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Genetics

A protein required for RNA processing and splicing in *Neurospora* mitochondria is related to gene products involved in cell cycle protein phosphatase functions

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ABSTRACT **The *Neurospora crassa cyt-4* mutants have pleiotropic defects in mitochondrial RNA splicing, 5′ and 3′ end processing, and RNA turnover. Here, we show that the *cyt-4*$^+$ gene encodes a 120-kDa protein with significant similarity to the SSD1/SRK1 protein of *Saccharomyces cerevisiae* and the DIS3 protein of *Schizosaccharomyces pombe*, which have been implicated in protein phosphatase functions that regulate cell cycle and mitotic chromosome segregation. The CYT-4 protein is present in mitochondria and is truncated or deficient in two *cyt-4* mutants. Assuming that the CYT-4 protein functions in a manner similar to the SSD1/SRK1 and DIS3 proteins, we infer that the mitochondrial RNA splicing and processing reactions defective in the *cyt-4* mutants are regulated by protein phosphorylation and that the defects in the *cyt-4* mutants result from failure to normally regulate this process. Our results provide evidence that RNA splicing and processing reactions may be regulated by protein phosphorylation.**

The processing and splicing of mitochondrial RNAs play a key role in expression of mitochondrial genes in *Neurospora crassa* and other organisms. In *N. crassa*, mutants defective in splicing the group I intron in the mitochondrial large rRNA have been identified among strains deficient in cytochromes *b* and aa_3. In an initial screen, we identified eight such mutations, which mapped to three nuclear genes, *cyt-18*, *cyt-19*, and *cyt-4* (1, 2). More recently, we found an additional mutant in the *cyt-4* gene, which others had mapped erroneously to the *cyt-1* gene (A. J. Snook and A.M.L., unpublished data). The mutations in the *cyt-18*, *cyt-19*, and *cyt-4* genes are recessive to their wild-type alleles and complement each other in heterokaryons (2). Thus, the genetic analysis indicates that at least three trans-acting polypeptides are required for splicing the group I intron in the mitochondrial large rRNA *in vivo*.

Of the genes involved in mitochondrial RNA splicing, the one studied in the greatest detail has been the *cyt-18* gene, which encodes a mitochondrial tyrosyl-tRNA synthetase that functions in both splicing and aminoacylation (3). Temperature-sensitive mutants in the *cyt-18* gene are defective in mitochondrial protein synthesis and in splicing the mitochondrial large rRNA intron and other group I introns in mitochondrial mRNAs (1, 4). The mitochondrial tyrosyl-tRNA synthetase binds directly to the intron RNA and is by itself sufficient to splice the mitochondrial large rRNA intron *in vitro* (5, 6). The *cyt-19-1* mutant is defective in splicing the same group I introns as the *cyt-18* mutants and is presumed to be defective in a second component that functions directly in splicing, perhaps by contributing to the correct folding of the precursor RNAs or by facilitating binding of the CYT-18 protein (7, 8). The mutations in the *cyt-18* and *cyt-19* genes specifically affect the splicing of group I introns and have relatively little effect on other mitochondrial RNA processing reactions (1, 4, 7).

By contrast, the *cyt-4* mutants have a complex phenotype with defects in a number of different aspects of mitochondrial RNA metabolism in addition to RNA splicing (9, 10). The five *cyt-4* mutants are cold sensitive; they grow slowly at 25°C and more rapidly at 37°C. Initial studies focusing on the mitochondrial large rRNA showed that the *cyt-4* mutants are defective in both splicing and 3′ end synthesis and that the splicing defect is more pronounced at lower temperatures (9). At 25°C, the *cyt-4* mutants are defective in splicing the mitochondrial large rRNA intron and accumulate an unspliced 35S pre-rRNA that contains a 110-nucleotide 3′ end extension, whereas at 37°C the mutants no longer accumulate unspliced 35S pre-rRNA but remain tightly defective in 3′ end synthesis. At both 25°C and 37°C, the *cyt-4* mutants also accumulate aberrant RNAs derived from the 35S pre-rRNA, including a truncated 2.0-kilobase (kb) intron RNA and 0.6- and 1.1-kb RNAs that contain sequences in the 3′ exon. The defects in 3′ end synthesis and accumulation of aberrant RNAs are specifically related to mutations in the *cyt-4* gene and are not characteristic of *cyt-18* and *cyt-19* mutants, indicating that they are not secondary effects of defective splicing (9).

