MOLECULAR AND CELLULAR BIOLOGY, Feb. 1992, p. 499–511
0270-7306/92/020499-13$02.00/0

Vol. 12, No. 2

The Mitochondrial Tyrosyl-tRNA Synthetase of *Podospora anserina* Is a Bifunctional Enzyme Active in Protein Synthesis and RNA Splicing

UTE KÄMPER,[1,2]† ULRICH KÜCK,[1] ANDREW D. CHERNIACK,[2] AND ALAN M. LAMBOWITZ[2]*

Lehrstuhl für Allgemeine Botanik, Ruhr-Universität Bochum, D-4630 Bochum 1, Germany,[1] and Departments of Molecular Genetics and Biochemistry and the Biotechnology Center, The Ohio State University, 484 West Twelfth Avenue, Columbus, Ohio 43210[2]

Received 30 August 1991/Accepted 6 November 1991

The *Neurospora crassa* mitochondrial tyrosyl-tRNA synthetase (mt tyrRS), which is encoded by the nuclear gene *cyt-18*, functions not only in aminoacylation but also in the splicing of group I introns. Here, we isolated the cognate *Podospora anserina* mt tyrRS gene, designated *yts1*, by using the *N. crassa cyt-18* gene as a hybridization probe. DNA sequencing of the *P. anserina* gene revealed an open reading frame (ORF) of 641 amino acids which has significant similarity to other tyrRSs. The *yts1* ORF is interrupted by two introns, one near its N terminus at the same position as the single intron in the *cyt-18* gene and the other downstream in a region corresponding to the nucleotide-binding fold. The *P. anserina yts1*$^+$ gene transformed the *N. crassa cyt-18-2* mutant at a high frequency and rescued both the splicing and protein synthesis defects. Furthermore, the YTS1 protein synthesized in *Escherichia coli* was capable of splicing the *N. crassa* mt large rRNA intron in vitro. Together, these results indicate that YTS1 is a bifunctional protein active in both splicing and protein synthesis. The *P. anserina* YTS1 and *N. crassa* CYT-18 proteins share three blocks of amino acids that are not conserved in bacterial or yeast mt tyrRSs which do not function in splicing. One of these blocks corresponds to the idiosyncratic N-terminal domain shown previously to be required for splicing activity of the CYT-18 protein. The other two are located in the putative tRNA-binding domain toward the C terminus of the protein and also appear to be required for splicing. Since the *E. coli* and yeast mt tyrRSs do not function in splicing, the adaptation of the *Neurospora* and *Podospora* spp. mt tyrRSs to function in splicing most likely occurred after the divergence of their common ancestor from yeast.

The mitochondrial (mt) genome of the ascomycete *Podospora anserina* contains an extraordinarily large number of introns (17). In race s of *P. anserina*, the mitochondrial DNA (mtDNA) contains 31 group I introns and 2 group II introns; these are found in the mt large rRNA and all protein-coding genes except *ND2* and *ATPase6*. The different group I introns can be classified into subgroups having closely related structures (51). The large number of group I introns in *P. anserina* mtDNA implies that there is either an efficient mechanism for intron dispersal or an inefficient mechanism for intron removal.

The splicing of group I introns occurs via RNA catalyzed transesterification reactions initiated by the addition of guanosine to the 5′ end of the intron RNA (12, 34). These splicing reactions are RNA catalyzed, and a number of group I introns have been shown to self-splice in vitro. Nevertheless, genetic analysis of mtRNA splicing in *Neurospora crassa* and *Sacharomyces cerevisiae* indicates that the splicing of many, if not all, group I introns requires protein factors, which may facilitate correct folding of the catalytically active intron RNAs in vivo (41). Even group I introns that are self-splicing in vitro require protein factors for efficient splicing in vivo (24, 25, 66).

A number of proteins required for splicing group I introns have been identified by analysis of *N. crassa* and yeast mutants defective in mtRNA splicing (41). Some of these proteins, called maturases, are encoded by the introns themselves, whereas others are encoded by nuclear genes. Many of the splicing factors identified thus far appear to be specific for a particular organism, and they include aminoacyl-tRNA synthetases and other host proteins that also function in protein synthesis. On the basis of these findings, we suggested that the adaptation of host proteins to function in splicing may have occurred relatively recently in evolution, possibly reflecting the recent dispersal of the introns themselves (41).

The presence of a large number of group I introns in *P. anserina* mtDNA raises the questions of how these introns are spliced and whether their splicing might be regulated. The splicing of mt introns in *P. anserina* may also be relevant to the phenomenon of senescence, which is influenced by both nuclear and mt genes (36). During aging, the fungus accumulates amplified circular (plasmid-like) DNAs consisting of different regions of the mt genome containing group I or group II introns (16, 50, 55, 67). From work with nuclear longevity mutants lacking amplified plasmid-like molecules (65), it was suggested that nuclear-encoded factors required for mtRNA processing may control the generation of these amplified DNA molecules (37).

Thus far, there has been no information about protein factors required for RNA processing in *P. anserina*, although recently self-splicing of a group I intron (*cytb*-I2) and a group II intron (plDNA) have been demonstrated in vitro (29, 60, 61). In *N. crassa*, a filamentous fungus closely related to *P. anserina*, an important protein required for splicing group I introns is the mitochondrial tyrosyl-tRNA synthetase (mt tyrRS), which is encoded by the nuclear gene *cyt-18* (2). Two temperature-sensitive mutants in the *cyt-18*

* Corresponding author.
† Present address: The Biotechnology Center, Rightmire Hall, The Ohio State University, Columbus, OH 43210-1002.

gene, *cyt-18-1* and *cyt-18-2*, are defective in splicing the mt large rRNA intron and other group I introns as well as in mt tyrRS activity. In the present work, we used the *N. crassa cyt-18* gene as a hybridization probe to clone the cognate *yts1* gene encoding the mt tyrRS from *P. anserina*. We show that the *P. anserina yts1* gene encodes a mt tyrRS, which is also a bifunctional protein active in both mt protein synthesis and RNA splicing.

MATERIALS AND METHODS

Strains and growth conditions. *P. anserina* s (ATCC 26003) was maintained as described previously (20). *N. crassa* strains were wild-type 74*A* (74-OR23-1*A*; FSGC 2489) and mutant *cyt-18-2* (289-67 GG al-2 *A*) (7). Procedures for maintaining *N. crassa* strains, preparing conidia, and growing cells in liquid culture were as described previously (19). For the analysis of mtRNA and cytochrome spectra, *N. crassa* strains were grown at 37°C. Wild-type 74*A* was grown for 11 h. Rapidly growing transformants (i.e., those showing good complementation of the *cyt-18-2* mutation) were also grown for 11 h, whereas slowly growing transformants (i.e., those showing poor complementation of the *cyt-18-2* mutation) were grown for 25 h.

Escherichia coli K803 (21) was used for the amplification of the recombinant λ EMBL4 phage. *E. coli* SMH50 was used to grow the single-stranded M13mp19 phage for dideoxy sequencing (71). *E. coli* JM83 (49) was used for cloning of DNA with the bacterial vectors pUC19 (71), pBS(−) (Stratagene, La Jolla, Calif.), or pT7-7 (64). *E. coli* BL21::DE3(pLysS) or *E. coli* K38 containing plasmid pGP1-2 were used for expression of the YTS1 protein (57, 64).

Recombinant plasmids and bacteriophage. The *P. anserina* genomic library was constructed by ligating *Eco*RI partial digest fragments of nuclear DNA from *P. anserina* s to EMBL4 arms. Recombinant phage were packaged with a commercial DNA packaging system (Gigapack-plus; Genofit, Heidelberg, Germany) and used to infect *E. coli* K803.

Recombinant plasmid pUH1 contains the *yts1* gene (the 5.5-kb *Bam*HI fragment from phage LC54) cloned into the *Bam*HI site of pUC19 (71). pBP6 was constructed by cloning the same 5.5-kb *Bam*HI fragment from phage LC54 into pBS(−) and then deleting a 2.6-kb *Hin*dIII fragment containing *P. anserina* sequences upstream of the 5′ end of the *yts1* open reading frame (ORF).

Recombinant plasmids containing C-terminal truncations of the *yts1* ORF were obtained by digesting pBP6 with the indicated restriction enzyme or with *Bal 31* and recloning the appropriate *yts1* fragment in pUC19. For *Bal 31* treatment, pBP6 was linearized with *Stu*I and digested with *Bal 31* (Boehringer GmbH, Mannheim, Germany) (58). The digests were phenol extracted, 5′ protruding ends were filled in with the Klenow fragment of DNA polymerase I, and the plasmids were religated. The resulting plasmids were then sequenced to determine the extent of degradation. The following plasmids contain the indicated DNA fragments and truncations, where the nt (nucleotide) refers to the position in Fig. 2, and aa (amino acid) refers to the last codon of the *yts1* gene retained in the clone: pBP6-4 (2.3-kb *Sph*I-*Bam*HI, nt 2184, aa 578); pBP6-3 (2.0-kb *Bam*HI-*Stu*I, nt 1884, aa 468); pBP2-45 (*Bal 31* treatment, nt 1814, aa 444); pBP2-55 (*Bal 31* treatment, nt 1721, aa 413); pBP2-29 (*Bal 31* treatment, nt 1638, aa 386); pBP6-2 (1.55-kb *Hin*dIII, nt 1552, aa 358); pBP6-1 (1.4-kb *Eco*RI, nt 1212, aa 246).

Preparation and analysis of DNA. Nuclear DNA was isolated from *P. anserina* and from *N. crassa* transformants as described previously (38). Standard methods were used for isolation of phage DNA (62), preparation of plasmid DNA (9), isolation of single-stranded M13 DNA, restriction enzyme analysis, and Southern hybridization (58). DNA probes were ^{32}P labeled by nick translation (56) or by the random hexamer method (22).

Plaque hybridization. The *P. anserina* genomic library was screened by plaque hybridization with ^{32}P-labeled plasmid p337, which contains the *N. crassa cyt-18* gene (6, 13).

DNA sequencing. For sequencing, restriction fragments of λ clone LC54 were subcloned in M13mp19 to give the following M13 clones: M12-2 and M12-5 (contains a 2.1-kb *Bgl*II-*Bam*HI fragment in both orientations), M14-0 (contains a 1.6-kb *Eco*RI-*Bam*HI fragment), and M16-0 (contains a 1.2-kb *Hin*dIII-*Eco*RI fragment). Nested deletions of the double-stranded M13 clones were constructed by the T4 DNA polymerase method (18), and sequences were determined by the dideoxy method (8, 59). Sequencing was from a universal M13 primer or synthetic oligonucleotides, as shown in Fig. 1, with deoxyadenosine 5′-[α-^{35}S]thiotriphosphate (1,000 mCi/mmol; Amersham Buchler, Braunschweig, Germany), by using Klenow fragment of DNA polymerase I (Boehringer), Sequenase (United States Biochemical Corp., Cleveland, Ohio), or Taq polymerase (Biozyme, Hameln, Germany) (32). Sequences were analyzed by using the computer programs of Lipman and Pearson (44) and Mount and Conrad (53). The complete DNA sequences of both DNA strands were determined experimentally.

