Cytochrome Oxidase Subunit 2 Gene in *Neurospora crassa* Mitochondria*

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The nucleotide sequence of the cytochrome oxidase subunit 2 (COX2) gene has been obtained from cloned mitochondrial DNA segments of *Neurospora crassa*. The coding sequences have been identified on the basis of protein sequence homology with the subunit 2 of cytochrome oxidase from yeast and man. The postulated precursor of the *N. crassa* subunit 2 protein is 250 amino acids long, with a molecular weight of 28,700. As in the tRNA and rRNA genes, the subunit 2 gene is flanked by G+C-rich palindromic sequences, which are highly conserved in *N. crassa* mitochondria. Three major transcripts have been detected by Northern blot hybridization. A transcript of 1100 bases is tentatively considered the fully processed mRNA. Furthermore, S1 nuclease protection experiments have revealed that the putative subunit 2 mRNA has a 330 nucleotide long 5' leader sequence.

Known mitochondrial genomes from different sources exhibit very important differences in their organization. Mammalian mitochondrial genomes differ from those of lower eukaryotes in gene organization, in number of genes, and in the absence of split genes and intergenic regions (1-5). The mitochondrial genomes of *Saccharomyces cerevisiae* and *Aspergillus nidulans* show a very low G+C content, mainly because of the high A+T content of the intergenic regions, and also because of the strong bias for A+T-containing codons. The size of the mitochondrial genome of *N. crassa* is 60 kb, and is about the same size as that of *S. cerevisiae*, while its G+C base composition (40%) is more similar to that of mammalian mitochondrial DNA. The higher G+C content of the mitochondrial genome of *N. crassa* might reflect a different codon usage, a different structure of intergenic regions, or a higher number of genes.

The overall organization of the mitochondrial genome of *N. crassa* has been investigated by using yeast mitochondrial gene-specific probes (6, 7). The present work reports the sequence of the gene for the cytochrome oxidase subunit 2 (COX2) and its flanking regions. The gene for this subunit has been sequenced in yeast (11), mammals (1-3), and maize (12). The amino acid sequence is conserved, but an intron has been found in the maize gene. In *N. crassa*, this gene has no introns. Work by RajBhandary and co-workers has demonstrated the existence of peculiar structures in the intergenic regions around the rRNA (8), several tRNA (9), and COX3 genes (10) of *N. crassa* mitochondrial DNA. Similar structures have been observed in the flanking regions of the COX2 gene. To investigate the possible function of these structures, a transcript analysis has also been performed.

**MATERIALS AND METHODS**

**Plasmid Construction**—Plasmid pE2/35 contains the EcoRI fragment 4 of wild type strain SL74A *N. crassa* mtDNA, cloned into the EcoRI site of pBR322. This fragment was shown to contain the genes for subunit 2 of cytochrome oxidase and for subunits 6 and 9 of the mitochondrial ATPase (6, 7). Plasmids pP16/34 and pP2/36 were obtained by the subcloning of pE2/35 (digested with PstI) into the PstI site of pBR322. They contain, respectively, the 930- and 750-bp long PstI fragments of Fig. 1.

**DNA Hybridization**—Restriction digests of DNA were separated by electrophoresis on 1.5% agarose and then transferred to nitrocellulose (13). Mitochondrial DNA from the yeast petite DS 200A1 (Ref. 11 and Fig. 1), prepared according to Bonitz et al. (6) was used as the probe. Hybridization was performed at 42 °C in 50% formamide, 0.75 M NaCl, 75 mM sodium citrate, 0.1% polyvinylpyrrolidone (Mr = 40,000), 0.1% bovine serum albumin, 0.1% Ficoll (Mr = 400,000), 20 mM potassium phosphate, pH 6.5, and 10% dextran sulfate (14) for 16 h using 2 x 10^6 cpm of the 5' end-labeled probe.