In addition to the defects in synthesis of the mitochondrial large rRNA, the *cyt-4* mutants also have defects in a number of other mitochondrial RNA processing pathways, including those for cob coI, coII, and ATPase 6 mRNAs and for some tRNAs (10). These defects include inhibition of some 5′ and 3′ end processing reactions, defective splicing of both introns in the *cob* gene at both 25°C and 37°C, and accumulation of aberrant RNAs that may be degradation products. Again, these defects appear to be specifically related to the mutation in the *cyt-4* gene and were not found in other mutants defective in mitochondrial protein synthesis or energy metabolism. Defective splicing of the mitochondrial large rRNA intron in the *cyt-4* mutants could be a secondary effect of the defect in 3′ end synthesis, leaving 3′ end extensions that impede proper folding of the precursor RNA (9). However, defective splicing of the *cob* introns cannot be accounted for by defective 5′ or 3′ end processing of cob pre-mRNA, and the *cyt-4* mutation appears to affect a component that functions directly in splicing these introns (10). The phenotype of the *cyt-4* mutants, which involves defects in a number of otherwise unrelated RNA processing, splicing, and turnover reactions, suggests

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Abbreviations: ORF, open reading frame; RFLP, restriction fragment length polymorphism; RNP, ribonucleoprotein.

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that the *cyt-4* gene product is either a common component in these processes or is required for their regulation.

Here we show that the *cyt-4* gene encodes a protein with significant similarity to the SSD1/SRK1 protein of *Saccharomyces cerevisiae* and the DIS3 protein of *Schizosaccharomyces pombe*, which have been implicated in the function of protein phosphatases that regulate the cell cycle and mitotic chromosome segregation (11–13). This finding suggests that splicing and processing of mitochondrial RNAs are likewise regulated by protein phosphorylation, presumably coordinating these processes with other aspects of cellular metabolism.¶

MATERIALS AND METHODS

Strains of *N. crassa* and Growth Conditions. The *N. crassa* strains used in this study were wild-type 74-OR23-1*A* (designated 74*A*), *cyt-4-1* (AEG-193*a*), *cyt-4-5* (HS-47*a*), *cyt-18-2* GG *al-2 A* (289-67), and *cyt-19-1 pan-2a* (GK13*a*) (2, 14). The strain used for cloning the *cyt-4*$^+$ gene by sib selection, *cyt-4-1 qa-2 aro-9 inl a*, was obtained from a cross between FGSC 3952 (*qa-2 aro-9 inl A*) and *cyt-4-1* (AEG-193*a*).

Recombinant Plasmids. pRAL1-cyt-4 contains the *cyt-4*$^+$ gene cloned from the pRAL1 plasmid library (see below). Plasmid pDE6.4 contains a 6.4-kb *Eco*RI fragment of pRAL1-cyt-4, cloned in Bluescribe vector [pBS(+); Stratagene]. The insert contains 5.8 kb of *N. crassa* sequences and 0.6 kb of the pRAL1 vector. pDEK1 was derived from pDE6.4 by deleting the region between the *Kpn* I site of the pBS(+) vector and the *Kpn* I site within the *N. crassa* sequence. pDEB1 was derived from pDE6.4 by deleting a 2.7-kb *Bam*HI fragment containing nucleotides 1–2155 of the *cyt-4*$^+$ sequence. pDSS7 contains a 3.8-kb *Sph* I/*Sna*BI fragment of pRAL1-cyt-4 cloned into pBS(+). pDH74-1 contains a 3.5-kb *Hin*dIII fragment of pDE6.4 cloned into pBS(+). pBML contains the *N. crassa* benomyl-resistance gene subcloned from pBC1 into pBS(+) (15, 16).

Cloning the *cyt-4*$^+$ Gene. The *cyt-4*$^+$ gene was cloned by complementation of a *cyt-4-1 qa-2 aro-9 inl a* host strain by sib selection using the pRAL1 plasmid library of *N. crassa* wild-type 74*A* genomic DNA (17). Transformed spheroplasts were suspended and plated in 3% agar containing Vogel's medium, 1 M sorbitol, 0.02% inositol, 2% sorbose, 0.05% fructose, and 0.05% glucose, and *cyt-4*$^+$/*qa-2*$^+$ transformants were selected by growth at 20°C in the absence of aromatic amino acids. Transformants were visible after 7 days as large colonies against a background of small colonies.