Synthesis of oligonucleotides. Oligonucleotides were synthesized by the β-cyanoethyl phosphoramidite method (63), by using an Applied Biosystems model 381A DNA synthesizer. Deprotected oligonucleotides were purified by high-pressure liquid chromatography by using a C18RP analytical column. The following oligonucleotides were used, with the numbers in parenthesis corresponding to their positions in Fig. 2:

5′ GAGCGCATATGAGCATGAGCAG ′ (335–356)
5′ GATCGGTGGCGCGACGGCCA 3′ (829–848)
5′ CCACCAATCCCAACCTTGCAT 3′ (1254–1234)
5′ CTGGATTCCATTTGCCTGATA 3′ (1281–1261)
5′ GCTCGTTATCACGGACGA 3′ (1360–1343)
5′ GTTACTTTGTGCGGCGATCC 3′ (1517–1536)
5′ GCTTGGTGCACGGCATGAACGCG 3′ (1670–1641)
5′ GGCCTGGTGGTTCAAGGC 3′ (1722–1705)
5′ ATGTAGGCACCTTGCTGGGT 3′ (1943–1924)
5′ CTCCTGGGGGAACCAGAG 3′ (2040–2022)
5′ CTGATCCGGAAACTTCATAA 3′ (2310–2291)
5′ GCCAATATCGTACAACTCAG 3′ (2475–2455)

Transformation of *N. crassa*. *N. crassa cyt-18-2* was transformed by using spheroplasts made with Novozym 234 as described previously (1, 13). Spheroplasts were cotransformed with plasmids containing the *yts1*$^+$ or *cyt-18*$^+$ genes plus pBML, which contains the *N. crassa* benomyl resistance (*Bml*^{r}) gene as a second selectable marker. Transformed spheroplasts were suspended in 4 ml of top agar (0.6% Bacto agar [Difco, Detroit, Mich.] containing Vogel's minimal medium, 500 mM $MgSO_4$, 2% sorbose, 0.05% fructose, 0.05% glucose, and 0.01% inositol). The top agar containing the transformed spheroplasts was then overlaid on 1.5% agar plates containing Vogel's minimal medium, 2% sorbose, 0.05% fructose, 0.05% glucose, 0.01% inositol, and 0.5 mg of benomyl per ml. Plates were incubated at room temperature for 10 h to permit regeneration of spheroplasts

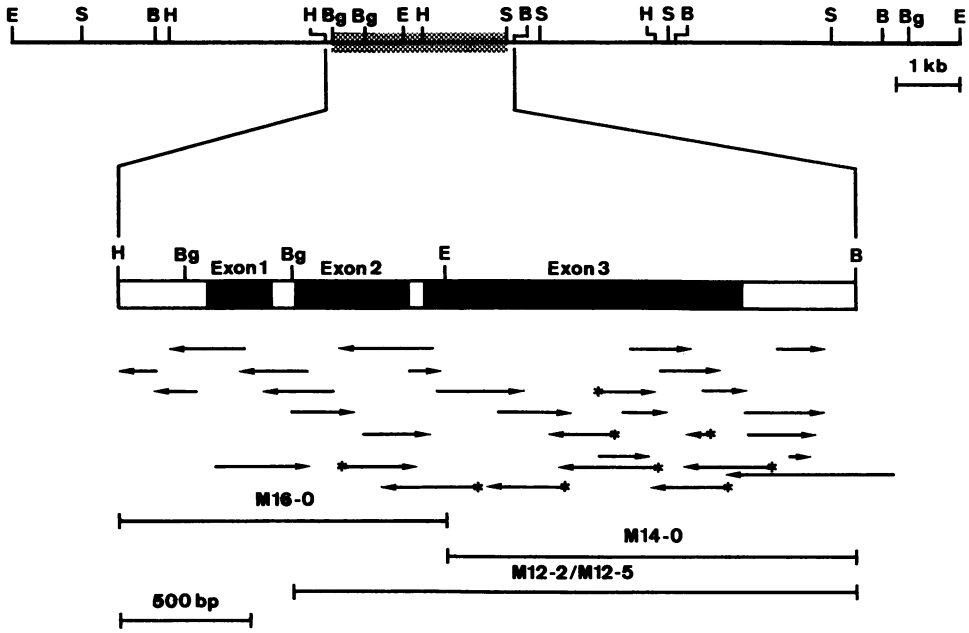

FIG. 1. Physical map of the *P. anserina* genomic region containing the *yts1* gene and strategy for DNA sequence determination. At the top is a restriction map of the 14.6-kb genomic region of λ clone LC54. The shaded fragment corresponds to the region of homology to the *N. crassa cyt-18*$^+$ gene. Below are the 2.8-kb *Hin*dIII-*Bam*HI fragment and subclones used for sequence analysis. Exons are shown in black; arrows indicate the direction and extent of sequencing; synthetic oligonucleotides are indicated by an asterisk. Other sequences were obtained from nested deletion clones by using an M13 primer. Abbreviations: B, *Bam*HI, Bg, *Bgl*II, E, *Eco*RI, H, *Hin*dIII, S, *Sal*I.

in the absence of selection and then transferred to 38.5°C for 4 days (13).

Isolation and analysis of mtRNA. Mitochondria were isolated by the modified flotation gradient method (40), and mtRNA was extracted by the UNSET procedure (25). RNA was denatured with glyoxal and analyzed by electrophoresis in 1.4% agarose gels (48). Gels were stained with ethidium bromide and photographed under 365-nm UV light.

Cytochrome spectra. Reduced − oxidized difference spectra of mitochondria were obtained essentially as described by Nargang et al. (54), by using a Beckman DU-7 spectrophotometer.

Analysis of YTS1 protein synthesized in *E. coli*. The YTS1 protein was expressed in *E. coli* from plasmid pT7-*yts1*, which contains a cDNA analog of the *yts1* gene cloned downstream of the bacteriophage T7 gene φ10 promoter in the expression vector pT7-7 (64). The construction of this plasmid by polymerase chain reaction procedures is described in detail elsewhere (29). pT7-*yts1* was expressed in *E. coli* BL21::DE3(pLysS), which contains an integrated, IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible bacteriophage T7 RNA polymerase gene (57). Induction of the YTS1 protein was followed by immunoblotting, by using anti-CYT-18 antibody C18-2 (45). For unknown reasons, the yield of the YTS1 protein was relatively low compared with that of CYT-18 protein, using either *E. coli* BL21::DE3 (pLysS) or *E. coli* K38 containing plasmid pGP1-2, in which the T7 RNA polymerase gene is expressed from the bacteriophage λ P_L promoter regulated by the temperature-sensitive repressor cI857 (64). The yield did not improve when the *yts1* cDNA was recloned in the pEX501 vector used routinely to express CYT-18 protein (33). The *yts1* cDNA was sequenced completely and found to have no errors introduced during polymerase chain reaction cloning.

For splicing assays, the YTS1 protein synthesized in *E. coli* was partially purified by chromatography in 1 ml of heparin-Sepharose columns, essentially as described for the CYT-18 protein synthesized in *E. coli* (33), except that approximately three times the amount of protein was loaded to compensate for poorer expression of YTS1. Column fractions (0.5 M KCl) containing YTS1 protein were assayed for splicing activity, by using a 698-nucleotide (nt) in vitro transcript containing a 583-nt derivative of the *N. crassa* mt large rRNA intron that is not self-splicing in vitro (27). The in vitro transcript substrate was synthesized from *Ban*I-linearized pHX9422, by using bacteriophage T3 RNA polymerase (GIBCO BRL, Gaithersburg, Md.) as described previously (27). Splicing reactions were carried out by incubating 1 μg of in vitro transcript with 40 μCi of [α-^{32}P]GTP (3,000 Ci per mmol; Dupont, NEN Research Products, Boston, Mass.) and 4 μl of YTS1 column fraction (0.1 μg of protein) in 20 μl of reaction medium containing 100 mM KCl, 20 mM Tris-HCl (pH 7.5), 5 mM dithiothreitol, and 0.25 U of Inhibitase (5 Prime→3 Prime, West Chester, Pa.), and varying concentrations of $MgCl_2$, as indicated in Fig. 8. As a positive control, reactions were carried out with CYT-18 protein (a 2-μl column fraction containing 0.3 μg of protein) synthesized in *E. coli* from plasmid pEX550 and purified in parallel with the YTS1 protein. Reactions were initiated by the addition of protein preparation, incubated for 15 min at 37°C, and terminated by phenol extraction and ethanol precipitation. Products were analyzed by electrophoresis in a 4% polyacrylamide–8 M urea gel followed by autoradiography.

