Transcripts and transcript-binding proteins in mitochondria of *Neurospora crassa*

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Abstract

We analyzed expression elements of three disparate groups of mitochondrial genes in *Neurospora crassa*, apocytochrome b (*COB*), cytochrome c oxidase 1 (*COX1*), and the clustered *ATP8-ATP6-mtATP9-COX2*. To identify promoter sequences we employed the published *N. crassa* consensus sequence for *COB* and rRNA genes, and we found closely related sequences within the 5'-regions of both *COX1* and the *ATP8-COX2* transcriptional units. We determined that the mature *COX1* RNA includes two flanking unassigned reading frame (URF) sequences, but the 3'-flanking *ND1* is not included in the *COX1* mRNA. The *ATP8-ATP6-mtATP9-COX2* polycistronic transcript does not include an adjacent 5'-URF sequence. Primer extension analysis showed one likely 5'-end for the *COX1* transcript, which is 73 nucleotides downstream of the consensus promoter sequence and is the first nucleotide 3' of the sequence for the tRNA *cys*. Primer extension analysis and S1 nuclease mapping of the *ATP8-COX2* RNA showed that the 5'-end for this transcript is the first nucleotide 3' of the consensus promoter sequence. We performed gel-shift experiments to detect proteins in mitochondria that bind to transcripts as possible regulatory proteins. The 5'-untranslated region (UTR) RNAs of *COB*, *COX1*, and *ATP8-COX2* appear to bind both unique proteins and an overlapping group of two to four proteins of ~155–45 kDa. We successively deleted regions of the RNA 5'-UTRs to identify sequences that bound these proteins. Similar predicted stem-loop secondary structures were detected in the protein-binding regions of all three UTRs.

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1. Introduction

The regulation of expression of mitochondrial genes, encoding proteins of the inner mitochondrial (mt) membrane, remains poorly understood and incompletely analyzed by both genetic and biochemical approaches. Mitochondria are prokaryotic in origin, but their genetic regulatory and protein synthesis systems show little resemblance to their ancestors. Primitive, bacteriophage-like RNA polymerases and simple promoter elements have been identified in mitochondria, but there is no evidence of modulation of gene transcription by *trans*-acting factors. In mammalian mitochondria, the entire genome is transcribed as two partially overlapping

Abbreviations: mt, mitochondrial; UTR, untranslated region; COB, apocytochrome b; COX, cytochrome c oxidase; ATP, ATPase; ND, NADH dehydrogenase; URF, unassigned reading frame.

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transcriptional units with two distinct promoters that differ sharply in their activities (Attardi and Schatz, 1988; Montoya et al., 1982). In *Saccharomyces cerevisiae* transcription of the mt genome depends upon at least 20 conserved promoters adjacent to individual genes, and extensive processing of transcripts precedes translation (Constanzo and Fox, 1990; Grivell, 1995; Jang and Jaehning, 1994). In *Neurospora crassa*, many fewer consensus promoter sequences have been identified, and these have been proposed to be responsible for transcription of polygenic sequences (Kennell and Lambowitz, 1989; Kubelik et al., 1990). As others have argued (Tzagoloff and Myers, 1986), it is unlikely that expression of mitochondrial genes is regulated transcriptionally. Many *Saccharomyces* pet strains (mutant in nuclear genes required for development of respiratory-competent mitochondria) have been examined, and none has been found to be affected in mt transcriptional activity (Tzagoloff and Myers, 1986).

Gene expression in mitochondria likely is regulated at a post-transcriptional step, either through translational control or through processing or stabilization of transcripts. In *S. cerevisiae*, mRNA-specific translational activators, such as the PET factors (Constanzo and Fox, 1990) encoded by nuclear genes, bind to the 5'-untranslated regions (5'-UTRs) of mt transcripts to promote their translation. In *N. crassa* the product of the cya-5 gene, which shares partial homology to the yeast Pet30p, has been identified as a likely translational activator of the cytochrome *c* oxidase 1 (COX1) mRNA (Coffin et al., 1997), and a homologous protein has been identified in maize chloroplasts (Fisk et al., 1999). The sequences of other characterized yeast PET translational activators are poorly conserved between species, and related sequences do not appear (unpublished observation) in the *N. crassa* genome (Neurospora Sequencing Project, [Assembly v. 3 (2002) Whitehead Institute/MIT Center for Genome Research (www-genome.wi.mit.edu)]; German *Neurospora* Genome Project, MIPS-NcDB [(2002) mips.gsf.de/proj/neurospora]).

Yeast mitochondria also contain factors, such as the abundant p40 (Papadopoulou et al., 1990; Dekker et al., 1992), that bind to all mt mRNA leader sequences examined. This protein is not essential for translation of the mRNA (Mulero and Fox, 1993), but it binds to a region of the 5'-leader that potentially interacts through base-pairing with the initiator AUG, thereby possibly regulating ribosome access (Dekker et al., 1992).

Stabilization of transcripts could be an additional mechanism of regulation of mt gene expression. In yeast COX3 or apocytochrome *b* (COB) mRNAs, deletions near the 5'-end caused major decreases in their abundance, but the residual mRNAs were functional (Mittelmeier and Dieckmann, 1993; Wiesmberger et al., 1995). Our earlier studies showed that some specific mt transcripts in *N. crassa* could be retained, untranslated, in a highly stable form throughout conidial dormancy and during spore germination, a process during which they were recruited into the mt polyribosomes, long after their synthesis, along with newly transcribed mRNAs from other mt genes (Bittner-Eddy et al., 1994).

In this study we identified some new elements that likely are involved in transcription and translation of mt mRNAs in *N. crassa*, we mapped the 5'-ends of transcripts of two mt genes, COX1 and the ATP8-COX2 cluster (Fig. 1A), and we asked if there are proteins that bind to these mt mRNAs, as well to the previously mapped COB transcript, that could provide a means of regulating mt gene expression. We have now detected three proteins that appear to bind uniquely to the 5'-UTRs of specific transcripts, and we have found two to four mt proteins, identified by size, that appear to be shared by the three mt transcripts. We have delimited the regions within the 5'-UTRs of the transcripts that bind these proteins. These binding regions of the transcripts are all characterized by a similar predicted stem-loop structure.

2. Materials and methods

2.1. Plasmids for generation of 5'-UTRs of RNAs

Total genomic DNA (Stevens and Metzenberg, 1982) was used as a template for generating the 802 bp 5'-UTR sequence of the COB gene and the 1212 bp 5'-UTR sequence of ATPase subunit 8 (*ATP8*), each of which contained some coding sequence. The pUCB3 plasmid containing *Eco RI*-fragment 3 from *N. crassa* mt DNA (Fungal Genetics Stock Center; Kansas City, KN) was the template for generating the
1366 bp 5'-UTR of COX1, also containing some coding sequence.