**RNA Hybridization**—*N. crassa* mitochondrial RNA was extracted by the method of Bonitz et al. (5) from mitochondria of the wild type strain SL74A, prepared by the method of Terpstra et al. (15). The mitochondrial RNA denatured with glyoxal (16) was electrophoresed on 1.5% agarose gels at 7 V/cm for 4-5 h, stained with ethidium bromide, and photographed. The RNA was transferred to diazobenzoxymethyl paper by the method of Alwine et al. (14). Nick-translated DNA fragments were hybridized to diazobenzoxymethyl strips overnight.

**DNA Sequencing**—Restriction fragments were labeled at their 5' ends with [gamma-32P]ATP (specific activity 2000-3000 Ci/mmol, from New England Nuclear), in the presence of T4 polynucleotide kinase, and [gamma-32P]ATP and then separated into single strands on 6 or 8% polyacrylamide gels and sequenced by the method of Maxam and Gilbert (17).

**S1 Nuclease Mapping**—The 930-bp long DNA fragment of Fig. 2 was cleaved with HinfI restriction endonuclease. The resulting fragments were labeled at their 5' ends with T4 polynucleotide kinase and [gamma-32P]ATP and then separated into single strands on 6% polyacrylamide gels. About 5-10 ng of HindIl-PstI fragment, representing the coding strand (see Fig. 6) was hybridized for 2 h at 45 °C with 10 μg of *N. crassa* mtRNA and then treated with 600 units of S1 nuclease (Boehringer Mannheim) in 100 μl at 37 °C for 60 min. The S1-resistant fragments were fractionated on 6% polyacrylamide, 8 M urea gels, alongside a guanine-specific reaction (17) of 5' end-labeled DNA of known sequence.

**RESULTS**

**Localization of the COX2 Gene**—This gene was localized on the EcoRI fragment 4 of *N. crassa* mitochondrial DNA by heterologous hybridization with a yeast probe (6, 7). This

* The abbreviation used is: bp, base pairs.
Fig. 1. Mapping of the COX2 gene within the cloned fragment EcoRI fragment 4 of N. crassa mitochondrial DNA. a, restriction map of the EcoRI fragment 4. b, restriction map of the yeast gene oxi I (11), 1 and 2 represent the probes used to map the COX2 gene of N. crassa. c, hybridization of probes 1 (track 1) and 2 (track 3) to EcoRI fragment 4 cloned in pBR322 (pE2/35), digested with PstI and transferred to nitrocellulose (track 2). The arrow represents the direction of transcription, which is the same as that for other N. crassa mitochondrial genes (27-29).

Fig. 2. Restriction map and sequencing strategy of the two cloned PstI fragments, pP16/34 (a) and pP2/36 (b), bearing the two portions of the COX2 gene. All restriction sites were confirmed by sequencing. The arrows represent the sites, the direction, and the extent to which the sequences were derived. The sequences starting from the PstI sites were also confirmed by labeling these sites at their 3′ ends.

The DNA sequence of the COX2 gene and its flanking regions is shown in Fig. 3. The regions discussed below can be distinguished in this sequence.

(a) A G + C-rich region starts from the PstI site at the 5′ end of the sequence and extends for 200 nucleotides. The EcoRI fragment 4 was cloned in pBR322 (pE2/35, see "Materials and Methods"). To more accurately localize the gene within this fragment, the clone pE2/35 was digested with PstI and analyzed by Southern blotting and hybridization with the previously described probe. A PstI fragment 930 bases long that showed positive hybridization with the probe was cloned (pP16/34) and sequenced. The nucleotide sequence showed that this fragment contained only the region corresponding to 180 amino acids of the NH2-terminal end of the gene.

To identify the adjacent PstI fragment that contained the remaining part of the gene, a probe carrying the COOH-terminal portion of the yeast gene was prepared from the same petite (probe 2, Fig. 1). Hybridization of the probe to a 750-bp fragment permitted the identification of the second part of the gene (Fig. 1). This latter fragment was cloned (pP2/36) and sequenced.