Nucleotide sequence accession number. The EMBL accession number for the sequence presented in this report is X54981.

```
   1  AAGCTTCGGGTCGGACCGGATTTTCTAGACCCCGAGCAGCACCCCTCCAGACGTCACTGTCACAACGCCAGAGCGGGCCCAGTGCGGGGACCCTGTTCTGGGTGGGAGGTG

 112  GCCCGAGCTTTTGGCCCAAAATTTCGAGCTTGGAGCCCGCGACAGCGTCCATCGAGACGTCTGGCGGGTCTTGAATCTAGACGTTTCACTCAACATATCGACATATTACAC

 223  GAGCTCTCACCCTTTTTCAGCAGGTCAAGGTGCCAGATCAGATCTTCGTAGGACTTCGTTGGCGGTAAAGCAACTTCACCACATTCAGCCGCCGTAATGGCGTTCGGAAAA

   1                Met Ser Met Ser Arg Gly Ser Val Cys Arg Arg Cys Leu Leu Thr Met Lys Ser Met Ala Gly Gly Gly Pro Thr
 334  GGAGCGCTC    ATG AGC ATG AGC AGA GGC TCC GTC TGC CGG AGA TGC CTG TTA ACG ATG AAG TCC ATG GCT GGT GGA GGA CCA ACA

  26  Ser Thr Tyr Ala Gln Gln Arg Gly Lys Lys Thr Trp His Gly Pro Lys Tyr Gln Ala Lys Ile Asp Gln Ala Gln Ala Asp Trp
 418  TCA ACA TAC GCT CAA CAA CGC GGC AAA AAG ACA TGG CAC GGC CCC AAG TAC CAG GCG AAG ATT GAC CAG GCA CAG GCT GAT TGG

  54  Glu Glu Arg Ala Glu Lys Ile Lys Lys Gly Glu Ile Gln His Thr Trp Asp Met Phe Val Glu Arg Gly Tyr Val Lys Asp Thr
 502  GAA GAG CGG GCT GAA AAG ATC AAG AAG GGG GAG ATT CAA CAC ACA TGG GAT ATG TTT GTT GAG AGA GGC TAT GTG AAG GAC ACA

  82  Ala Gly                                                                                                 Ser His Glu Thr
 586  GCT GG gtaggtctacaaagtctggcgctgaggtaaacttgaagagcactgttttccggagcagattggctgacttcaacttgggagaaag  A TCT CAT GAA ACT

  88  Ile Arg Lys Leu Met Leu His Lys Arg Ile Gly Ala Tyr Thr Gly Ile Asp Pro Thr Ala Pro Ser Leu His Ile Gly His Leu
 689  ATT CGC AAA CTG ATG CTT CAC AAA CGG ATT GGC GCC TAC ACT GGC ATT GAC CCA ACG GCG CCC TCT CTA CAC ATT GGC CAT TTG

 116  Leu Pro Leu Met Pro Ile Phe Trp Met Tyr Met His Gly Tyr Ala Gly Tyr Thr Leu Ile Gly Gly Ala Thr Ala Lys Ile Gly
 773  CTC CCG CTC ATG CCG ATT TTC TGG ATG TAT ATG CAC GGT TAC GCT GGT TAC ACT CTG ATC GGT GGC GCG ACG GCC AAG ATT GGC

 144  Asp Pro Thr Asp Arg Leu Val Ser Arg Thr Pro Leu Lys Arg Thr Asp Leu Thr Met Asn Leu Thr Lys Ile His Tyr Gln Leu
 857  GAC CCT ACT GAC CGG TTG GTC AGC CGC ACG CCT CTC AAA AGG ACC GAC CTC ACC ATG AAT TTG ACC AAG ATA CAC TAC CAA CTC

 172  Lys Ala Leu Trp Met Asn Val Glu Glu Gln Ala Arg Arg Arg Gly Phe Glu Lys Asp Trp Ala Trp Lys Arg Ala Val Val Asn
 941  AAG GCC CTC TGG ATG AAT GTG GAA GAA CAG GCA AGG AGG CGG GGC TTC GAG AAG GAT TGG GCA TGG AAA CGG GCT GTT GTG AAC

 200  Asn Ser Thr Trp Trp Asn Ser Leu Pro Leu Ile Glu Val Leu Lys Arg Leu Gly Asp Ser Met Arg Met Gly Pro Leu Leu Ser
1025  AAC TCT ACA TGG TGG AAC TCG CTT CCT CTG ATC GAG GTT CTC AAG AGG TTA GGG GAT AGT ATG AGA ATG GGT CCC TTG CTG TCC

 228  Arg Asp Thr                                                                  Val Lys Asn Lys Met Ser Lys Gly Asp Gly
1109  CGC GAT AC gtaagtgttgatttcaggcattcatcggcaggcgcattgctgaccttctctcacag G   GTC AAG AAC AAA ATG TCG AAA GGC GAT GGC

 241  Met Ser Phe Ser Glu Phe Thr Tyr Pro Leu Met Gln Gly Trp Asp Trp Trp His Met Tyr Gln Ala Asn Gly Ile Gln Met Gln
1204  ATG TCC TTC TCA GAA TTC ACC TAC CCG CTC ATG CAA GGT TGG GAT TGG TGG CAT ATG TAT CAG GCA AAT GGA ATC CAG ATG CAG

 269  Ile Gly Gly Ser Asp Gln Tyr Gly Asn Ile Val Thr Gly Val Glu Thr Val Lys Val Val Arg Asp Asn Glu Pro Asp Pro Ala
1288  ATC GGT GGC TCC GAC CAG TAC GGA AAC ATC GTG ACC GGC GTC GAG ACG GTG AAA GTC GTC CGT GAT AAC GAG CCA GAT CCG GCG

 297  Lys Lys Ile Glu Gly Gly Pro Phe Asn Asp Pro Val Gly Phe Thr Val Pro Leu Leu Thr Asp Ser Ala Gly Val Lys Phe Gly
1372  AAA AAA ATT GAA GGT GGT CCC TTC AAC GAT CCG GTC GGC TTC ACC GTC CCC CTC TTA ACA GAC TCA GCC GGT GTC AAG TTT GGA

 325  Lys Ser Ala Gly Asn Ala Val Trp Leu Asp Lys Phe Gln Thr Ser Glu Phe Asp Leu Tyr Gly Tyr Phe Val Arg Arg Ser Asp
1456  AAG AGC GCC GGC AAT GCC GTT TGG CTT GAC AAG TTC CAG ACT TCC GAG TTC GAC CTT TAC GGT TAC TTT GTG CGG CGA TCC GAT

 353  Gln Glu Val Glu Lys Leu Leu Lys Leu Phe Thr Phe Leu Pro Met Glu Asn Ile Asn Glu Ala Met Lys Ile His Ser Glu Asn
1540  CAA GAG GTC GAG AAG CTT CTC AAG CTC TTC ACC TTC CTG CCT ATG GAG AAT ATC AAC GAG GCC ATG AAG ATC CAC AGC GAG AAC

 381  Pro Ala Arg Arg Val Ala Gln His Leu Leu Ala Phe Glu Val Val Gly Leu Val His Gly Met Asn Ala Ala His Arg Thr Ala
1624  CCC GCC CGA CGA GTT GCT CAA CAT CTG CTG GCC TTT GAG GTA GTC GGC TTG GTG CAC GGC ATG AAC GCG GCG CAT AGG ACC GCC

 409  Leu Asn His Gln Ala Arg Tyr Gly Lys Gln Ile Asp Ile Pro Gly Val Thr Leu Arg Met Pro Lys Ala Ala Thr Glu Asp Thr
1708  TTG AAC CAC CAG GCC AGG TAC GGA AAG CAA ATC GAC ATC CCC GGC GTC ACG CTC AGG ATG CCC AAG GCA GCA ACC GAG GAC ACG

 437  Pro Pro Ser Ile Leu Asp Ala Pro Lys Met Asp Met Gln Leu Pro Glu Ser Leu Ile Met Gly Lys Ser Ile Gly Arg Ile Leu
1792  CCA CCC TCC ATT CTA GAC GCC CCC AAG ATG GAC ATG CAA CTG CCC GAA TCT CTC ATC ATG GGC AAA TCC ATC GGC CGC ATC CTC

 465  Tyr Ala Ala Gly Leu Ala Lys Ser Ala Ser Glu Gly His Arg Leu Ala Thr Gln Gln Gly Ala Tyr Ile Gly Ala Met Pro Gly
1876  TAC GCC GCA GGC CTC GCA AAG AGC GCC TCA GAA GGC CAC CGC CTC GCC ACC CAG CAA GGT GCC TAC ATT GGC GCC ATG CCT GGC

 493  His Lys Arg Thr Glu Asp Asn Lys Val Met Asp Tyr Ser Gln Leu Ser Phe Thr Pro Ile Lys Leu Trp Phe Pro Gln Glu Thr
1960  CAC AAG CGC ACC GAG GAC AAC AAG GTG ATG GAC TAC TCC CAG CTC AGC TTC ACA CCC ATC AAG CTC TGG TTC CCC CAG GAG ACG

 521  Arg Asn Tyr Leu Ile Asp Gly Lys Leu Leu Ile Leu Arg Lys Gly Lys Val Gln Ile Arg Val Ile Glu Met Val Ser Asp Glu
2044  AGG AAC TAC CTC ATC GAC GGC AAA TTG CTC ATC CTC CGC AAG GGC AAG GTC CAG ATT CGC GTC ATT GAG ATG GTC AGC GAC GAG

 549  Glu Trp Lys Glu Ser Gly Gln Thr Tyr Pro Gly Glu Pro Gly Thr Gly Ala Leu Arg Met Leu Arg Gln Gln Leu Lys Met Leu
2128  GAG TGG AAG GAG TCT GGT CAG ACG TAC CCT GGC GAG CCG GGG ACC GGT GCG CTG CGC ATG CTT CGC CAG CAG TTG AAG ATG CTG

 577  Lys Ser Gly Met Leu Thr Pro Asp Glu Val Lys Ala Asn Leu Lys Asn His Val Glu Glu Glu Ala Pro Pro Pro Gly Phe Met
2212  AAG TCG GGG ATG CTG ACG CCG GAC GAG GTC AAG GCC AAC TTG AAG AAC CAT GTC GAG GAG GAG GCG CCG CCG CCT GGG TTT ATG

 605  Lys Phe Pro Asp Gln Asp Ser Tyr Ala Ile Arg Arg Ala Thr Gln Glu Leu Met Asp Glu Ile His Gln Lys Glu Val Gly Gly
2296  AAG TTT CCG GAT CAG GAC TCT TAT GCT ATT AGG AGG GCG ACT CAG GAG CTG ATG GAT GAG ATT CAC CAG AAG GAG GTA GGG GGT

 633  Asp Ser Pro Arg Glu Glu Arg Arg Glu  *   *   *
2380  GAT TCG CCG AGG GAA GAG AGG AGG GAA TGA TGA TGA GTTGTAGAGGGGGATAGCCATCGTTGATCTGGGTTTTCATCTGAGTTGTACGATATTGGCTTG

2479  TATTATATCAGATGCATTATACTCTATTGTAGATTTTCGTATGTGTGCACATGAACTGGGATGAAGAGTTGGAGGTAGAAACGTAGGTAGATAAAAAAGCTCCAATCCAAA

2590  TACGTTTGCGGACCAAGATAAAAGAAAGATTGCTACTCCAAATAGGTAGGCCTCCTCGCTGCTAGACCTTCTCAGTATTTTCCCCCAGAGATTTCTGATCTTTTTTTCCGA

2701  AAGCCTACATCATCCCTTTGCCCGCATCATCAGCGATGGAGCACCGTCAAATCCCCCGCCGG
```

FIG. 2. Sequence of the *yts1*$^+$ gene and flanking regions. Exon sequences are indicated by capital letters, introns are indicated in small letters, and termination codons are marked by asterisks.