Transformation with subclones or *in vitro* mutants of the *cyt-4* gene was carried out with spheroplasts prepared from *cyt-4-1* (AEG-193*a*). The spheroplasts were cotransformed with the *cyt-4* plasmid and pBML, which contains the *N. crassa* benomyl-resistance gene. Transformed spheroplasts were suspended in top agar (0.6% agar, 2% sorbose, 0.05% fructose, 0.05% glucose, Vogel's minimal medium, and 0.5 M $MgSO_4$) and overlaid onto 1.5% agar plates containing 2% sorbose, 0.05% fructose, 0.05% glucose, Vogel's minimal medium, and 0.5 μg of benomyl per ml (18). Transformants were selected for rapid growth at 20°C in the presence of benomyl.

Chromosomal Location of the Cloned *cyt-4*$^+$ Gene. The chromosomal location of the putative *cyt-4*$^+$ clone was determined by restriction fragment length polymorphism (RFLP) mapping (19). In the standard mapping cross between the Oakridge-derived strain RLM1-33*a cot-1 al-2 arg-12 nuc-2 inl* (FGSC 4411) and Mauriceville-1c *A*, an *Xba* I RFLP that hybridized to ^{32}P-labeled pRAL1-cyt-4 segregated 17/18 with the linkage group I markers *al-2* and mating type. In a second mapping cross between Mauriceville-1c *A* and RLM47-34*a nuc-1 ad-9 al-2 nic-1*, an Oakridge-derived strain containing additional linkage group I markers, the *Xba* I RFLP segregated with *nuc-1* in 16/16 progeny analyzed.

Sequencing of the *cyt-4*$^+$ Gene. We determined the sequence of a 4.5-kb region of pRAL1-cyt-4, which contains the *cyt-4*$^+$ gene. The 3.5- and 1.8-kb *Hin*dIII fragments of pRAL1-cyt-4 were subcloned in both orientations in M13mp19 and M13mp18, and nested deletions were generated by the exonuclease III method (20). Single-stranded M13 clones were sequenced by the dideoxynucleotide chain-termination method (21), using the Klenow fragment of DNA polymerase I (GIBCO/BRL-Life Technologies, Inc., Gaithersburg, MD). Gaps in the sequence were filled by sequencing double-stranded subclones of pRAL1-cyt-4 in pBS(+) (22), using synthetic oligonucleotide primers and Sequenase (USB, Cleveland, OH). The DNA sequences of both DNA strands were determined experimentally for the entire region encoding the 1117-amino acid open reading frame (ORF) and 808 base pairs (bp) downstream of the ORF.

Analysis of the *cyt-4-1* Mutant Allele. The *cyt-4-1* mutant allele was cloned from a partial genomic library containing 5- to 6-kb *Kpn* I/*Eco*RI fragments of *cyt-4-1* (AEG-193*a*) DNA in the vector pBS(+). The library was screened by colony hybridization, using an internal fragment of the *cyt-4*$^+$ gene (^{32}P-labeled 1-kb *Sst* I/*Bam*HI fragment from pDSS7) as a probe. Transformation of the *cyt-4-1* mutant with hybrid genes containing different segments of the *cyt-4*$^+$ and *cyt-4-1* mutant alleles localized the mutation to a 1.4-kb *Bst*EII fragment, whose only alteration in *cyt-4-1* is a 5-bp deletion (B.T., K.F.D., and A.M.L., unpublished data).

Analysis of CYT-4 Protein. Rabbit antibodies were raised against two regions of the *cyt-4* ORF expressed as TrpE/CYT-4 fusion proteins in pATH vectors, using published procedures (5, 23). Plasmid pC4-3 contains a 0.4-kb *Bgl* II/*Pst* I fragment of pRAL1-cyt-4 (amino acids 115–251) cloned in pATH3, and pC4-5 contains a 1-kb *Sst* I/*Bam*HI fragment of pRAL1-cyt-4 (amino acids 270–595) cloned in pATH11. IgG antibodies were purified from sera by batch adsorption to DEAE-Sephacel, chromatography through affinity columns containing TrpE and other insoluble proteins from induced *Escherichia coli* RR1 containing a pATH vector, and adsorption to protein A-Sepharose (5). Procedures for preparation of mitochondria and mitochondrial ribonucleoprotein (RNP) particles, SDS/polyacrylamide gel electrophoresis, immunoblotting, and glycerol gradient centrifugation were as described (5, 24).