With one exception, polymerase chain reaction (PCR) products were cloned initially into pGEM-T (Promega) before being cloned into the transcription vector pGEM3Zf(+) (Promega); the p50utrCOB was generated via PCR with (sense primer) 5'-CTGGTACCGAGAGGGAGTGGCGTTTAAA and (antisense primer) 5'-CTGGATCCGTTACTAGGTTGTGAAGCGT, and the PCR product was cloned into pGEM-T and used directly for transcription. Two oligonucleotides, (sense primer) 5'-CGTGAATTCCTTTAGGGTTAGCCTATAGC and (antisense primer) 5'-CGTGGATCCTCATCTTTCAGTTCAAATCGA, were employed to generate PCR products for directional cloning into the EcoRI and BamHI sites of pGEM3Zf(+), yielding p5utrCOX1. The oligonucleotides used for construction of p50utrATP8, the 5'-UTR sequence of the ATP8 gene, were (sense primer) 5'-CAGAGCTCCCCAAGGGATCATATAAGTTGCA and (antisense primer) 5'-CAGGGATCCGAA-TAAACGAACAAATCTAGGT.

DNA sequences not in GenBank were provided by A. Lambowitz and R.A. Collins (personal communication). Some sequences were verified through BLAST search of the data of the Neurospora Sequencing Project, [Assembly v. 3 (2002). Whitehead Institute/MIT Center for Genome Research (www-genome.wi.mit.edu)].

2.2. Preparation of RNA transcripts

Linearized p5utrCOB was generated by digestion of the plasmid with BamHI, DpnI, ScaI, XmaI, or EcoRI to obtain templates for synthesis of full-length 5'-UTR RNA (BamHI) as well as progressively truncated templates to synthesize 5'-UTR RNAs of 670, 498, 340, and 291 b, respectively. Linearized p5utrCOX1 was generated by digestion of the plasmid with BamHI, FokI, HindIII, DdeI, or BsaHI to obtain DNA templates for transcription of full-length 5'-UTR RNA (BamHI), as well as progressively truncated templates to transcribe 5'-UTR RNAs of 816, 496, 444, and 310 b, respectively. Linearized p5utrATP8 was generated by digestion of the plasmid with BamHI, DdeI, SpeI, StyI, or EcoRV to obtain templates for transcription of full-length 5'-UTR RNA as well as progressively truncated RNAs of 1017, 785, and 315 b, respectively. In vitro transcription of these DNAs was performed with T7 RNA polymerase (Promega) at 37°C for 1 h in the presence of 50 μCi of [α-32P]UTP. Restriction of these templates and transcription of appropriately sized RNAs was monitored by gel electrophoresis. For each full-length RNA tested, unlabeled RNA was synthesized and used for competition assays. After transcription, template DNA was removed with DNase I (RNase-free) (Promega). The transcripts were extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated with 0.3 M sodium acetate (pH 5.2) and ethanol.

2.3. S1 nuclease experiments

Two single-stranded DNAs for S1 nuclease analysis of the ATP8 transcript were products of asymmetric PCR (McCabe, 1990). Oligonucleotide pairs (sense primer) 5'-CTGCATGATCCGAA-TAAACGAACAAATCTAGGT and (antisense primer) 5'-CAGGGATCCGAAAUTR of COX1, also containing some coding sequence.