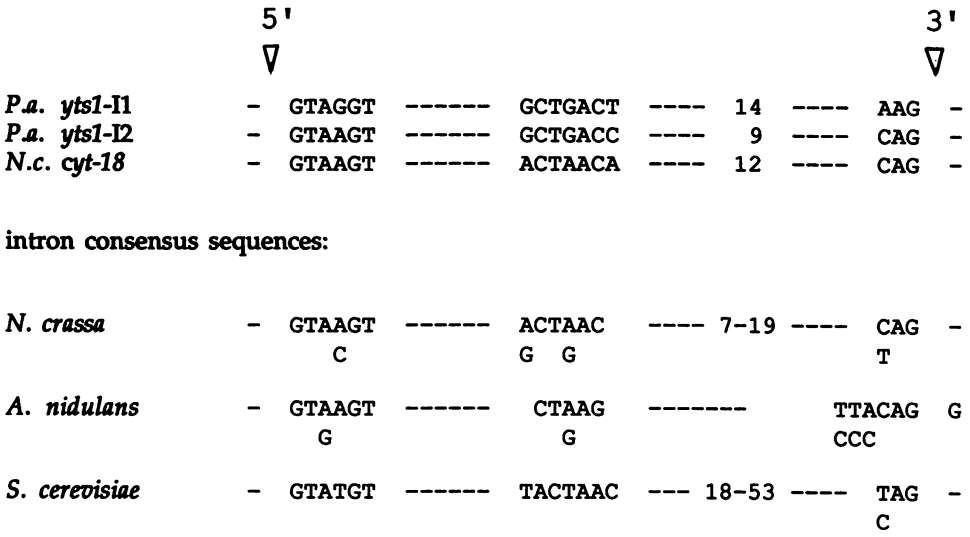

FIG. 3. Comparison of intron sequences in the *yts1* gene with those in other fungi. The intron sequence for the *N. crassa cyt-18* gene is from Akins and Lambowitz (2). Intron consensus sequences are shown for *N. crassa* (26), *Aspergillus nidulans* (47), and *S. cerevisiae* (42). Arrows show the positions of exon-intron boundaries, and numbers give the nucleotides between the internal conserved sequence and the 3' splice site. Abbreviations: P.a., *P. anserina*; N.c., *N. crassa*.

RESULTS

***yts1* gene encodes the mt tyrRS.** To isolate the *P. anserina* mt tyrRS gene, we constructed a bacteriophage λ library consisting of 4×10^5 independent genomic DNA fragments from *P. anserina* s. The library was screened by plaque hybridization under fairly stringent conditions (0.2 mM NaCl, 45% formamide; 37°C) with ^{32}P-labeled plasmid p337, which contains the *N. crassa cyt-18* gene. This screening led to the isolation of two λ clones, LC54 and LC71, which have identical restriction maps and contain the *yts1* gene (Fig. 1). We confirmed by Southern hybridization that ^{32}P-labeled p337 hybridized to the same sizes of *Eco*RI, *Bgl*II, *Sal*I, and *Bam*HI fragments in both of the λ clones and in genomic DNA from *P. anserina* s, indicating that this region of the clones has not undergone substantial rearrangement relative to the genomic DNA (not shown).

Additional Southern hybridizations localized the region homologous to the *cyt-18* gene to a 2.8-kb *Hin*dIII-*Bam*HI fragment. For sequencing, the three fragments of LC54 shown at the bottom of Fig. 1 were cloned in M13mp19 and nested deletions were constructed by using the T4 DNA polymerase method (18). The deletion clones were then sequenced by the dideoxy method (8, 59) as diagrammed in Fig. 1. Gaps were closed by sequencing from synthetic oligonucleotides, whose sequences were chosen from the DNA sequence already determined. In this way, we obtained a continuous DNA sequence of 2,762 bp (Fig. 2). The sequences of both DNA strands were determined experimentally.

Analysis of the DNA sequence revealed an ORF of 641 amino acids interrupted by two short introns (85 and 56 bp) identified by conformity to intron consensus sequences in *N. crassa* and other fungi (Fig. 3) and by the break in the homology to the *N. crassa* CYT-18 protein. The amino acid sequence of the ORF has significant homology to tyrRSs, including 32% identity to the *Bacillus stearothermophilus* tyrRS (70) and 55% identity to the *N. crassa* CYT-18 protein (2, 13). These findings suggested strongly that we had cloned the *P. anserina* mt tyrRS gene, and we designated this gene *yts1*. The first intron in the *P. anserina* gene is located in the codon for Gly-83 at exactly the same position as the single intron in the *N. crassa cyt-18* gene. However, the two introns have little sequence similarity and even the splice site and branch point sequences appear to have diverged somewhat (Fig. 3). The second intron in the *yts1* gene is located in the codon for Thr-230.

Comparison of YTS1 with other tyrRS protein sequences. Figure 4 shows an amino acid sequence alignment between the *P. anserina* YTS1 protein, the *B. stearothermophilus* tyrRS protein, and the *N. crassa* CYT-18 protein. The N-terminal 33 amino acids of the YTS1 protein and 32 amino acids of the CYT-18 protein have the characteristics of a mitochondrial targeting sequence (i.e., a preponderance of basic and hydroxylated amino acids and no acidic amino acids) (68, 69). In the case of the *N. crassa* CYT-18 protein, N-terminal protein sequencing showed that the leader is cleaved after Arg-32–Gly-33, which fits the consensus Arg-X for a protein processing site in *N. crassa* mitochondria (33). This sequence is conserved in the *yts1* ORF and is likely to correspond to the processing site.

The *B. stearothermophilus* tyrRS, whose structure has been partially determined by X-ray crystallography, consists of three domains: an N-terminal nucleotide-binding fold or α/β domain (aa 1 to 220), an α-helical domain (aa 248 to 318), and a C-terminal domain, required for binding the tRNA (aa 319 to 419) (4, 10, 11). The regions that are most strongly conserved between the *B. stearothermophilus* tyrRS, the YTS1 protein, and the CYT-18 protein are located in the α/β and α-helical domains (Fig. 4, 5; see Fig. 7). The YTS1 and CYT-18 proteins contain a "HIGH" sequence, which has been shown to be part of the ATP-binding site in bacterial synthetases (Fig. 5A), as well as additional amino acids identified in bacterial tyrRSs as being involved in binding of ATP (e.g., *B. stearothermophilus* Asp-38, Thr-40, and Lys-82) or tyrosine (e.g., Tyr-169, Gln-173, and Asp-176) (10, 43). In the *B. stearothermophilus* tyrRS, a number of amino acids involved in binding $tRNA^{Tyr}$ have been identified by analysis of in vitro mutants (4, 5, 39). Figure 4 shows that the YTS1

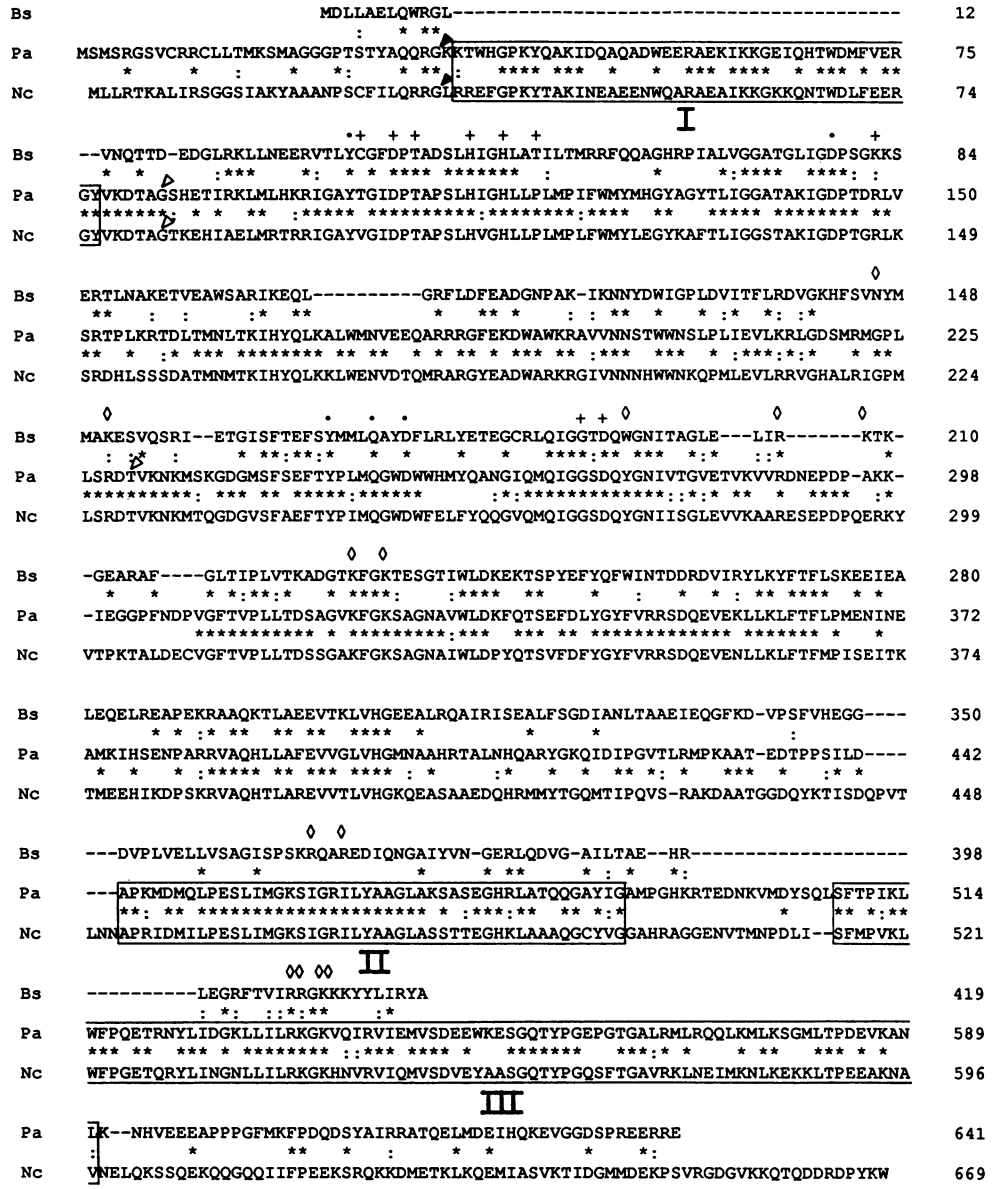

FIG. 4. Alignment of amino acid sequences of *B. stearothermophilus* (Bs) tyrRS, the *N. crassa* (Nc) CYT-18 protein, and the YTS1 protein of *P. anserina* (Pa). Asterisks indicate identical amino acids; colons indicate conservative amino acid changes according to Miyata et al. (52); solid arrowheads show the putative proteolytic cleavage sites of targeting sequences; and open arrowheads indicate the positions of introns. Amino acids involved in binding tRNA (◇), ATP (+), and tyrosine (●) in *B. stearothermophilus* tyrRSs are indicated. I, II, and III indicate the three regions that are highly conserved only in the *P. anserina* and *N. crassa* mt tyrRSs, which function in splicing.

and CYT-18 proteins contain clear cognates of some (e.g., *B. stearothermophilus* Arg-407 and Lys-410), but not all of these amino acids (e.g., Asn-146, Trp-196, Arg-368, and Lys-411).

Downstream of the putative nucleotide-binding fold, we identified three regions (aa 309 to 326, 359 to 400, and 524 to 540) that are conserved in five different tyrRSs (Fig. 5B to D). The region in Fig. 5B includes amino acids that are in the proximity of the CCA terminus of the tRNA in bacterial tyrRSs, and the region in Fig. 5D contains amino acids involved in binding $tRNA^{Tyr}$ (4). Interestingly, the tRNA binding sequence RRGK (Fig. 5D) is located at the C termini of the bacterial and yeast mt tyrRSs, but a cognate of this sequence (RKGK) appears to be displaced by an insertion in the YTS1 and CYT-18 proteins and is followed by an additional segment of more than 100 amino acids.