RESULTS

Cloning of the *cyt-4*$^+$ Gene. The *cyt-4*$^+$ gene was cloned by complementation of the *cyt-4-1* mutant by the sib selection procedure, using the pRAL1 plasmid library of wild-type 74A genomic DNA. Subdivision of the library led to the isolation of a single plasmid, pRAL1-cyt-4, which contains a 6.4-kb insert of *N. crassa* genomic DNA and gave >6000 *cyt-4*$^+$/*qa-2*$^+$ transformants per μg of DNA. Randomly selected *cyt-4-1* transformants had wild-type growth rates, cytochrome spectra, and mitochondrial RNA profiles (data not shown). In addition, the pRAL1-cyt-4 plasmid transformed the *cyt-4-5* mutant as efficiently as it did *cyt-4-1*. RFLP mapping showed that the putative *cyt-4* clone is closely linked to *nuc-1* on linkage group I, in good agreement with the previously determined map location of the *cyt-4* gene (cf. ref. 25). The finding that transformation of the *cyt-4* mutants with the *cyt-4*$^+$ clone restored a fully wild-type phenotype indicates that the pleiotropic defects in these mutants result from mutations in a single gene.

The *cyt-4*$^+$ Gene Encodes an ORF of 1117 Amino Acids. Fig. 1 shows a restriction map of the *N. crassa* genomic DNA insert in pRAL1-cyt-4. We determined the sequence of a

¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. M80735).

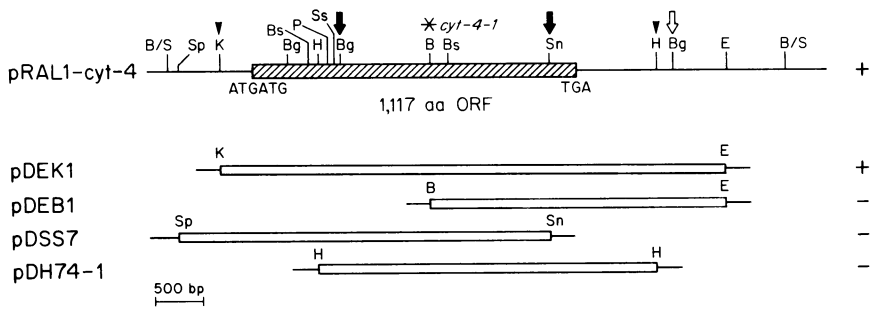

FIG. 1. Restriction map of pRAL1-cyt-4 and localization of the *cyt-4*$^+$ gene within the cloned sequence. The 1117-amino acid ORF is hatched. Solid arrows indicate sites at which insertion of a *Spe* I linker resulted in loss of *cyt-4*$^+$ transforming activity. Open arrow indicates the site at which insertion of a *Spe* I linker had no effect on *cyt-4*$^+$ transforming activity. Asterisk indicates location of the 5-bp deletion in the *cyt-4-1* mutant. Arrowheads indicate *Kpn* I and *Hin*dIII sites at the boundaries of the sequenced region. B, *Bam*HI; Bg, *Bgl* II; Bs, *Bst*EII; E, *Eco*RI; H, *Hin*dIII; K, *Kpn* I; P, *Pst* I; S, *Sau*3AI; Sn, *Sna*BI; Sp, *Sph* I; Ss, *Sst* I. Subclones used to transform the *cyt-4-1* mutant are shown below the restriction map. Open bars represent *N. crassa* genomic sequences. The presence (+) or absence (−) of *cyt-4*$^+$ transforming activity is indicated on the right.

4529-bp region that extends from the *Kpn* I to *Hin*dIII sites indicated by the arrowheads in Fig. 1 and contains the *cyt-4*$^+$ gene. Analysis of the sequence revealed a long ORF of 1117 amino acids beginning with two ATG codons at position 369 and terminating with a TGA codon at position 3720. The ORF region contains no matches for the *N. crassa* intron consensus sequences (26).