With one exception, polymerase chain reaction (PCR) products were cloned initially into pGEM-T (Promega) before being cloned into the transcription vector pGEM3Zf(+) (Promega); the p50utrCOB was generated via PCR with (sense primer) 5'-CTGGTACCGAGAGGGAGTGGCGTTTAAA and (antisense primer) 5'-CTGGATCCGTTACTAGGTTGTGAAGCGT, and the PCR product was cloned into pGEM-T and used directly for transcription. Two oligonucleotides, (sense primer) 5'-CTGGTACCGAGAGGGAGTGGCGTTTAAA and (antisense primer) 5'-CTGGATCCGTTACTAGGTTGTGAAGCGT, and the PCR product was cloned into pGEM-T and used directly for transcription. Two oligonucleotides, (sense primer) 5'-CTGGTACCGAGAGGGAGTGGCGTTTAAA and (antisense primer) 5'-CTGGATCCGTTACTAGGTTGTGAAGCGT, and the PCR product was cloned into pGEM-T and used directly for transcription. Two oligonucleotides, (sense primer) 5'-CTGGTACCGAGAGGGAGTGGCGTTTAAA and (antisense primer) 5'-CTGGATCCGTTACTAGGTTGTGAAGCGT, and the PCR product was cloned into pGEM-T and used directly for transcription. Two oligonucleotides, (sense primer) 5'-CTGGTACCGAGAGGGAGTGGCGTTTAAA and (antisense primer) 5'-CTGGATCCGTTACTAGGTTGTGAAGCGT, and the PCR product was cloned into pGEM-T and used directly for transcription. Two oligonucleotides, (sense primer) 5'-CTGGTACCGAGAGGGAGTGGCGTTTAAA and (antisense primer) 5'-CTGGATCCGTTACTAGGTTGTGAAGCGT, and the PCR product was cloned into pGEM-T and used directly for transcription. Two oligonucleotides, (sense primer) 5'-CTGGTACCGAGAGGGAGTGGCGTTTAAA and (antisense primer) 5'-CTGGATCCGTTACTAGGTTGTGAAGCGT, and the PCR product was cloned into pGEM-T and used directly for transcription. Two oligonucleotides, (sense primer) 5'-CTGGTACCGAGAGGGAGTGGCGTTTAAA and (antisense primer) 5'-CTGGATCCGTTACTAGGTTGTGAAGCGT, and the PCR product was cloned into pGEM-T and used directly for transcription. Two oligonucleotides, (sense primer) 5'-CTGGTACCGAGAGGGAGTGGCGTTTAAA and (antisense primer) 5'-CTGGATCCGTTACTAGGTTGTGAAGCGT, and the PCR product was cloned into pGEM-T and used directly for transcription. Two oligonucleotides, (sense primer) 5'-CTGGTACCGAGAGGGAGTGGCGTTTAAA and (antisense primer) 5'-CTGGATCCGTTACTAGGTTGTGAAGCGT, and the PCR product was cloned into pGEM-T and used directly for transcription. Two oligonucleotides, (sense primer) 5'-CTGGTACCGAGAGGGAGTGGCGTTTAAA and (antisense primer) 5'-CTGGATCCGTTACTAGGTTGTGAAGCGT, and the PCR product was cloned into pGEM-T and used directly for transcription. Two oligonucleotides, (sense primer) 5'-CTGGTACCGAGAGGGAGTGGCGTTTAAA and (antisense primer) 5'-CTGGATCCGTTACTAGGTTGTGAAGCGT, and the PCR product was cloned into pGEM-T and used directly for transcription. Two oligonucleotides, (sense primer) 5'-CTGGTACCGAGAGGGAGTGGCGTTTAAA and (antisense primer) 5'-CTGGATCCGTTACTAGGTTGTGAAGCGT, and the PCR product was cloned into pGEM-T and used directly for transcription. Two oligonucleotides, (sense primer) 5'-CTGGTACCGAGAGGGAGTGGCGTTTAAA and (antisense primer) 5'-CTGGATCCGTTACTAGGTTGTGAAGCGT, and the PCR product was cloned into pGEM-T and used directly for transcription. Two oligonucleotides, (sense primer) 5'-CTGGTACCGAGAGGGAGTGGCGTTTAAA and (antisense primer) 5'-CTGGATCCGTTACTAGGTTGTGAAGCGT, and the PCR product was cloned into pGEM-T and used directly for transcription. Two oligonucleotides, (sense primer) 5'-CTGGTACCGAGAGGGAGTGGCGTTTAAA and (antisense primer) 5'-CTGGATCCGTTACTAGGTTGTGAAGCGT, and the PCR product was cloned into pGEM-T and used directly for transcription. Two oligonucleotides, (sense primer) 5'-CTGGTACCGAGAGGGAGTGGCGTTTAAA and (antisense primer) 5'-CTGGATCCGTTACTAGGTTGTGAAGCGT, and the PCR product was cloned into pGEM-T and used directly for transcription. Two oligonucleotides, (sense primer) 5'-CTGGTACCGAGAGGGAGTGGCGTTTAAA and (antisense primer) 5'-CTGGATCCGTTACTAGGTTGTGAAGCGT, and the PCR product was cloned into pGEM-T and used directly for transcription. Two oligonucleotides, (sense primer) 5'-CTGGTACCGAGAGGGAGTGGCGTTTAAA and (antisense primer) 5'-CTGGATCCGTTACTAGGTTGTGAAGCGT, and the PCR product was cloned into pGEM-T and used directly for transcription. Two oligonucleotides, (sense primer) 5'-CTGGTACCGAGAGGGAGTGGCGTTTAAA and (antisense primer) 5'-CTGGATCCGTTACTAGGTTGTGAAGCGT, and the PCR product was cloned into pGEM-T and used directly for transcription.
CGAT and (antisense primer) 5'-CAGTTCA-GCAGGGGGAAGCTA and (sense primer) 5'-GCTCTGGAAGATAGATTGAGGT and (antisense primer) 5'-GTAGATCGGGCAGTGGATCAT were used for the PCR. The template was total N. crassa DNA. S1 nuclease digestion was performed according to Sambrook et al. (1989) with 100–400 units of enzyme (Calbiochem; San Diego, CA). The S1 nuclease reactions were resolved on a 1.0% agarose alkaline gel (Sambrook et al., 1989). The DNA was then transferred to MagnaGraph nylon membrane (MSI; Westboro, MA), and the membrane was probed with double-stranded DNA generated by PCR using the same primer pairs as the asymmetric PCR. The double-stranded DNA was labeled with [α-32P]dCTP by random hexamer priming (Prime-A-Gene Labeling System; Promega), using the vendor-supplied protocol.

2.4. Isolation of RNA

Total RNA was isolated from germinating conidia with a method based on guanidine-HCl extraction and LiCl purification (Bittner-Eddy et al., 1994).

2.5. Primer extension

Primer extension was used to define the 5'-end of the COX1 transcript, using total cellular RNA and the oligonucleotide primer 5'-TCGAGCCCCGAGGGGT CTTTT, complementary to sequence approximately 300 nt from the most likely end of the transcript. Similar primer extension analysis of the ATP8 transcript employed the oligonucleotide 5'-CCGGACCTAATCTCGAAGTC, complementary to sequence approximately 90 nt from the 5'-end of the transcript according to S1 nuclease analysis. The AMV reverse transcriptase primer extension system (Promega) was used according to the vendor’s protocol. The primer extension reactions were analyzed on both a denaturing 8 M urea-6% acrylamide (35 × 43 cm) gel and a denaturing 7 M urea-8% acrylamide (16 × 18 cm) gel.

2.6. Northern analyses

For Northern hybridization analysis, 20 μg of total cellular RNA were heated to 60°C in a formaldehyde-formamide buffer, cooled on ice, separated electrophoretically in formaldehyde-agarose gels, and transferred to MagnaGraph nylon membrane. The membrane hybridization and washing (Bittner-Eddy et al., 1994) was modified from that of Church and Gilbert (1984). The membranes were exposed to Kodak XAR-5 film. The oligonucleotides (below) were 5'-end labeled with 10 units T4 polynucleotide kinase (Promega) in the presence of 60 μCi [γ-32P]dATP at 37°C.

The antisense oligonucleotides used for investigation of the COX1 transcript are as follows (italicized probe numbers refer to Figs. 2 and 4):

1. 3'′-COB: 5′-GCGAAGCTCGCTCGAGGGAA (includes the XhoI site downstream of the COB gene)
2. COX1 consensus promoter sequence: 5′-CTGATCCCTTGCTAA (the consensus promoter upstream of the tRNA cys gene)
3. tRNA cys: 5′-GGTAAACCATTTACATCGTA (internal to the tRNA cys gene)
4. 3′-tRNA cys-UTR boundary: 5′-GGCTAACCC-TAAAGGATTACGTA
5. 3′-tRNA cys: 5′-GGCTATAGGCTAACCCTA (sequence immediately downstream of tRNA cys gene)
6. URFn: 5′-GACGCCTCGCGCTCGGATTT (sequence within the URFn)
7. COX1: 5′-GCTGTTCCAAGTAACCCAG (internal to COX1 coding sequence)
8. URFn: 5′-TTGTTTCTCATCTCCAGT (sequence within the URFn)
9. ND1: 5′-GGGACCGCATCCTGCTCGGATTT (internal to ND1 coding sequence).

The antisense oligonucleotides used for investigation of the ATP8-ATP6-mtATP9-COX2 polycistronic transcript are:

3. 5′-ATP8 consensus promoter sequence: 5′-
TTCTCGAACTAAATCG (sequence immediately upstream of consensus promotor)

4. ATP8 consensus promoter sequence: 5'TTCCCTTCTAA
5. 3'-ATP8 consensus promoter sequence: 5'TTGCAACTTATATGATCC (sequence immediately downstream of the consensus promotor)
6. ATP8: 5'-GAATAAACGAACAAATCTAGGT (internal to the ATP8 coding sequence).