In addition to sequences that are conserved in the bacterial tyrRSs, the *Podospora* YTS1 and the *N. crassa* CYT-18 proteins have three additional sequence blocks (I, II, and III; Fig. 4) that are conserved only between these two tyrRSs. Region I (aa 35 to 77) corresponds to the idiosyncratic N-terminal domain, which was shown previously to be required for RNA splicing activity of the CYT-18 protein (13). Regions II and III (aa 443 to 488 and 508 to 590 of YTS1) are in the C-terminal domain, which is required for both splicing and tyrRS activity of the CYT-18 protein (33),

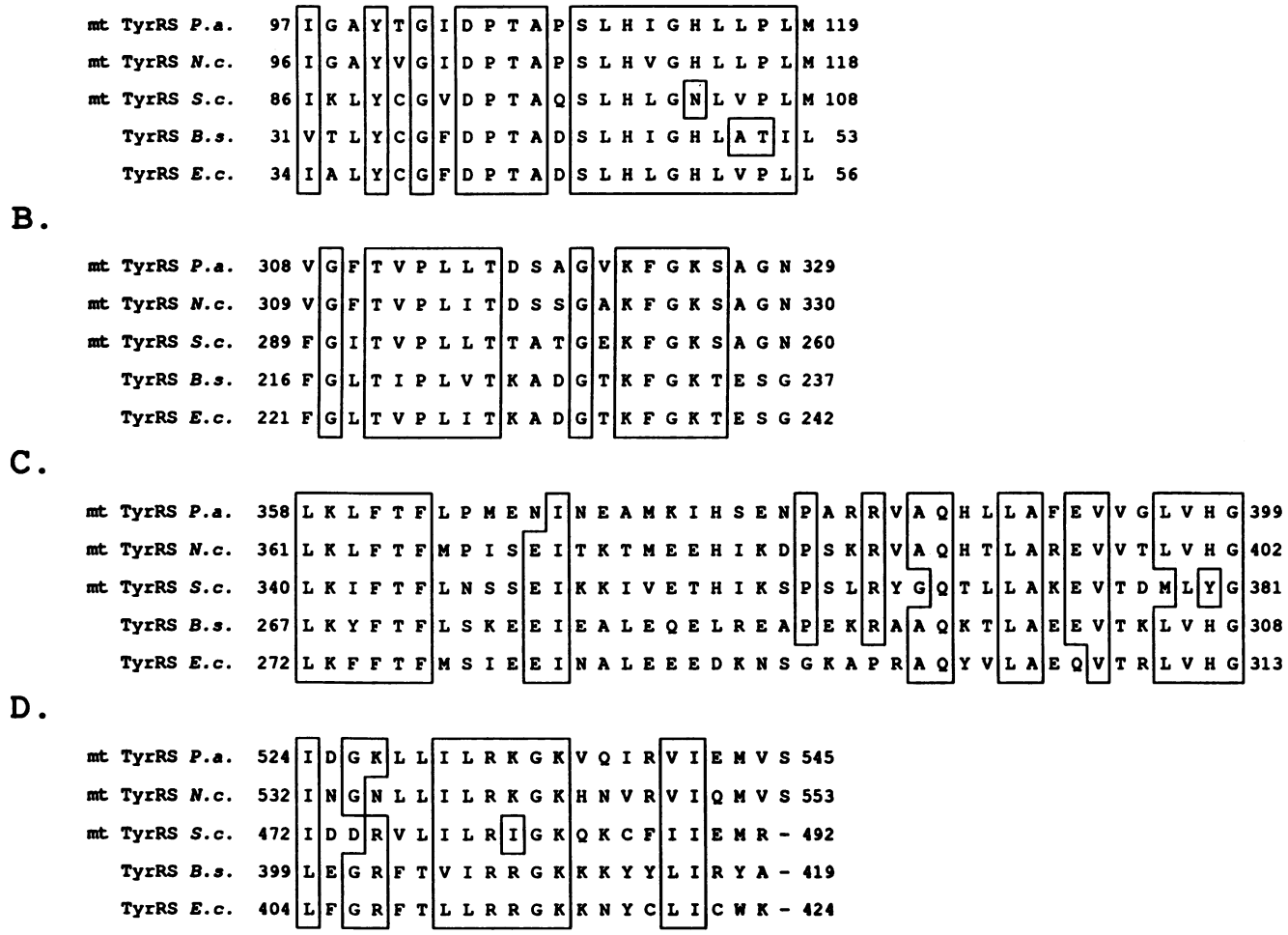

FIG. 5. Alignment of conserved sequences from different tyrRSs. TyrRS sequences compared are from *P. anserina* mitochondria (P.a.) (this work), *N. crassa* mitochondria (N.c.) (2), *S. cerevisiae* mitochondria (S.c.) (31), *B. stearothermophilus* (B.s.) (70), and *E. coli* (E.c.) (3). (A) Region containing the HIGH sequence; (B and C) conserved regions downstream of the nucleotide-binding fold; (D) C-terminal region containing the R(R/K)GK motif. Boxes enclose regions that contain identical or related amino acids (52) in at least four of the proteins.

and region III surrounds the short sequence identified above as a potential cognate of the RRGK sequence involved in tRNA binding in the bacterial tyrRSs.

Other characteristics of the *yts1* gene. As found frequently for other fungal genes, the region up to −200 nt from the ATG codon of the *yts1* gene lacks good matches for the TATA or CAAT sequences characteristic of promoters in higher eukaryotes, and the 3′-flanking region lacks a good match for the AATAAA sequence comprising part of a polyadenylation signal (28). The codon usage in the *yts1* gene shows biases similar to those reported for other genes of filamentous fungi (e.g., preference for a pyrimidine, especially C, in the third position and preference for G over A if a purine is used in the third position). However, these biases are not very strong and rare codons, such as AGN or UCA, are relatively frequent. The *N. crassa* genes encoding the mt tyrRS, mt leuRS, and mt valRS also show only moderate codon biases (2, 14, 35).

***yts1*$^+$ complements *N. crassa* mutant *cyt-18-2*.** The *P. anserina yts1*$^+$ gene was tested for its ability to transform the *N. crassa cyt-18-2* mutant, which has temperature-sensitive defects in both splicing and aminoacylation leading to impaired growth at temperatures above 37°C (2, 46). In these experiments, the *cyt-18-2* mutant was cotransformed with plasmids containing the *yts1*$^+$ or *cyt-18*$^+$ genes (pBP6 or p337, respectively) plus pBML to provide *Bml*^{r} as a second selectable marker. *cyt-18*$^+$ *Bml*^{r} transformants were scored for the ability to grow at 38.5°C on medium containing benomyl. In *N. crassa*, most transformants result from the integration of transforming DNA at ectopic chromosomal locations and are not dependent on extensive homology between the transforming DNA and the chromosomal target (23). pBP6, which contains the *yts1*$^+$ gene, efficiently transformed the *cyt-18-2* mutant, as judged by the ability to restore wild-type like growth at 38.5°C (Table 1).

The *cyt-18-2* mutant used as the host in the transformation experiments accumulates unspliced 35S pre-rRNA and is grossly deficient in cytochromes *b* and aa_3, as expected for a deficiency in mitochondrial protein synthesis. As shown in Fig. 6, transformants obtained with pBP6 had wild-type mt RNA profiles and cytochrome spectra similar to those of wild-type 74*A*, indicating that the *yts1*$^+$ gene rescued both the splicing and aminoacylation defects in the mutants. The ability of the *P. anserina yts1*$^+$ gene to transform the *N.*

TABLE 1. Transformation of the *cyt-18-2* mutant with the cloned *yts1*$^+$ gene[a]

Plasmid(s)	No. of transformants (avg ± SD) at:	
	25°C	38.5°C
pBP6 (*yts1*$^+$) + pBML (*Bml*^{r})	7,080 ± 32	3,113 ± 42
p337 (*cyt-18*$^+$) + pBML (*Bml*^{r})	4,187 ± 66	1,340 ± 29
pBML (*Bml*^{r})	1,760 ± 41	0
None	0	0

[a] *cyt-18-2* spheroplasts were cotransformed with either the *yts1*$^+$ gene (pBP6) or the *cyt-18*$^+$ gene (p337) plus plasmid pBML, which contains the *N. crassa Bml*^{r} gene. Transformed spheroplasts were plated at 25 or 38.5°C in the presence of benomyl, as described in Materials and Methods. Transformation frequencies are averages +/− the standard deviations for five different experiments. Transformation frequencies at 25°C are the number of transformants per microgram of pBML. Transformation frequencies at 38.5°C are the number of transformants per microgram of pBP6 DNA or p337 DNA, respectively.

crassa mutant at a high frequency, without prior modification of the promoter, RNA splicing, or polyadenylation signals, suggests that sequence elements required for efficient expression of this gene are interchangeable between the two fungi.

Carboxy terminus of YTS1 is necessary for restoration of synthetase activity but not splicing activity. In order to investigate the function of the C terminus of the YTS1 polypeptide, we constructed a series of plasmids with progressively larger truncations of the C terminus of the ORF and tested them for the ability to transform the *cyt-18-2* mutant (Fig. 7). In each experiment, we compared the transformation frequencies of the deletion clones with those of the full-length clone, pBP6, and individual transformants were characterized with respect to the cytochrome spectrum and mtRNA profile (Fig. 6). Transformants of plasmids pBP6-1 and pBP6-2, which were unable to complement the *cyt-18-2* mutant, were identified among benomyl-resistant transformants grown at 25°C by Southern hybridization, by using ^{32}P-labeled *yts1* plasmid to detect integrated *yts1* sequences (not shown). As shown in Fig. 7, truncations that remove progressively more amino acids from the C terminus of the ORF resulted in progressively lower transformation frequencies, with a truncated gene encoding 358 aa of the *yts1* ORF unable to transform the *cyt-18-2* mutant.

