. Fersht, and G. Winter. 1985. Reversible dissociation of dimeric tyrosyl-tRNA synthetase by mutagenesis at the subunit interface. *Biochemistry* 24:5852-5857.
20. Kleid, D. G., D. Yansura, B. Small, D. Dowbenko, D. M. Moore, M. J. Grubman, P. D. McKercher, D. O. Morgan, B. H. Robertson, and H. L. Bachrach. 1981. Cloned viral protein vaccine for foot-and-mouth disease: responses in cattle and swine. *Science* 214:1125-1128.
21. Kreike, J., M. Schulze, T. Pillar, A. Korte, and G. Rodel. 1986. Cloning of a nuclear gene *MRS1* involved in the excision of a single group I intron (bI3) from the mitochondrial *COB* transcript in *S. cerevisiae*. *Curr. Genet.* 11:185-191.
22. Labouesse, M., C. J. Herbert, G. Dujardin, and P. P. Slonimski. 1987. Three suppressor mutations which cure a mitochondrial RNA maturase deficiency occur at the same codon in the open reading frame of the nuclear NAM2 gene. *EMBO J.* 6:713-721.
23. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature (London)* 227:680-685.
24. Lambowitz, A. M. 1979. Preparation and analysis of mitochondrial ribosomes. *Methods Enzymol.* 59:421-433.
25. Lambowitz, A. M., R. A. Akins, G. Garriga, M. Henderson, A. R. Kubelik, and K. A. Maloney. 1985. Mitochondrial introns and mitochondrial plasmids of *Neurospora*, p. 237-247. In E. Quagliariello, E. C. Slater, R. Palmieri, C. Saccone, and A. M. Kroon (ed.), *Achievements and perspectives of mitochondrial research*, vol. 2: biogenesis. Elsevier Science Publishers B.V., Amsterdam.
26. Lazowska, J., C. Jacq, and P. P. Slonimski. 1980. Sequence of introns and flanking exons in wild-type and *box3* mutants of cytochrome *b* reveals an interlaced splicing protein coded by an intron. *Cell* 22:333-348.
27. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
28. 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. USA* 76:2635-2639.
29. Merrill, C. R., D. Goldman, S. A. Sedman, and M. H. Ebert. 1981. Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. *Science* 211:1437-1438.
30. O'Farrell, P. Z., H. M. Goodman, and P. H. O'Farrell. 1977. High resolution two-dimensional electrophoresis of basic as well as acidic proteins. *Cell* 12:1133-1142.
31. Pittenger, T. H., and D. J. West. 1979. Isolation and characterization of temperature-sensitive respiratory mutants in *Neurospora crassa*. *Genetics* 93:539-556.
32. Rietveld, K., C. W. A. Pleij, and L. Bosch. 1983. Three-dimensional models of the tRNA-like 3' termini of some plant viral RNAs. *EMBO J.* 2:1079-1085.
33. 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.
34. Spindler, K. R., D. S. E. Rosser, and A. J. Berk. 1984. Analysis of adenovirus transforming proteins from early regions 1A and 1B with antisera to inducible fusion antigens produced in *Escherichia coli*. *J. Virol.* 49:132-141.
35. Szostak, J. W. 1986. Enzymatic activity of the conserved core of a group I self-splicing intron. *Nature (London)* 322:83-86.
36. Tabak, H. J., and L. A. Grivell. 1986. RNA catalysis in the excision of yeast mitochondrial introns. *Trends Genet.* 2:51-55.
37. von der Haar, F. 1973. Affinity elution as a purification method for aminoacyl-tRNA synthetases. *Eur. J. Biochem.* 34:84-90.
38. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* 244:4406-4412.