Two DNA fragments were also used to probe Northern blots for the ATP8-ATP6-mtATP9-COX2 transcript: (1.) EcoRI-fragment 8; and (2.) the first 1600 bp of EcoRI-fragment 9 (subcloned after cutting with EcoRI and HincII) from cloned N. crassa mtDNA (Fungal Genetics Stock Center). These fragments were labeled with [α-32P]dCTP by random hexamer primers (Prime-A-Gene Labeling System; Promega), using the vendor-supplied protocol.

2.7. Processing and lysis of mitochondria

N. crassa (strain 74A) cells (0.75–2.0 g) were homogenized mechanically for 30 s in 10 ml disruption buffer (0.25 M sucrose, 1 mM EDTA, 50 mM Tris; pH 7.5), and the mt fraction was isolated by differential centrifugation (Brambl, 1980; Plesofsky--Vig and Brambl, 1990). Mitochondria were suspended in 500 μl of 20 mM Tris (pH 7.5), 10% glycerol. This preparation was aliquoted and frozen at −20°C. Mitochondria were lysed at 2 μg/μl in 1% Triton X-100, 20 mM Tris (pH 8.0), 100 mM NaCl, 5 mM EDTA, 10% glycerol, 300 units RNasin (Promega, Madison, WI). The lysate was placed on ice for 15 min and mixed frequently. The supernatant was collected by centrifugation at 13,000 × g for 12 min at 4°C.

2.8. RNA binding assay

Soluble mt lysates were incubated with the radiolabeled RNAs in a buffer containing 20 mM Tris (pH 8.0), 100 mM NaCl, 5 mM EDTA, 5 mM MgCl2, 10% glycerol, 3 mM DTT. Typically, lysates containing 30 μg of protein were incubated with at least 2 × 105 cpm of [α-32P]UTP-labeled RNA (~15 ng) in a total volume of 25 μl. Binding reactions were carried out at 25°C for 15 min. Competition reactions with cold RNAs (~50 × molar excess) were carried out for 10 min prior to the addition of the 32P-labeled RNA. Heparin sulfate (Sigma; St. Louis, MO) at a final concentration of 5 mg/ml was added with the 32P-labeled RNAs. After the binding reactions, 3000 units of RNase T1 (Ambion; Austin, TX) were added to the reaction mixture and incubated at 25°C for 20 min.

2.9. UV Cross-linking

The RNA was covalently cross-linked on ice to the proteins by a 5 × 10⁶ μJ exposure to short-wavelength UV light (254 nm) at 6 cm distance, using a Stratalinker 1800 (Stratagene; La Jolla, CA) (Hel et al., 1996). The samples were resolved on either a 5% polyacrylamide-Tris/borate/EDTA gel or a sodium dodecyl sulfate (SDS) 10% denaturing polyacrylamide gel (Laemmli, 1970), and dried gels were autoradiographed.

3. Results

3.1. Conserved promoter sequences in three mitochondrial genes

Others (Kennell and Lambowitz, 1989; Kubelik et al., 1990) have identified five likely transcriptional
promoters in the \( N. \) crassa mt genome, based upon a consensus sequence of

\[(AT-rich)_{15-27}TTAG(A/T)RR(G/T)(G/C)N(A/T)\]

These include a promoter sequence for \( COB \), -(AT-rich)\(_{23}\)TTAGAGGGGA-, which begins \(-730\) bp upstream from the ATG start codon, and three promoter sequences adjacent to the mt small \( rRNA \) gene and one adjacent to the large \( rRNA \) gene. We sought to find similar consensus sequences near other protein-encoding genes of interest in this present study. We found closely related sequences within the 5'-upstream regions of both \( COX1 \) and the \( ATP8-ATP6-mtATP9-COX2 \) transcriptional unit (Fig. 1B). The consensus sequence for the \( COX1 \) promoter (matching 10/11 bases) is \(-1416\) bp from the ATG and immediately 5' to the \( tRNA^{cyt} \). The consensus sequence for the \( ATP8-COX2 \) promoter (matching 10/11 bases) is \(-1106\) bp upstream of the ATG, between \( URF_k \) and \( ATP8 \). The two putative promoter sequences are preceded by AT-rich regions of 15/17 upstream from the ATG, between \( URF_k \) and \( ATP8 \). The two putative promoter sequences are preceded by AT-rich regions of 15/17 upstream from the ATG, between \( URF_k \) and \( ATP8 \). We wished to determine what sequences were initially included sequences for all four genes. Here, \( COB \) was not included in the 5'-end of the mature \( COX1 \) RNA. Our results thus agree with others’ (Burger et al., 1985), showing that the 5'-end of the mature \( COX1 \) RNA is determined by cleavage from the \( tRNA^{cyt} \), a conclusion further strengthened by primer extension analysis (below). As expected, a probe (#5) to sequence between \( tRNA^{cyt} \) and the \( URF_n \) did hybridize to an RNA the same size as \( COX1 \) RNA (probe #7, Fig. 2B), thus confirming this juncture as the site of processing to yield the 5'-end of the mature \( COX1 \) RNA. This result shows that the URMs are included within the transcript, but the adjacent \( ND1 \) coding sequence is not, since its predominant 3.2 kb RNA is smaller (Burger and Werner, 1985). The consensus sequence for the putative promoter for the \( COX1 \) gene lies immediately upstream of the \( tRNA^{cyt} \) gene.

3.2. Mapping of the 5'-ends of RNAs

We wished to determine what sequences were included in the 5'-UTRs of the transcripts for \( COX1 \) and the \( ATP8-ATP6-mtATP9-COX2 \) cluster. We employed combinations of Northern analysis, primer extension, and S1 nuclease to define the 5'-ends of these RNAs and to help confirm the identification of putative promoter sequences (above).