The ability of the *yts1* gene to rescue the splicing defect is indicated by the ratio of unspliced 35S pre-rRNA to mature large rRNA in the transformants grown at 37°C. Transformants obtained with plasmid pBP6, which contains the

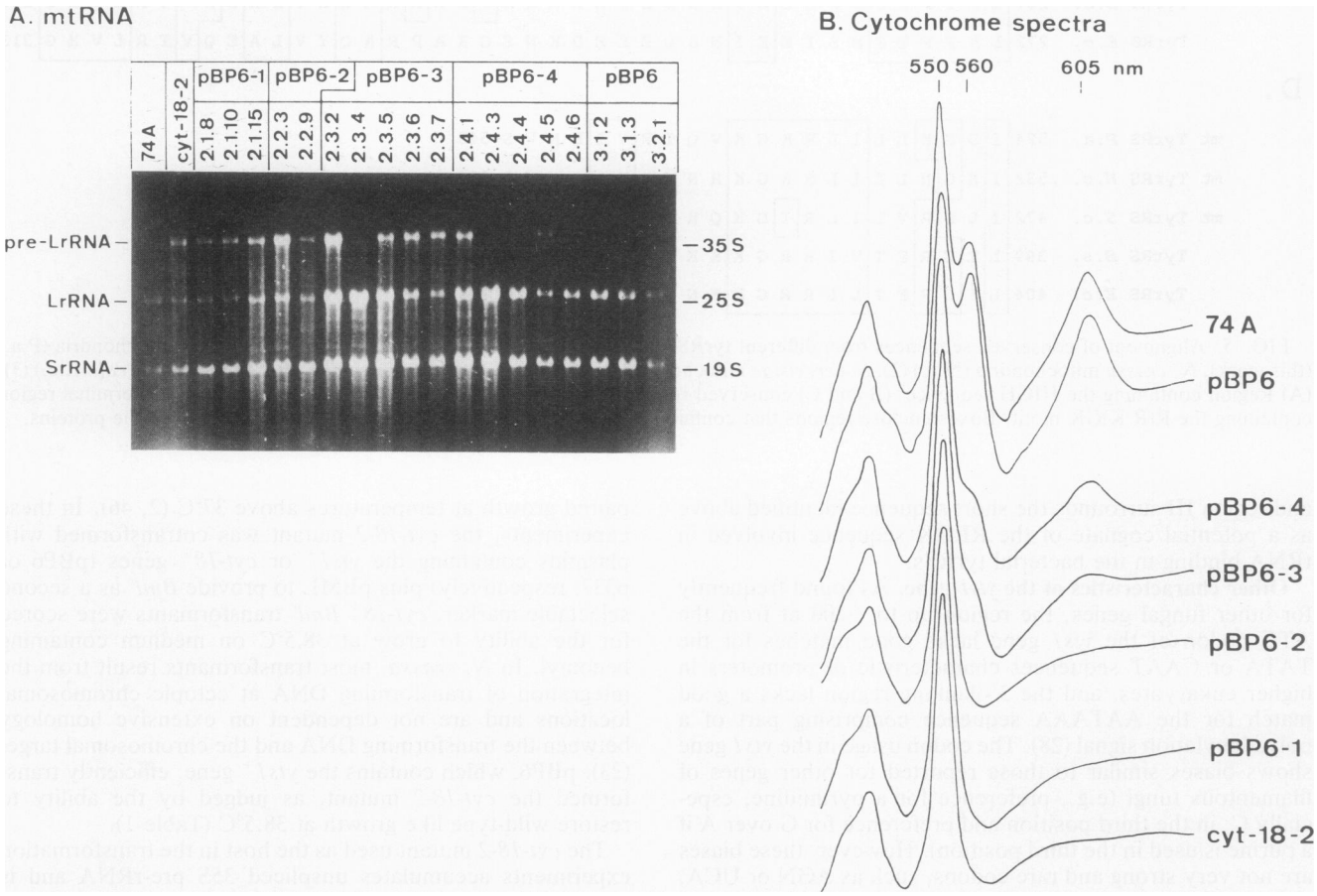

FIG. 6. Analysis of mtRNAs and cytochrome spectra of transformants of the *N. crassa cyt-18-2* mutant. (A) Gel electrophoretic analysis of whole mtRNA from wild-type 74*A*, mutant *cyt-18-2*, and *cyt-18-2* transformants obtained with indicated plasmids. All strains were grown at 37°C. MtRNA was isolated, denatured with glyoxal, and analyzed by electrophoresis in a 1.4% agarose gel (48). (B) Cytochrome spectra of strains 74*A* and *cyt-18-2* and transformants obtained with plasmids pBP6 (transformant 3.1.3), pBP6-4 (transformant 2.4.4), pBP6-3 (transformant 2.3.2), pBP6-2 (transformant 2.2.9), and pBP6-1 (transformant 2.1.15). The peaks at 550, 560, and 605 nm are the α peaks of cytochromes *c*, *b*, and aa_3, respectively.

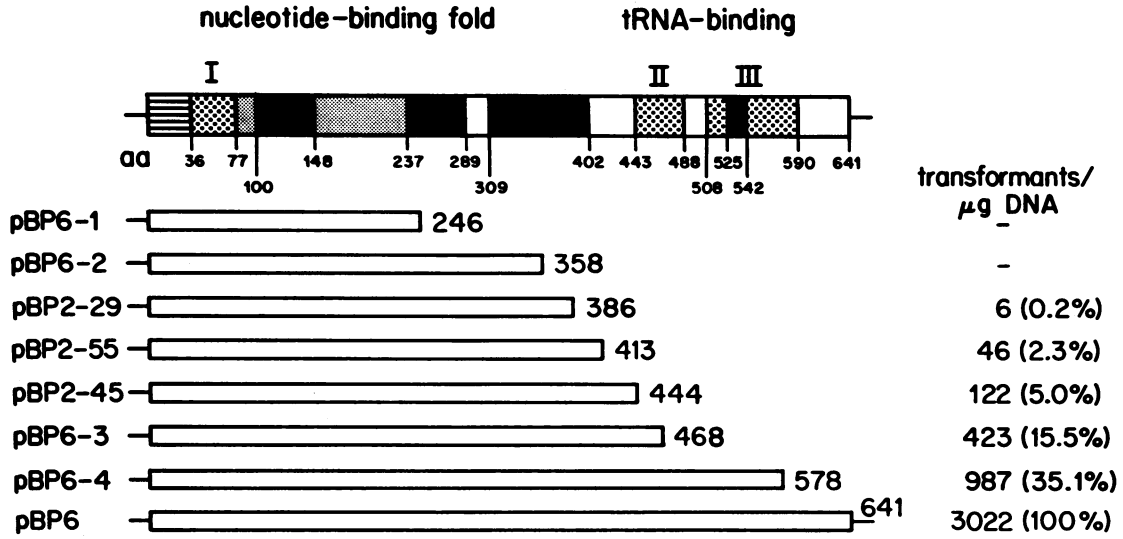

FIG. 7. Map of the *yts1* ORF and transformation of the *N. crassa cyt-18-2* mutant with *yts1* genes having C-terminal truncations. The map of the *yts1* ORF is shown at the top. Nucleotide-binding fold and tRNA-binding domains identified by sequence comparison with the *B. stearothermophilus* and *E. coli* tyrRSs are indicated. The striped area is N-terminal mitochondrial targeting sequence; regions conserved in YTS1 and the bacterial tyrRSs are shown in black (50% or more identity) or gray (20% or more identity); regions conserved only between YTS1 and CYT-18 proteins are dotted. The first and last amino acids of different regions are indicated. Constructs used for transformation experiments together with the number of encoded amino acids are shown below. Percent values indicate transformation frequencies of the construct relative to that of the full-length clone in the same experiment. Data are mean values of nine (pBP6 and pBP6-1, -2, -3, and -4) or five (pBP2-29, -45, and -55) transformation experiments.

complete *yts1*$^+$ gene, and three of five transformants obtained with plasmid pBP6-4, which has a truncation of 63 aa, had essentially wild-type mtRNA profiles, with no detectable accumulation of unspliced 35S pre-rRNA (Fig. 6). The remaining pBP6-4 transformant also showed substantial amelioration of the splicing defect, as judged by decreased amounts of 35S pre-rRNA relative to the mutant. Transformants with pBP6-3, which lacks 173 aa at the C terminus of the ORF, appeared to be partially restored for splicing, whereas transformants obtained with plasmids having larger truncations generally showed greater amounts of unspliced 35S pre-rRNA. The finding that different transformants obtained with the same plasmid show some variability in phenotype is not uncommon for filamentous fungi and may reflect different levels of expression of the *yts1* gene due to different ectopic locations and/or different copy numbers resulting from multiple integrations or the generation of long tandem repeats at the integration sites (23).

Representative cytochrome spectra of transformants are shown in Fig. 6B. Only transformants obtained with the whole gene clone, pBP6, had wild-type cytochrome spectra. Transformants obtained with plasmids having C-terminal truncations had progressively lower ratios of cytochromes *b* and aa_3 relative to cytochrome *c*. Interestingly, transformants obtained with pBP6-4, which were completely restored for splicing of the large rRNA intron, had concentrations of cytochrome *b* and aa_3 that were only about 50% that of the wild-type concentration. The inability to fully restore wild-type levels of cytochrome aa_3 in these pBP6-4 transformants is most likely to be due to a deficiency in protein synthesis and not defective splicing, since the genes encoding the three mitochondrially synthesized subunits of cytochrome aa_3 do not contain introns in this strain of *N. crassa* (15). Barring some currently unknown intron in a gene required for mitochondrial protein synthesis, the most likely possibility is that the C-terminal 63 aa of the YTS1 protein are required to completely rescue the defect in protein synthesis but not the defect in RNA splicing. One possible explanation is that sequences near the C terminus of the protein are required for aminoacylation, but not splicing. However, an alternative explanation is that the truncated YTS1 protein forms heterodimers with the resident *N. crassa cyt-18-2* mutant protein, and these heterodimers are more active in splicing than aminoacylation. It has been shown previously that *B. stearothermophilus* tyrRS proteins having mutations in different regions can complement each other via formation of heterodimers (5).

YTS1 protein synthesized in *E. coli* is active in splicing. To test directly whether the YTS1 protein can function in splicing group I introns, we expressed functional YTS1 protein in *E. coli*. A plasmid designated pT7-*yts1*, which contains a cDNA analog of the *yts1* gene cloned downstream of the bacteriophage T7 gene ϕ10 promoter, was constructed by PCR techniques and expressed in *E. coli* as described in Materials and Methods. After induction, immunoblots of *E. coli* lysates probed with anti-CYT-18 antibody showed a band of the expected size, approximately 70 kDa, which was not present in uninduced cells (not shown). For unknown reasons, the yield of YTS1 protein was relatively low compared with that of CYT-18 protein synthesized in *E. coli* (see Materials and Methods).