The following evidence indicates that the 1117-amino acid ORF corresponds to the *cyt-4*$^+$ gene. First, as shown in Fig. 1, a subclone of pRAL1-cyt-4 that contained the ORF transformed the *cyt-4-1* mutant, whereas subclones in which the ORF was truncated failed to transform the mutant. Second, the insertion of *Spe* I linkers, containing stop codons in every reading frame, at two sites within the 1117-amino acid ORF abolished *cyt-4*$^+$ transforming activity, whereas insertion of a *Spe* I linker downstream of the ORF had no effect on *cyt-4*$^+$ transforming activity (Fig. 1). Finally, cloning and characterization of the *cyt-4-1* mutant allele showed that it contains a 5-bp deletion (CTGGA; nucleotides 2153–2157; asterisk in Fig. 1), which would result in a frameshift at codon 596 of the ORF and lead to a truncated protein of 606 amino acids. Analysis of hybrid genes containing different segments of the wild-type and mutant alleles confirmed that this deletion corresponds to the *cyt-4-1* mutation (B.T., K.F.D., and A.M.L., unpublished data).

The predicted CYT-4 protein has a mass of 125.1 kDa and a pI of 6.5. The N-terminal 57 amino acids of the *cyt-4* ORF contain a preponderance of basic, hydrophobic, and hydroxylated amino acids and only one acidic amino acid, as expected for a mitochondrial targeting sequence (27). As found previously for *N. crassa* nuclear genes encoding components involved in mitochondrial protein synthesis, the *cyt-4* ORF does not show strong codon biases found in highly expressed *N. crassa* genes, and the sequence preceding the putative initiation codon (GCAAGT) is a relatively poor match to the consensus found upstream of initiation codons in *N. crassa* genes encoding cytosolic components [R(A/C/T)CA(A/C)(A/C); cf. ref. 28]. In Northern blots of whole cell RNA from wild-type 74*A*, we detected very low levels of a putative *cyt-4* mRNA of ≈3.8 kb (data not shown).

Immunological Detection of the CYT-4 Protein. To determine whether the *cyt-4*$^+$ ORF is expressed and to identify the protein that it encodes, immunoblots of mitochondrial proteins from wild-type 74*A* and mutant strains were probed with antibodies against two different regions of the *cyt-4*$^+$ ORF expressed as TrpE/CYT-4 fusion proteins (see *Materials and Methods*). As shown in Fig. 2, in wild-type 74*A*, both antibody preparations detected a protein of 120 kDa, in good agreement with the size expected for the protein encoded by the *cyt-4*$^+$ ORF. The 120-kDa protein was absent in both the *cyt-4-1* and *cyt-4-5* mutants, and, in its place, the *cyt-4-1* mutant consistently showed a lighter band at ≈69 kDa, the size of the protein expected to result from the 5-bp deletion and frameshift at codon 596 of the *cyt-4* ORF (predicted size, 68.4 kDa). The *cyt-18-2* and *cyt-19-1* mutants contained high amounts of the 120-kDa protein, indicating that the deficiency of this band in the *cyt-4* mutants is due specifically to the *cyt-4* mutations and is not a secondary effect of defective mitochondrial metabolism or RNA splicing.

In addition to the 120-kDa band, the C4-5 antibodies, and to a lesser extent the C4-3 antibodies, bound to an abundant protein of 55 kDa. Competition experiments showed that binding of both antibody preparations to the 120-kDa band and binding of the C4-5 antibody preparation to the 55-kDa band could be blocked by competition with the appropriate fusion protein but not by the other fusion protein or bovine serum albumin at the same concentrations (data not shown). However, the 55-kDa protein was present in the same amounts in all strains, including the *cyt-4* mutants, and thus does not appear to be *cyt-4* specific. The most likely possibility is that the 55-kDa band corresponds to an otherwise unrelated protein that has one or more epitopes in common with the CYT-4 protein.