To characterize the \( COX1 \) transcript, we prepared oligonucleotide probes (Fig. 2A) to the protein-coding regions of \( COX1 \) and NADH dehydrogenase subunit 1 (\( ND1 \)), to two adjacent reading frames (\( URF_n \) and \( URF_n \)) of unknown significance, and to sequences within and flanking the \( tRNA^{cyt} \) (a \( tRNA \) gene situated between the upstream \( COB \) gene and the downstream \( URF_n-CoX1-URF_n-ND1 \) sequence). The results of these Northern analyses (Fig. 2B) show that a 5.5 kb RNA [the size of \( COX1 \) RNA detected in mt polyribosomes (Bittner-Eddy et al., 1994)] containing coding sequence for the \( COX1 \) protein also hybridizes with the \( URF_n \) and \( URF_n \) probes, but not with the \( ND1 \) probe. This result shows that the URMs are included within the transcript, but the adjacent \( ND1 \) coding sequence is not, since its predominant 3.2 kb RNA is smaller (Burger and Werner, 1985). The consensus sequence for the putative promoter for the \( COX1 \) gene lies immediately upstream of the \( tRNA^{cyt} \) gene. Others have shown that the processing of the \( tRNA^{cyt} \) generates the 5'-end of the \( COX1 \) mRNA (Burger et al., 1985). These same studies (Burger et al., 1985), however, concluded that this processing step to remove the \( tRNA^{cyt} \) also generated the 3'-end of \( COB \) mRNA, itself proposed to be a part of a longer primary transcript that included \( COX1 \) mRNA. As shown in Fig. 2B, a probe (#1) to sequence 3' to \( COB \) gives a pattern of hybridization that is that of \( COB \) (Bittner-Eddy et al., 1994). A 15-nt probe (#2) that encompassed the putative promoter sequence for \( COX1 \) (immediately 5' of the \( tRNA^{cyt} \) did not hybridize to an RNA. A positive control for this hybridization result is a 14 nt junction probe (#4, see below) that gave a clear signal under these experimental conditions. A probe internal to \( tRNA^{cyt} \) (#3) did not hybridize to a large transcript-, but a probe (#4) that encompassed the \( tRNA^{cyt} 
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to that of a probe to a protein-coding region for ATP8 (#6). A short oligonucleotide probe (#4) that contained only sequence for the consensus promoter also did not hybridize. Probes prepared against sequences 5’ to the predicted promoter (probes #2 and #3) would not be expected to hybridize to RNA, whereas a probe 3’ to the promoter (#5) would be expected to hybridize; these are the results we obtained (Fig. 3B), strengthening our conclusion that this promoter sequence is functional.

We employed primer extension analysis of the 5’-ends of the transcripts of COX1 and of the ATP8-ATP6-mtATP9-COX2 cluster (Fig. 4). The analysis (Fig. 4) of COX1, with a primer beginning at −1011 b, yielded a DNA of 323 nt in comparison to a sequencing ladder, showing that there was only one likely 5’-end for the COX1 gene transcript, at nt −1333 (with respect to the AUG start codon), which is 73 nt downstream of the consensus promoter sequence and the first nt 3’ of the sequence for the intervening tRNA cys.

Primer extension analysis of the 5’-end of the ATP8-COX2 sequence, with a primer beginning at −1002 b, yielded a cluster of DNAs of 72–76 nt (possibly due to nuclease activity) and a longer DNA of 94 nt, based on comparison to a sequencing ladder (Fig. 4). The major 5’-end for this transcript, therefore, is likely at nt −1095 with respect to the AUG, and this end is the first nt 3’ of the consensus promoter sequence. Further, we mapped the 5’-terminus of the ATP8-COX2 polycistronic RNA with S1 nuclease. A complementary DNA, encompassing both the coding region of ATP8 as well as the 5’-UTR of the RNA from +202 to −666 b, was completely protected by the transcript (data not shown). We found that another complementary DNA (extending from −641 to −1435 b of the 5’-UTR) was partially protected by the transcript (approximately 450 b of a 794 b total), and we conclude that the 5’-end of the transcript is at the end of this protected fragment.

3.3. Identification of RNA-binding proteins

A central objective of this study was to identify trans-acting proteins that would bind to 5’-UTRs of COB, COX1, and ATP8-ATP6-mtATP9-COX2 transcripts and to determine if individual transcripts likely associated with distinct arrays of proteins or if they bound to the same proteins. We performed gel-shift experiments to detect soluble proteins in mitochondria that would bind to individual transcripts and could be specifically displaced. In these experiments, a transcript that included the complete 5’-UTR of each mRNA was synthesized and radiolabeled in vitro with α-[32P]UTP. The radiolabeled transcript was incubated with a soluble mt lysate, and after the binding reaction, RNase T1 was added, followed by covalent cross-linking of proteins to the protected RNA.

When a transcript for the 5’-UTR of COB was incubated with the mt lysate and the cross-linked products were subjected to SDS-polyacrylamide gel electrophoresis, we detected four proteins that bound to this RNA, with apparent sizes of 155, 132, 126, and 112 Mr (Fig. 5A). These proteins were blocked from binding to the radiolabeled COB RNA if they were pre-incubated with a 50-fold excess of unlabeled COB RNA, but protein binding was not blocked by a 50-fold excess of unlabeled 5’-UTR transcripts for COX1 or ATP8-COX2.

When a transcript for the 5’-UTR of COX1 was incubated with mt lysate, five proteins bound to this RNA of 155, 132, 126, and 45 Mr (Fig. 5B). These proteins were blocked from binding to the radiolabeled COX1 RNA if they were pre-incubated with a 50-fold excess of unlabeled COX1 RNA, and binding
was not blocked by unlabeled 5'-UTR transcripts for COB or for ATP8-ATP6-mtATP9-COX2.

A full-length 5'-UTR transcript for ATP8-ATP6-mtATP9-COX2 was synthesized and incubated with a mt lysate, and this RNA bound to four proteins with apparent sizes of 138, 110, 98, and 50 Mr (Fig. 5C). The binding of proteins to the radiolabeled probe was diminished by pre-incubation with 50-fold excess of unlabeled ATP8-COX2 5'-UTR RNA. The binding of most of these proteins was also prevented by 50-fold excess of unlabeled transcripts for COB or COX1, with binding of the larger proteins especially being blocked by COX1. With all three 5'-UTR transcripts described here, binding of proteins to the radiolabeled RNAs was abolished by the treatment of the reaction mixtures with Proteinase K.

It seems likely that the mRNAs for COB, COX1, and ATP8-COX2 share certain proteins as possible regulatory factors, rather than each binding a distinct group of proteins. Some of these proteins seem to be related in size, and they may have overlapping specificities, based upon their capacity to be competed off by multiple UTRs. The four proteins that bind the ATP8-COX2 5'-UTR RNA are all slightly larger than four that bind the 5'-UTR of COX1, but they may represent the same proteins whose sizes differ slightly because of the residual RNAs that remain cross-linked to them. Two of these proteins, of 130/138 Mr, and 105/110 Mr, may be the same as the 132 Mr, and 112 Mr, proteins found to bind to the COB 5'-UTR RNA. Furthermore, two other binding proteins (93/98 Mr, and 45/50 Mr) may be shared by the 5'-UTR RNAs of COX1 and the ATP8-COX2 cluster. In contrast, two proteins of 155 and 126 Mr, appear to bind uniquely to the COB 5'-UTR RNA, and an 88 Mr protein bound only to the 5'-UTR of COX1.
3.4. Protein-binding regions of transcript 5'-UTRs

We sought to identify the regions of the transcript 5'-UTRs that bound these proteins. We prepared for each transcript a series of deletions from the 3'-end of the UTR, with the original 5'-termini remaining in all truncated 5'-UTRs.