The YTS1 protein synthesized in *E. coli* BL21::DE3 (pLysS) was partially purified by heparin-Sepharose chromatography and used for in vitro splicing assays, essentially as described for the *N. crassa* CYT-18 protein synthesized in *E. coli* (33). Splicing was assayed by using a 698-nt in vitro transcript from pHX9422, which contains a 583-nt derivative of the *N. crassa* mt large rRNA intron along with flanking exons. This transcript, which is not self-splicing, was incubated with column fractions containing YTS1 protein and

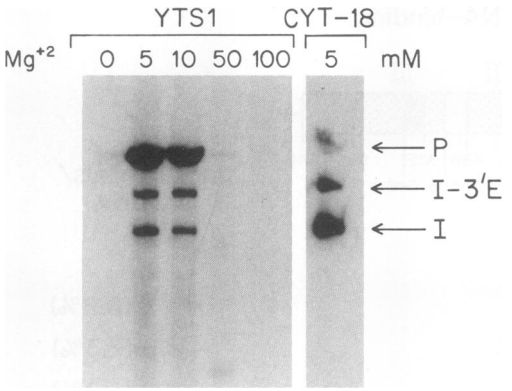

FIG. 8. The YTS1 protein splices a group I intron in vitro. Splicing was assayed by using a 698-nt in vitro transcript from plasmid pHX9422, which contains a 583-nt derivative of the *Neurospora* mt large rRNA intron. The transcript was incubated with *E. coli*-synthesized YTS1 or CYT-18(pEX550) protein in the presence of α-[^{32}P]GTP in reaction media containing different $MgCl_2$ concentrations. Splicing is indicated by the appearance of [^{32}P]GTP-labeled bands of 584 and 634 nt corresponding to the intron (I) and intron-3′ exon intermediate (I-3′E). The precursor RNA band (P) is also labeled because of a putative end-labeling activity in the protein preparations from *E. coli* (33). This activity is more pronounced in the YTS1 preparation because of overloading of the column necessary to obtain sufficient YTS1 protein to detect splicing activity. As discussed previously, however, only part of the activity could be reproducibly separated from the *E. coli*-synthesized CYT-18 protein, and the remainder may be associated with the protein or the intron RNA (33).

[^{32}P]GTP in reaction media containing different concentrations of Mg^{2+}, and products were compared with those obtained by using the *N. crassa* CYT-18 protein. As shown in Fig. 8, the YTS1 protein was in fact capable of splicing the *N. crassa* mt large rRNA intron, as judged by the appearance of [^{32}P]GTP-labeled bands of 584 nt, corresponding to the excised intron, and 634 nt, corresponding to the splicing intermediate resulting from GTP-dependent cleavage at the 5′ splice site. As in the case of the *N. crassa* CYT-18 protein, the maximum extent of splicing was found between 5 and 10 mM Mg^{2+} (33). No splicing was observed in the absence of the column fraction or with comparable column fractions from *E. coli* that does not express YTS1 protein (33; results not shown). These results provide direct evidence that the YTS1 protein is capable of splicing a group I intron.

DISCUSSION

Our results show that the *P. anserina* mt tyrRS, like the *N. crassa* mt tyrRS, is capable of splicing group I introns. The *P. anserina yts1*$^+$ gene transforms the *N. crassa cyt-18-2* mutant and rescues both the splicing and aminoacylation defects, and the YTS1 protein expressed in *E. coli* splices the *N. crassa* mt large rRNA intron in vitro. The *cyt-18-2* mutant is defective in splicing not only the mt large rRNA intron but also a number of additional group I introns in mt mRNAs (15, 46). The finding that transformants of the *cyt-18-2* mutant obtained with the *yts1*$^+$ gene have wild-type cytochrome spectra and RNA profiles suggests that the YTS1 protein also functions in splicing these other group I introns in *N. crassa* mitochondria. On the basis of our results, it seems likely that the YTS1 protein functions as well in splicing group I introns in *P. anserina* mitochondria, and this can now be tested by gene replacement experiments, by using a mutant *yts1* gene that carries the *cyt-18-2* temperature-sensitive mutation. As in *N. crassa*, the presence of a mt tyrRS that functions in splicing a number of different group I introns may provide a means of coordinately regulating the mitochondrial genes containing these introns.

The finding that the efficient transformation of the *N. crassa cyt-18-2* mutant by the *P. anserina yts1*$^+$ gene did not require modification of the promoter, splicing, or polyadenylation signals suggests that sequence elements required for expression of the mt tyrRS gene are functionally similar in the two fungi. If this is generally true, it may be possible to isolate many additional *P. anserina* genes directly by transformation of the corresponding *N. crassa* mutants.

The *P. anserina yts1* gene is the third mt tyrRS gene whose sequence has been determined. Interestingly, the *P. anserina* gene contains two introns, the *N. crassa* gene contains one intron, and the yeast mt tyrRS gene contains no introns. The first intron in the *P. anserina* gene is located at the same position as the single intron in the *N. crassa cyt-18* gene, immediately downstream of the idiosyncratic N-terminal domain required for splicing activity. This location raises the possibility that the N-terminal domain was acquired by exon shuffling after the divergence of the common ancestor of *N. crassa* and *P. anserina* from *S. cerevisiae*. Despite being at the same location, however, the *N. crassa* and *P. anserina* introns have minimal sequence homology and have presumably diverged to a greater extent than the surrounding protein coding sequences.

The second intron in the *yts1* gene is located in a region encoding a connecting peptide, CP1, which separates the two halves of the nucleotide-binding fold (11). The amino acid sequence in this region is highly conserved between the *N. crassa* and *P. anserina* tyrRSs but not in the other tyrRSs. Since other amino acyl-tRNA synthetases do not contain an intron in this region, it seems likely that the intron was acquired recently by the *P. anserina* gene. Otherwise, it would be necessary to postulate that the intron was lost independently from other tyrRs but was for some reason retained in *P. anserina*.

The *P. anserina* and *N. crassa* tyrRSs are the only two that are known to function in splicing. The *E. coli* tyrRS is not capable of splicing the *N. crassa* mt large rRNA intron in vitro, and deletion mutants in the yeast mt tyrRS are not defective in splicing the mt large rRNA intron in yeast cells (41). In addition to regions that are conserved between all tyrRSs and are presumably related to tyrRS function, the *N. crassa* and *P. anserina* mt tyrRSs have three additional regions, one at the N terminus and two toward the C terminus, that are conserved only between these two tyrRSs (Fig. 4 and 7, top). The N-terminal domain (designated I) was shown previously to be required for splicing, but not for aminoacylation of *E. coli* $tRNA^{Tyr}$ (13). Comparison of this region in the *P. anserina* and *N. crassa* proteins shows conservation of a number of basic amino acids, which might be involved in RNA binding, as well as other amino acids identified as being functionally important by analysis of second site revertants (Phe-71 and Ala-81) or mutated proteins (Gly-38–Tyr-41) (13).

The other two regions that are conserved only between the *P. anserina* and *N. crassa* mt tyrRSs, designated II and III, correspond to aa 443 to 488 and 508 to 590 of the YTS1 protein. The first region has no detectable similarity to bacterial tyrRSs or yeast mt tyrRS, whereas the second region is centered around a small stretch (18 aa; Fig. 5D) that

is also conserved at the C termini of the bacterial and yeast mt tyrRSs and has been implicated in tRNA-binding in the *B. stearothermophilus* tyrRS (4, 5). The functional importance of regions II and III is supported by analysis of a limited number of in vitro mutants of the *N. crassa* CYT-18 protein synthesized in *E. coli* (33). Thus, C-terminal truncations that extend into region III and a four-amino-acid insertion after aa 470 in region II completely abolished both splicing and mt tyrRS activity, and a single amino acid deletion, Asn-544 in region III abolished mt tyrRS activity and strongly inhibited splicing activity. By contrast, a four-amino-acid insertion in a relatively nonconserved part of the C-terminal region of the CYT-18 protein (Tyr-417) had no substantial effect on either activity (33).

In the present work, transformation of the *N. crassa cyt-18-2* mutant with truncated *yts1* plasmids pBP6-4 and pBP6-3 completely or partially restored splicing activity, even though pBP6-4 deletes part of region III and pBP6-3 deletes all of region III and part of region II. These findings could indicate that sequences in regions II and III are not required for splicing in vivo, despite being required in vitro in the *N. crassa* protein (33). However, an alternate interpretation is that the YTS1 protein forms functional heterodimers with the resident mutant CYT-18 protein and that only one subunit in the heterodimer needs to retain these sequences. As indicated previously, *E. coli* tyrRS polypeptides having mutations in different regions of the protein have been shown to complement via the formation of heterodimers (5).

The finding that splicing activity of the *N. crassa* and *P. anserina* mt tyrRSs may depend on three uniquely shared regions not found in other tyrRSs raises the question of how these regions were acquired or differentiated from other tyrRSs. The presence of the idiosyncratic N-terminal domain in the *N. crassa* and *P. anserina* mt tyrRSs, but not in bacterial or *S. cerevisiae* mt tyrRS, suggests that it was acquired after the divergence of the common ancestor of *Neurospora* and *Podospora* spp. from *S. cerevisiae* (13). The alternate possibility, that this domain was present in the ancestral tyrRS, would require the uneconomical assumption that it was lost separately from the bacterial and *S. cerevisiae* mt tyrRSs. A similar argument could be made for regions II and III toward the C terminus of the protein. However, because these regions are also required for tyrRS activity, it is also possible that their conservation in *N. crassa* and *P. anserina* reflects some feature of the interaction with $tRNA^{Tyr}$ which has diverged in other organisms.

Proteins involved in splicing group I and group II introns are idiosyncratic in different organisms and include not only aminoacyl-tRNA synthetases, but also other host proteins that function in protein synthesis (41). In *S. cerevisiae*, the mt tyrRS does not appear to be capable of functioning in splicing, but the mt leuRS functions in splicing two specific group I introns, apparently acting in concert with a maturase encoded by these introns (30). In other organisms and perhaps also in other organelles, such as chloroplasts, the role played by the *N. crassa* and *P. anserina* mt tyrRSs in splicing may be filled by other aminoacyl-tRNA synthetases or different host RNA-binding proteins. Alternatively, some feature of the tyrRS may make it more readily adaptable to function in splicing.

ACKNOWLEDGMENTS

U. Kämper and U. Kück thank G. Isowitz and I. Godehardt for technical assistance, H. Rathke for preparing figures, M. Walz for his intellectual support, and K. Esser for his interest during the initial steps of this investigation. A.C. and A.M.L. thank Janet Gianelos for technical assistance.

U. Kämper was on a part-time stipend of the Deutscher Akademischer Austausch Dienst, Bonn-Bad Godesberg, Germany. Oligonucleotide synthesis in Bochum was supported by a grant of the Minister für Wissenschaft und Forschung des Landes Nordrhein-Westfalen. Work in A.M.L.'s laboratory was supported by NIH grant GM37951.