The findings that the 120-kDa protein binds specifically to antibodies against two different regions of the CYT-4 protein and is deficient in two *cyt-4* mutants provide strong evidence that this protein is the *cyt-4*$^+$ gene product. The wild-type 74*A* and *cyt-18-2* and *cyt-19-1* mutant strains showed an additional light band of ≈115 kDa, which was absent in the *cyt-4* mutants and thus also appears to be *cyt-4* specific. Moreover, on close inspection, the major 120-kDa CYT-4 band is seen to be a closely spaced doublet. These findings raise the

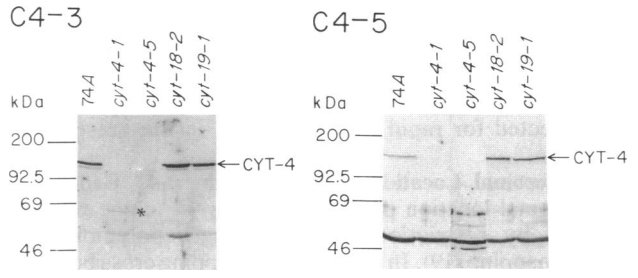

FIG. 2. Immunological detection of the CYT-4 protein. Proteins in mitochondrial lysates (35 μg) from wild-type 74*A* and the indicated mutant strains were separated by electrophoresis in SDS/7.5% polyacrylamide gels, with a 3% stacking gel, transferred to nitrocellulose, and probed with antibody preparation C4-3 or C4-5. Positions of the molecular size marker proteins (rainbow markers; Amersham) are indicated on the left.

possibility that the CYT-4 protein might be subject to post-translational modification.

Characterization of the CYT-4 Protein. The CYT-4 protein is not tightly associated with mitochondrial RNA. In several experiments, only ≈1% of the CYT-4 protein present in mitochondrial lysates was recovered in preparations of RNP particles from either wild-type 74*A* or the *cyt-18-2* and *cyt-19-1* mutants, which accumulate unspliced precursor RNAs (data not shown). Furthermore, the small amount of CYT-4 protein recovered in the RNP particles did not remain associated with the particles during a subsequent sucrose gradient centrifugation and instead remained at the top of the gradients. In glycerol gradients, the CYT-4 protein from wild-type 74*A* mitochondrial lysates sedimented at ≈200 kDa, suggesting that it might be a dimer or part of a small complex (data not shown).

The CYT-4 Protein Is Related to the SSD1/SRK1 and DIS3 Proteins. Computer analysis showed that the CYT-4 protein lacks RNA1, RNA2, zinc finger-like, arginine-rich, DEAD, or DEAH motifs characteristic of known RNA binding proteins and helicases. However, comparison of the predicted amino acid sequence of the CYT-4 protein with the GenBank data base using the FASTA program (29) detected significant similarity to the SSD1/SRK1 protein of *S. cerevisiae*. This protein and the related DIS3 protein of *Sc. pombe* have been implicated in protein phosphatase functions that control the cell cycle and mitotic chromosome separation (11–13).

Fig. 3*A* shows an amino acid sequence alignment of the CYT-4 and SSD1/SRK1 proteins. The SSD1/SRK1 protein has a predicted molecular mass of 140 kDa, similar to that of the CYT-4 protein (125 kDa). The alignment shows that the two proteins have 18% identity and 65% similarity throughout their length, including at least three correctly ordered sequence blocks with relatively high similarity distributed throughout the proteins (I, II, and III in Fig. 3*A*). By comparison, the *SSD1/SRK1* and *dis3*$^+$ gene products have 21% identity over a 600-amino acid region (13). When compared over this same region, the CYT-4 and DIS3 sequences also have 21% identity, with the most highly conserved regions corresponding to sequence blocks II and III in Fig. 3*A*. (Sequence block I was not included in the published part of the DIS3 sequence.) A three-way alignment of the CYT-4, SSD1/SRK1, and the published part of the DIS3 sequences revealed a highly conserved sequence, which is located near the C termini of all three proteins and may be a signature sequence for this class of proteins (Fig. 3*B*).

DISCUSSION

The phenotype of the *cyt-4* mutants, which have pleiotropic defects in otherwise unrelated mitochondrial RNA processing, splicing, and turnover reactions, raised the possibility that the *cyt-4* gene product has a regulatory function (10). Here we find that the *cyt-4*$^+$ gene encodes a 120-kDa protein with significant similarity to the *S. cerevisiae* SSD1/SRK1 and the *Sc. pombe* DIS3 proteins, which have been implicated in protein phosphatase functions. The CYT-4 protein is truncated or deficient in two *cyt-4* mutants and has characteristics consistent with a regulatory role. It is localized to mitochondria, the presumed site of its protein targets, but is not tightly associated with mitochondrial RNA particles, as might be expected if it functioned directly in RNA processing or splicing. Furthermore, the CYT-4 protein lacks amino acid sequence motifs characteristic of known RNA binding proteins. Assuming that the CYT-4 protein functions in a manner similar to the SSD1/SRK1 and DIS3 proteins, we infer that the mitochondrial RNA splicing and processing reactions that are defective in the *cyt-4* mutants are ordinarily regulated by protein phosphorylation and that the defects in the *cyt-4* mutants result from failure to normally regulate this process.