When a full-length 5'-UTR (+75 – 2727) for the COB transcript was incubated with mt lysate, four proteins of 155, 132, 126, and 112 Mr were detected as binding to the RNA (Fig. 6A). These proteins also bound strongly to truncated 5'-UTR (–58 – 2727), 5'-UTR (–230 – 2727), and 5'-UTR (–388 – 2727), but the binding of all four proteins was very diminished or eliminated with a truncated 5'-UTR (–437 – 2727). Therefore, it seems likely that a region of the COB 5'-UTR between –388 and –437 nt from the AUG codon is necessary for protein binding and likely contains binding sites of these four proteins.

A full-length 5'-UTR (+33 – 1333) for the COX1 transcript was incubated with mt lysate, and it bound five proteins of 130, 105, 93, 88, and 45 Mr (Fig. 6B), which also bound strongly to a truncated COX1 5'-UTR (–518 – 1333). However, these five proteins bound only weakly to truncated COX1 5'-UTR (–838 – 1333), and they did not bind at all to truncated 5'-UTR (–890 – 1333) or 5'-UTR (–1024 – 1333). Therefore, a region within the COX1 5'-UTR between –518 and –838 nt from the AUG codon likely contains a required protein-binding site.

A full-length 5'-UTR (+117 – 1095) RNA of the ATP8-COX2 polycistron transcript bound proteins from the mt lysate of 138, 110, 98, and 50 Mr (Fig. 6C). These proteins also bound strongly to a truncated...
5'-UTR(-79 - 1095) and a truncated 5'-UTR(-311 - 1095). Whereas the 138, 110 and 98 M₉ proteins also bound to truncated 5'-UTR(-781 - 1095), the 50 M₉ protein did not bind to this smaller transcript. Thus, the region of the ATP8-ATP6-mtATP9-COX2 5'-UTR required to bind the three high molecular weight proteins is likely between -781 and -1095 with respect to the AUG codon, but the region between -311 and -781 is necessary for binding of the 50 M₉ protein.

3.5. Predictions of secondary structures within 5'-UTRs

We wished to determine if common structural features could be identified in the 5'-UTRs of these three transcripts which could represent possible binding sites for proteins. We employed mfold version 3.1 (Zuker, © 1995–2002), a secondary structure prediction algorithm, to evaluate the 5'-UTRs for possible stem-loop structures. This algorithm computes an RNA structure by free energy minimization of the folded molecule; many diverse foldings are possible that are close to the minimum energy. We sought to identify, both by this algorithm and by visual inspection, sequences in the three 5'-UTRs that could be folded into similar structures.

We found within the protein-binding regions of each of the three 5'-UTRs a stem-loop structure of 41–45 nt that exhibited common features, including a terminal hairpin loop, an interior loop, and in the hairpin stem between the interior and hairpin loops, a deformity created by a bulge loop (Fig. 7). These stem-loop structures were found between 2438 and 2394 b (with respect to the initiating AUG) of the COB 5'-UTR RNA, between 2722 and 2681 b of the COX1 5'-UTR RNA, and between 2437 and 2727 b of the ATP8-COX2 5'-UTR RNA. Reduced binding may be due to partial deletion of the binding site(s) or its destabilization or, alternatively, to the presence of a small amount of full-length transcript resulting from incompletely digested (but undetected) template DNA. (A) Proteins that bind to the 5'-UTR COB RNAs of full or reduced lengths. The COB RNAs assayed were +75 to -727 (full-length), -58 to -727, -230 to -727, -388 to -727, and -437 to -727. Binding was severely reduced or abolished for the shortest RNA tested, extending from -437 to -727, suggesting that a required binding site is located between -388 and -437 from the transcript AUG. (B) Proteins that bind to 5'-UTR COX1 RNAs of full or reduced lengths. The COX1 RNAs assayed were +33 to -1333 (full-length), -518 to -1333, -838 to -1333, -890 to -1333, and -1024 to -1333. Binding was strikingly reduced for the RNA extending from -838 to -1333 and for shorter RNAs, suggesting that a required binding site is located between -518 and -838 from the transcript AUG. (C) Proteins that bind to 5'-UTR ATP8-COX2 RNAs of full or reduced lengths. The ATP8-COX2 RNAs assayed were +117 to -1095 (full-length), -79 to -1095, -311 to -1095, and -781 to -1095. The three higher molecular weight proteins bound to all the RNAs tested, but the 50 M₉ protein did not bind detectably to the shortest RNA, extending from -781 to -1095. This suggests that a required binding site for the high M₉ proteins is located between -781 and -1095 from the AUG, but that the region between -311 and -781 contains a binding site for the 50 M₉ protein.
5′-UTR of the COX1 RNA, and between −1077 and −1034 b of the 5′-UTR of ATP8-COX2 polycistronic transcript. The free energies of the COB, COX1, and ATP8-COX2 structures were \( \Delta G = -11 \), \( \Delta G = -25.4 \), and \( \Delta G = -11.6 \), respectively. There were no similar stem-loop structures detected in the 5′-UTRs of the three RNAs within several hundred bases surrounding these predicted structures.

4. Discussion

4.1. General

Mitochondria are organelles, derived from prokaryotic ancestors, that contain a small, rudimentary genome; however, their mechanisms for gene expression are completely dependent upon the nucleus for both enzymic and regulatory components. It seems unlikely that these nucleus-encoded regulatory factors are involved in regulating mt gene expression transcriptionally. Instead, increasing evidence indicates that mt gene expression is regulated at the level of translation, with nuclear-encoded proteins binding to structural elements in the mt mRNA to activate translation, stabilize mRNAs, or repress their translation. In this present study of mt gene expression elements in N. crassa, we have identified two new, putative promoter elements, for COX1 and the ATP8-COX2 polycistron, that are related to the five that have previously been identified. We defined the 5′-UTRs of transcripts of these two gene clusters, mapping their 5′ ends to positions that are consonant with promoter element identification. We also identified two to four proteins that appear to bind to the 5′-UTRs of COB, COX1, and ATP8-COX2 transcripts, possibly through interaction with a stem-loop secondary structure with shared features. We have also detected proteins that

![Fig. 7. Predicted secondary structures in 5′-UTR RNAs. Stem-loop structures within the protein-binding regions of the 5′-UTR RNAs of COB (−438− − 394), COX1 (−722− − 681), and ATP8-COX2 (−1077− − 1034). These putative RNA structures, with free energies of −11, −25.4, and −11.6 \( \Delta G \), respectively, were predicted by mfold version 3.1 (Zuker, © 1995–2002).](image-url)
bind apparently uniquely to either the COB or the COX1 5'-UTR and that could be involved in other, individual regulatory steps.