REFERENCES

1. **Akins, R. A., and A. M. Lambowitz.** 1985. General method for cloning *Neurospora crassa* nuclear genes by complementation of mutants. Mol. Cell. Biol. **5**:2272–2278.
2. **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.
3. **Barker, D. G., C. J. Bruton, and G. Winter.** 1982. The tyrosyl-tRNA synthetase from *Escherichia coli*: complete nucleotide sequence of the structural gene. FEBS Lett. **150**:419–423.
4. **Bedouelle, H.** 1990. Recognition of $tRNA^{Tyr}$ by tyrosyl-tRNA synthetase. Biochimie **72**:589–598.
5. **Bedouelle, H., and G. Winter.** 1986. A model of synthetase/transfer RNA interaction as deduced by protein engineering. Nature (London) **320**:371–373.
6. **Benton, W. D., and R. W. Davis.** 1977. Screening λ-gt recombinant clones by hybridization to single plaques *in situ*. Science **196**:180–182.
7. **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.
8. **Biggin, M. D., T. J. Gibson, and G. F. Hong.** 1983. Buffer gradient gels and ^{35}S label as an aid to rapid DNA sequence determination. Proc. Natl. Acad. Sci. USA **80**:3963–3965.
9. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
10. **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 the tyrosyl adenylate intermediate. J. Mol. Biol. **208**:83–98.
11. **Burbaum, J. J., R. M. Starzyk, and P. Schimmel.** 1990. Understanding structural relationships in proteins of unsolved three-dimensional structure. Proteins Struct. Funct. Genet. **7**:99–111.
12. **Cech, T. R., A. J. Zaug, and P. J. Grabowski.** 1981. *In vitro* splicing of the ribosomal RNA precursor of *Tetrahymena*: involvement of a guanosine nucleotide in the excision of the intervening sequence. Cell **27**:487–496.
13. **Cherniack, A. D., G. Garriga, J. D. Kittle, Jr., 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.
14. **Chow, C. M., R. L. Metzenberg, and U. L. RajBhandary.** 1989. Nuclear gene for mitochondrial leucyl-tRNA synthetase of *Neurospora crassa*: isolation, sequence, chromosomal mapping, and evidence that the *leu-5* locus specifies structural information. Mol. Cell. Biol. **9**:4631–4644.
15. **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.
16. **Cummings, D. J., I. A. MacNeil, J. M. Domenico, and E. T. Matsuura.** 1985. Excision-amplification of mitochondrial DNA during senescence in *Podospora anserina*. J. Mol. Biol. **185**: 659–680.
17. **Cummings, D. J., K. L. McNally, J. M. Domenico, and E. T. Matsuura.** 1990. The complete DNA sequence of the mitochondrial genome of *Podospora anserina*. Curr. Genet. **17**:375–402.
18. **Dale, R. M. K., B. A. McClure, and J. P. Houchins.** 1985. A rapid single-stranded cloning strategy for producing a sequential series of overlapping clones for use in DNA sequencing: appli-

cation to sequencing the corn mitochondrial 18S rDNA. Plasmid 13:31–40.
19. **Davis, R. H., and F. J. de Serres.** 1970. Genetic and microbiological research techniques for *Neurospora crassa*. Methods Enzymol. **17A**:79–143.
20. **Esser, K.** 1974. *Podospora anserina*, p. 531–551. *In* R. C. King (ed.), Handbook of genetics, vol. I. Plenum Press, New York.
21. **Fedoroff, N.** 1983. Comparison of host strains for cloning maize DNA in bacteriophage λ. Plant Mol. Biol. Rep. **1**:27–29.
22. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**:6–13.
23. **Fincham, J. R. S.** 1989. Transformation in Fungi. Microbiol. Rev. **53**:148–170.
24. **Gampel, A., M. Nishimiki, and A. Tzagoloff.** 1989. CBP2 protein promotes *in vitro* excision of a yeast mitochondrial group I intron. Mol. Cell. Biol. **9**:5424–5433.
25. **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.
26. **Germann, U. A., G. Müller, P. E. Hunziker, and K. Lerch.** 1988. Characterization of two allelic forms of *Neurospora crassa* laccase. Amino- and carboxyl-terminal processing of a precursor. J. Biol. Chem. **263**:885–896.
27. **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.
28. **Gurr, S. J., S. E. Unkles, and J. R. Kinghorn.** 1987. The structure and organization of nuclear genes of filamentous fungi, p. 93–139. *In* J. D. Kinghorn (ed.), Gene structure in eukaryotic microbes. IRL Press, Oxford.
29. **Heinen, U.** 1991. Mitochondriale Genexpression bei Pilzen: molekulare Analysen zur nukleo-zytoplasmatischen Wechselwirkung. Diss. Bot. **166**:42–45.
30. **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.
31. **Hill, G., and A. Tzagoloff.** 1987. Personal communication.
32. **Innis, M. A., K. B. Myambo, D. H. Gelfand, and M. A. D. Brow.** 1988. DNA sequencing with *Thermus aquaticus* DNA polymerase and direct sequencing of polymerase-chain reaction amplified DNA. Proc. Natl. Acad. Sci. USA **85**:9436–9440.
33. **Kittle, J. D., Jr., G. Mohr, J. A. Gianelos, H. Wang, and A. M. Lambowitz.** 1991. 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. Genes Dev. **5**:1009–1021.
34. **Kruger, K., P. J. Grabowski, A. J. Zaug, J. Sands, D. E. Gottschling, and T. R. Cech.** 1982. Self-splicing RNA: autoexcision and autocyclization of the ribosomal RNA intervening sequence of *Tetrahymena*. Cell **31**:147–157.
35. **Kubelik, A. R., B. Turcq, and A. M. Lambowitz.** 1991. The *Neurospora crassa cyt-20* gene encodes cytosolic and mitochondrial valyl-tRNA synthetases and may have a second function in addition to protein synthesis. Mol. Cell. Biol. **11**:4022–4035.
36. **Kück, U.** 1989. Mitochondrial DNA rearrangements in *Podospora anserina*. Exp. Mycol. **13**:111–120.
37. **Kück, U., H.-D. Osiewacz, U. Schmidt, B. Kappelhof, E. Schulte, U. Stahl, and K. Esser.** 1985. The onset of senescence is affected by DNA rearrangements of a discontinuous mitochondrial gene in *Podospora anserina*. Curr. Genet. **9**:373–382.
38. **Kück, U., M. Walz, G. Mohr, and M. Mracek.** 1989. The 5′-sequence of the isopenicillin N-synthetase gene (*pcbC*) from *Cephalosporium acremonium* directs the expression of the prokaryotic hygromycin B phosphotransferase gene (*hph*) in *Aspergillus niger*. Appl. Microbiol. Biotechnol. **31**:358–365.
39. **Labouze, E., and H. Bedouelle.** 1989. Structural and kinetic bases for the recognition of $tRNA^{Tyr}$ by tyrosyl-tRNA synthetase. J. Mol. Biol. **205**:729–735.
40. **Lambowitz, A. M.** 1979. Preparation and analysis of mitochondrial ribosomes. Methods Enzymol. **59**:421–433.
41. **Lambowitz, A. M., and P. S. Perlman.** 1990. Involvement of aminoacyl-tRNA synthetases and other proteins in group I and group II intron splicing. Trends Biochem. Sci. **15**:440–444.
42. **Langford, C. J., F.-J. Klinz, C. Donath, and D. Gallwitz.** 1984. Point mutations identify the conserved, intron-contained TAC TAAC box as an essential splicing signal in yeast. Cell **36**:645–653.
43. **Leatherbarrow, R. J., A. R. Fersht, and G. Winter.** 1985. Transition-state stabilization in the mechanism of tyrosyl-tRNA synthetase revealed by protein engineering. Proc. Natl. Acad. Sci. USA **82**:7840–7844.
44. **Lipman, P., and W. R. Pearson.** 1985. Rapid and sensitive protein similarity searches. Science **227**:1435–1441.
45. **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.
46. **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.
47. **May, G. S., M. L. S. Tsang, H. Smith, S. Fidel, and N. R. Morris.** 1987. *Aspergillus nidulans* β-tubulin genes are unusually divergent. Gene **55**:231–243.
48. **McMaster, G. K., and G. G. Carmichael.** 1977. Analysis of single and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. Proc. Natl. Acad. Sci. USA **74**:4835–4839.
49. **Messing, J.** 1982. Notes on plasmids pUC8 and pUC9. Focus **4**:12.
50. **Michel, F., and D. J. Cummings.** 1985. Analysis of class I introns in a mitochondrial plasmid associated with senescence of *Podospora anserina* reveals extraordinary resemblance to the *Tetrahymena* ribosomal intron. Curr. Genet. **10**:69–79.
51. **Michel, F., and E. Westof.** 1990. Modelling of the three dimensional architecture of group I catalytic introns based on comparative sequence analysis. J. Mol. Biol. **216**:585–610.
52. **Miyata, T., S. Miyazawa, and T. Yasunaga.** 1979. Two types of amino acid substitutions in protein evolution. J. Mol. Evol. **12**:219–236.
53. **Mount, D. W., and B. Conrad.** 1986. Improved programs for DNA and protein sequence analysis on the IBM personal computer and other standard computer systems. Nucleic Acids Res. **14**:443–454.
54. **Nargang, F. E., H. Bertrand, and S. Werner.** 1978. A nuclear mutant of *Neurospora crassa* lacking subunit 1 of cytochrome c oxidase. J. Biol. Chem. **253**:6364–6369.
55. **Osiewacz, H. D., and K. Esser.** 1984. The mitochondrial plasmid of *Podospora anserina*: a mobile intron of a mitochondrial gene. Curr. Genet. **8**:299–305.
56. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labelling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. **113**:237–251.
57. **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 polymerase. Gene **56**:125–135.
58. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
59. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
60. **Schmidt, U.** 1990. Personal communication.
61. **Schmidt, U., B. Riederer, M. Mörl, C. Schmelzer, and U. Stahl.** 1990. Self-splicing of the mobile group II intron of the filamentous fungus *Podospora anserina* (COI I1) *in vitro*. EMBO J. **9**:2289–2298.
62. **Schwarz-Sommer, Z. S., A. Gierh, R. B. Klösgen, U. Wienand, A. A. Peterson, and H. Saedler.** 1984. The Spm (En) transpos-

able element controls the excision of a 2 kb DNA insert at the wx^{m-8} allele of *Zea mays*. EMBO J. **3**:1021–1028.

63. **Sinha, N. D., J. Biernat, J. McManus, and H. Köster.** 1984. Polymer supported oligonucleotide synthesis XVIII: use of β-cyanoethyl-N,N-diakylamino-/N-morpholino-phophoramidite of deoxynucleosides for the synthesis of DNA fragments simplifying deprotection and isolation of the final product. Nucleic Acids Res. **12**:4539–4557.
64. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82**:1074–1078.
65. **Tudzynski, P., U. Stahl, and K. Esser.** 1982. Development of a eukaryotic cloning system in *Podospora anserina*. Curr. Genet. **6**:219–222.
66. **van der Horst, G., and H. T. Tabak.** 1985. Self-splicing of yeast mitochondrial ribosomal and messenger RNA precursors. Cell **40**:759–766.
67. **Vierny-Jamet, C.** 1988. Senescence in *Podospora anserina*: a possible role for nucleic acid interacting proteins suggested by the sequence analysis of a mitochondrial DNA region specifically amplified in senescent cultures. Gene **74**:387–398.
68. **von Heijne, G.** 1986. Mitochondrial targeting sequences may form amphiphilic helices. EMBO J. **5**:1335–1342.
69. **von Heijne, G., J. Steppuhn, and G. Herrmann.** 1989. Domain structure of mitochondrial and chloroplast targeting peptides. Eur. J. Biochem. **180**:535–545.
70. **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.
71. **Yanish-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.