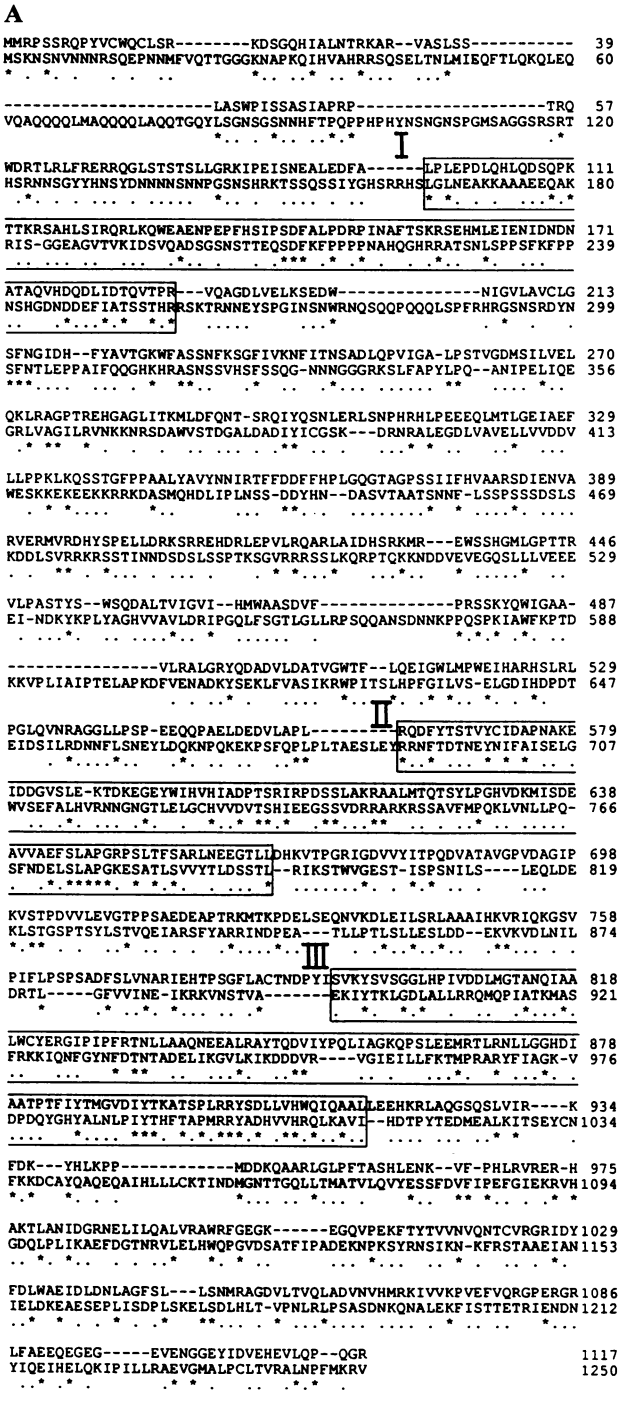

FIG. 3. (*A*) Alignment of CYT-4 (top line) and SSD1/SRK1 (bottom line) amino acid sequences. Sequences were aligned using the algorithm of Higgins and Sharp (30). I, II, and III indicate conserved blocks of amino acid sequences. (*B*) Conserved amino acid sequence in three-way alignment of *N. crassa* CYT-4, *S. cerevisiae* SSD1/SRK1, and *Sc. pombe* DIS3 proteins. Asterisks indicate identical amino acids; dots indicate conservative replacements (31).