4.2. Gene promoters/regulatory elements

The promoter element of S. cerevisiae mt genes was one of the first to be characterized. The yeast mt DNA contains about 20 transcriptional initiation sites distributed around the genome that contain the highly conserved nonanucleotide sequence 5'-A(T/T)TATAAGTA, the last A being the initiating nucleotide (reviewed in Grivell, 1995; Jang and Jaehning, 1994). This AT-rich element resembles the prokaryotic -10 element and the eukaryotic TATA box, but its position in relation to the transcriptional start site resembles that of bacteriophage genes (Jang and Jaehning, 1994). As defined through in vitro transcription analyses, five transcriptional promoters have been identified in the N. crassa mt genome, yielding a consensus sequence of (AT-rich) 15–27TTAG(A/T)RR(G/T)(G/C)N(A/T), that reflects a greater divergence of promoter sequences in comparison to yeast (Kennell and Lambowitz, 1989; Kubelik et al., 1990). These consensus elements include a promoter sequence for COB, (AT-rich)23TTAGAGAGGG, which begins -730 bp upstream from the ATG start codon. This is the only previously known N. crassa mt promoter sequence adjacent to a protein-encoding gene, the others being adjacent to the two rRNA-encoding genes (Kubelik et al., 1990).

In the present study we have identified putative promoter sequences for COX1 and for the ATP8-ATP6-mtATP9-COX2 polygenic cluster. The sequence for the polygenic cluster is at -1106– -1096 bp from the ATG, between URFk and ATP8, and matches the consensus sequence in 10/11 bases. This promoter sequence ends at the nt preceding the mapped 5' end of the transcript. Previous workers (Kennell and Lambowitz, 1989) concluded that the initiating nt in the Neurospora COB transcripts was the sixth nt of the 11-nt consensus promoter sequence. In contrast, for the ATP8-COX2 transcript we observed no hybridization to mt RNA with an 11-nt probe that matched this sequence. Instead, our primer extension analysis produced a DNA of 94 nt, which would initiate with the first nt 3' of the promoter, rather than falling within the consensus promoter sequence. We found a promoter consensus sequence for COX1 at -1416 bp from the ATG and almost immediately 5' to the tRNA\(^{\text{cys}}\), where transcription likely initiates. Our results make it unlikely that COB and COX1 are parts of the same transcriptional unit, as concluded previously (Burger et al., 1985).

4.3. Definition of primary transcripts and mRNAs

Based upon Northern hybridization experiments, it appears that the entire mt genome of Neurospora is transcribed (Burger et al., 1985; Breitenberger et al., 1985). However, because of the few consensus promoters identified so far, relative to Saccharomyces mtDNA, it has seemed likely that an individual promoter in Neurospora may be responsible for synthesis of polygenic transcripts that then undergo extensive processing (Kubelik et al., 1990). In addition, our previous studies indicated that the ATP8-ATP6-mtATP9-COX2 polygenic cluster yielded a single transcript (Bittner-Eddy et al., 1994).

The size and processing of the primary transcript containing COX1 sequence has been examined previously (Burger et al., 1985; de Vries et al., 1985). Northern analyses predicted transcription of the region encompassing COB-tRNA\(^{\text{cys}}\)-URFu-COX1-URFn-tRNA\(^{\text{arg}}\)-ND1 as a single precursor RNA of ~12.5 kb (Burger et al., 1985). However, our Northern analyses with oligonucleotide probes and our identification here of a likely transcriptional promoter between COB and COX1 are not consistent with a single transcript containing sequences for both these genes. Furthermore, transcripts of these two genes accumulated with disparate kinetics and unrelated size species during conidial germination (Bittner-Eddy et al., 1994), making it unlikely that they are derived from a common precursor, as previously proposed (Burger et al., 1985).

Primer extension analysis showed that the mature COX1 mRNA begins at the first nucleotide 3' to the tRNA\(^{\text{cys}}\) sequence, and the apparent cleavage site at the 3'-end of the tRNA\(^{\text{cys}}\) defines the 5'-end of the mature COX1 mRNA (Fig. 8), as described previously (Burger et al., 1985). Thus, the primary transcript
probably includes the tRNA\(^{cya}\), indicating that in \(N.\) crassa, like metazoans, the mt tRNA sequences may serve as transcript processing signals, as proposed previously (Burger et al., 1985; Breitenberger et al., 1985). In contrast, the mature 5'-end of transcript for the \(ATP8-COX2\) cluster, being the first nucleotide 3' to the consensus promoter sequence (Fig. 8), does not, apparently, result from processing.

4.4. Identification of proteins that bind to 5'-UTRs of mtRNAs

A major step of regulation of mt gene expression in \(S.\) cerevisiae is at the level of translation. Genetic and biochemical studies have identified several nucleus-encoded proteins whose association with specific mt mRNAs is required for their translation (Fox, 1996). The activation of mRNA translation of cytochrome \(c\) oxidase subunit peptide 3 (\(COX3\)) by Pet54p, Pet494p, and Pet122p requires the binding of these three proteins to sites within the 5'-leader sequence that are at least 172 nt upstream of the initiation codon (Constanzo and Fox, 1988). The product of the yeast \(CBS1\) gene, another mt translation activator, acts on the 5'-untranslated leader sequence of the apocytochrome \(b\) (\(COB\)) mRNA (Rödel and Fox, 1987). At least one of these activators, Pet309p, also functions in removal of introns from the transcript it activates (Manthey and McEwen, 1995; Manthey et al., 1998). Two yeast nuclear genes, \(NCA1\) and \(CBP1\), encode proteins that stabilize individual mRNAs for \(ATP9\) and \(COB\), respectively (Dieckmann and Staples, 1994). A nuclear gene, \(cya-5\), has been identified in \(Neurospora\) whose product is homologous to Pet309p and in whose absence the accumulated mRNA for \(COX1\) is not translated (Coffin et al., 1997). Related proteins with partial sequence homology have been found in higher plant chloroplasts (Fisk et al., 1999). The \(cya-5\) protein is predicted to be 130 kDa, and we have detected proteins of this size binding to RNAs. However, these size-related proteins bind to all three UTRs in our experiments, and the \(cya-5\) mutation affected translation of only \(COX1\) RNA (Coffin et al., 1997).

In \(S.\) cerevisiae there is an abundant 40 kDa protein that has been shown to bind to the 5'-UTRs of all major mt mRNAs (Papadopoulou et al., 1990; Dekker et al., 1992). This RNA-binding protein is now known to be the mt NAD\(^+\)-dependent isocitrate dehydrogenase, whose two subunits (of similar size) are both required for the dual functions (Elzinga et al., 1993). It has been proposed that this enzyme protein could act as a repressor of translational initiation (Dekker et al., 1992). The mRNA-specific translational activators, perhaps located in the inner membrane, would interact with the p40-mRNA complex and reverse the inhibition (Dekker et al., 1992). Homologues to the isocitrate dehydrogenase subunits are included in the \(Neurospora\) sequence database, but we find a protein of this size (\(\sim 40\) kDa) binding only to two of the three UTRs.

In the present study, using gel-shift assays, we identified several mt proteins that bind to the 5'-UTRs of the three transcripts evaluated. Each of the transcript 5'-UTRs bound to a large protein of apparent \(M_r\), 132 (\(COX1\)), 130 (\(COX1\)), or 138 (\(ATP8-COX2\)) and to a smaller protein of \(M_r\) 112, 105 or 110, respectively. Although similarity of size does not indicate identity, these two proteins may be the only species that are common to all three transcripts. Two other protein size-species were shared only between \(COX1\) and \(ATP8-COX2\): a protein with an apparent \(M_r\) of 93 (\(COX1\)) and 98 (\(ATP8-COX2\)) and a protein with an apparent \(M_r\) of 45 (\(COX1\)) and 50 (\(ATP8-COX2\)). The \(M_r\) 50 protein was unusual in that its binding capacity was lost early (and uniquely) in the 5'-UTR trimming experiments, suggesting that it bound to a different site within the 5'-UTR of \(ATP8-COX2\) transcripts than the other proteins. These proteins otherwise behaved as a cohort, binding or being displaced as a group. If any of the proteins that bind to
all 5′-UTRs were necessary for binding of the remaining proteins as a group, the capability of both the COB and COX1 5′-UTRs to compete off all proteins from the 5′-UTR of ATP8-COX2 would be explained.

4.5. Protein-binding regions of transcript 5′-UTRs and secondary structure predictions

We have identified regions of the 5′-UTRs of Neurospora mt transcripts that bind these proteins (Fig. 8), using a series of deletions from the 3′-end of the 5′-UTRs. In the COB transcript a 50 nt sequence between −388 and −437 from the AUG contained the binding site. In the COX1 transcript, a 321 nt sequence between −518 and −838 from the AUG bound the proteins, and in the ATP8-COX2 transcript a 315 nt sequence between −781 and −1095 is required for protein binding.

We evaluated these protein-binding regions and several hundred nt of flanking regions of the 5′-UTRs for possible stem-loop structures that could be involved in protein-RNA interaction. Using a free energy minimization algorithm to predict secondary structure (Zuker, 1990–2002), we found in each 5′-UTR a stem-loop structure of about 40 nt that showed a common overall pattern. These structures are predicted to form within the smallest regions of these 5′-UTRs found to be necessary for protein-binding (Fig. 8). We think it possible that these structures may be the sites which bind some or all of the proteins detected in the gel-shift assays, a proposal that could be tested directly in vitro by experimental alteration of the transcript structure. These potential RNA stem-loop structures, with loops and deforming bulges, generate major grooves in RNA helices that are sufficiently wide for interacting proteins to penetrate the normally inaccessible deep and narrow major groove of the A-form RNA helix (Draper, 1999). The predicted stem-loop structures and the regions required for protein binding are sufficiently distant from the initiating AUGs to make it unlikely that associated proteins would interact with ribosome binding sites or with the ribosome itself, as has been proposed for a −20/−35 RNA stem-loop structure within the COX2 mRNA that functions as a binding site for the yeast translational activator Pet111p (Dunstan et al., 1997). The prediction algorithm employed here to identify secondary structure does not predict RNA pseudoknots (Mathews et al., 1999), which may represent an additional regulatory motif, as proposed for translation of prokaryotic mRNA (Pleij, 1990).

Dunstan et al. (1997) have identified an octanucleotide sequence element, 5′-UAUAAAU, in the 5′-UTRs of yeast mt transcripts that they propose is a general ribosome recognition site. We found this same sequence in only one of the 5′-UTRs of the transcripts of interest in this study, at position −754−−747 with respect to the AUG of the ATP8-ATP6-mtATP9-COX2 transcriptional unit. However, we have found a closely related element, 5′-AUUAAAU(A/U), in each of the three 5′-UTRs, represented only once, and much closer to the AUG codon (Fig. 8). In the COB transcript, the 5′-AUUAAAU element is between nt −133 and −126, in the COX1 transcript the 5′-AUUAAAU element is between nt −58 and −51 (between URFu and the COX1 sequence), and in the ATP8-COX2 transcript the 5′-AUUAAAUU element is between nt −57 and −50, relative to the AUG initiating codon. A complementary sequence should be present in the small (19S) ribosomal RNA in a position where base-pairing interaction could occur. We found this predicted complementary sequence, 5′-TATTTAAT, occurring only once in the small mt rRNA gene, beginning at position +1243 of the Neurospora gene sequence. An identical complementary sequence also occurs once in the mt 19S rRNA of the closely related ascomycete Podospora anserina, beginning at position +1123. This sequence occurs within a mainly single-stranded bulge sequence between two predicted base-paired stems of the universally conserved region U5 of small rRNAs (Cummings et al., 1989). An alignment of this possible mRNA-binding motif shows identical sequences present in the mt small rRNAs of Aspergillus nidulans and several other fungi, in addition to N. crassa and P. anserina.

The entry of mRNA into the mt polyribosomes and translational apparatus in Neurospora apparently requires stabilization of transcripts and may require a regulated step that renders them translatable. Throughout vegetative spore (conidial) maturation, dormancy, and germination, the mt transcripts are individually regulated. The transcripts for COXI are maintained at a uniformly high level, whereas the
transcripts of COX2, undetectable in the dormant conidia, begin to accumulate only upon conidial activation (like the co-transcribed ATP6), and those of COX3, initially low, begin to increase after 30 min following initiation of germination (Bittner-Eddy et al., 1994). Nevertheless, these COX1, COX2, and COX3 transcripts are recruited into the polyribosomes uniformly and as a group after 60 min of germination (Bittner-Eddy et al., 1994). These apparently regulated steps of transcript recruitment into polyribosomes suggest that the mRNAs are withheld or admitted to the translational apparatus through their association with factors, such as negative regulatory elements and activator proteins. We believe that the transcript-associated proteins we have identified here could be a part of this device for transcript stabilization and translation regulation of organelle gene expression.

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