The *dis3* and *SSD1/SRK1* genes, to which the *cyt-4* gene is related, are involved in the regulation of various cellular processes in *S. cerevisiae* and *Sc. pombe*. *dis3* is one of three genes (*dis1*, *dis2*, and *dis3*) that are the sites of cold-sensitive mutations that block mitotic chromosome separation (32). A

role in protein phosphorylation was suggested by the findings that all three *dis* mutants are hypersensitive to caffeine, an inhibitor of cAMP phosphodiesterases, and that the *dis3* mutant has a phenotype similar to that resulting from mutations in the *dis2* gene, which encodes a putative type 1 protein phosphatase (11, 32). The finding that *dis2/dis3* double mutants are lethal, whereas neither *dis2* nor *dis3* mutations are lethal by themselves, further supports the idea that *dis3* is functionally similar to the *dis2*-encoded protein phosphatase (Kinoshita *et al.*, submitted, cited in ref. 13). The *SSD1/SRK1* gene suppresses mutations in the *SIT4* gene, which encodes a protein phosphatase implicated in regulation of pol II transcription and required for transition from late G_1 to the S phase of the cell cycle (12, 33). The *SSD1/SRK1* gene introduced on a plasmid also suppresses the phenotype associated with elevated levels of cAMP-dependent protein kinase activity resulting from mutations in the *PDE2* gene, encoding the low K_m cAMP phosphodiesterase, or the *bcy1/sra1* gene, encoding the regulatory subunit of cAMP-dependent protein kinase, as well as temperature-sensitive cell cycle arrest in the *ins1* mutants (12, 13). The DIS3 and SSD1/SRK1 gene products are not homologous to known protein phosphatases, and it is not known whether they are protein phosphatases of a different type or are only involved in regulating protein phosphatase function.

Unlike *dis3* mutants, the *cyt-4* mutants are not arrested in mitosis, nor are they hypersensitive to caffeine (B.T. and A.M.L., unpublished data; cf. ref. 11). The CYT-4 protein also differs from DIS3 and SSD1/SRK1 in its localization to the mitochondria; neither DIS3 nor SSD1/SRK1 is known to be a mitochondrial protein. It is possible that the CYT-4 protein belongs to a family of phosphatases or regulatory proteins, other members of which have a nuclear or cytosolic function. Alternatively, the CYT-4 protein may have additional as yet uncharacterized functions in the nucleus or cytosol.

The finding that the *cyt-4* mutants are defective in a number of different and apparently unrelated processes, including RNA splicing, 5′ and 3′ end processing, and RNA turnover, suggests that these processes are controlled by a common regulatory pathway. The different enzymes used in these processes may be regulated individually by CYT-4, or they may be part of a larger complex whose assembly is regulated by CYT-4. Alternatively, the different processes may require a common regulated component, or some processing reactions may be regulated sequentially by other processing reactions. Assuming that the CYT-4 protein functions in a manner similar to the DIS3 and SSD1/SRK1 proteins, we infer that hyperphosphorylation inhibits certain mitochondrial RNA processing and splicing reactions and that the CYT-4 protein is necessary for regeneration of the active dephosphorylated state. The CYT-4 protein may act on the RNA processing enzymes directly, or it may act on other regulatory molecules, which influence the activity or synthesis of these enzymes.

The mechanism by which the *cyt-4* mutations affect splicing is not clear. As indicated previously, a key protein required for splicing group I introns in *N. crassa* mitochondria is the mitochondrial tyrosyl-tRNA synthetase, which is encoded by nuclear gene *cyt-18*$^+$ (see Introduction). However, the *cyt-4* mutants produce functional CYT-18 protein, which is active in splicing the mitochondrial large rRNA intron *in vitro*, and the specific activity of the CYT-18 protein in the *cyt-4-1* mutant is not detectably lower than in wild-type 74*A* (ref. 5; Qingbin Guo and A.M.L., unpublished data). These findings suggest that the CYT-4 protein does not regulate the activity of the CYT-18 protein and may instead regulate some other protein factor required for splicing, perhaps the CYT-19 protein.

Finally, our results provide evidence that protein phosphorylation may be involved in regulating RNA splicing and processing reactions. The regulation suggested here by analysis of the *cyt-4* mutants may provide a mechanism for coordinating mitochondrial activity and RNA processing with the cell cycle and other aspects of cellular metabolism. A major issue for the future will be to ascertain whether this mode of regulation extends to other RNA splicing and processing reactions, including nuclear pre-mRNA splicing.

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