DNA Sequencing—The two fragments were sequenced using the restriction sites shown on the map in Fig. 2. In most cases, both complementary strands were sequenced and restriction sites were crossed. The sequence shown indicates a single PstI site within the gene. This site was generated by joining the sequence of the two fragments at the PstI site, assuming that no extra sequences occur between the two fragments. The comparison of the amino acid sequence obtained this way shows a high homology with the amino acid sequences of known subunit 2 genes (Fig. 4), implying that this assumption is correct.

The DNA sequence of the COX2 gene and its flanking regions is shown in Fig. 3. The regions discussed below can be distinguished in this sequence.

(a) A G + C-rich region starts from the PstI site at the 5′ end of the sequence and extends for 200 nucleotides. The sequence adjacent to the PstI site is complementary and inverted compared to that determined by Yin et al. (9) in a different region of this genome (see Fig. 5). The entire G + C-rich region shows several peculiarities: i.e. the sequence between nucleotides 82 and 140 contains a 10 nucleotide long sequence repeated six times with only minor modifications, and the region between nucleotides 150 and 190 contains several stretches of purines alternating with stretches of pyrimidines.

(b) The region from nucleotide 250 to 400, immediately preceding the gene, is highly A + T-rich. Similar A + T-rich sequences have been observed in the 5′ flanking regions of other genes in N. crassa mitochondrial DNA.

(c) The sequence contains a single open reading frame that

2 G. Macino and G. Morelli, unpublished observations.
Fig. 3. Nucleotide sequence of the N. crassa COX2 gene. Nucleotide numbering is given at the left margin. The nucleotide sequence is that of the nontranscribed strand. The reading frame is written in the three letter amino acid code. A sequence repeated six times in tandem is underlined. The palindromic sequences around PstI site are boxed. Dashed lines represent "PstI-like" sites.

shows a high degree of homology with the corresponding yeast and mammalian proteins (Fig. 4). The N. crassa COX2 protein (deduced from the nucleotide sequence) is 250 amino acids long and has $M_r = 28,700$. The amino acid sequence of the NH$_2$ terminus of the mature subunit 2 of cytochrome oxidase in N. crassa has been determined by Machleidt and Werner (18). The reading frame, derived from the nucleotide sequence, exactly matches the known amino acid sequence. However, there are two AUG initiator codons located 3 and 12 triplets upstream of the aspartic acid codon that represents the first residue of the mature protein, thus suggesting the existence of a precursor polypeptide.

(d) The sequence following the terminator codon is G + C rich and contains several palindromic regions, including two sequences that are highly homologous to the stem structures which contain PstI sites (Fig. 5).

Codon Usage—Table I shows the codon usage in the COX2 gene of N. crassa mitochondrial DNA. As already shown with the COX1 (19) and COX3 (10) genes of N. crassa, there is a strong bias for codons terminating in uridine or adenosine. A similar bias has been observed in yeast (5) and Aspergillus nidulans (20) mitochondrial DNA. The codon AUA, which in mammalian (1-3) and yeast (21) mitochondria probably codes for methionine, is here considered an isoleucine codon. Comparison of the COX2 coding regions of yeast and N. crassa shows that, 7 times out of 12, the codon AUA in N. crassa corresponds to AUU or AUC in yeast. It is interesting to note, moreover, that the CGN series of arginine codons is not used at all, as in the case with both yeast (5) and A. nidulans (20).

Transcript Analysis—Mitochondrial RNA from N. crassa was denatured with glyoxal, fractionated on agarose gels, transferred to diazobenzyloxymethyl paper, and analyzed by hybridization with a nick-translated probe from within the structural gene. The results, reported in Fig. 6a, show hybridization signals corresponding to RNA species of about 3200, 1850, and 1100 bases. Similar results were obtained by van den Boogaart et al. (22). To localize the 5' end of the 1100 nucleotide long transcript that is thought to be the mature mRNA, a nuclease S1 protection experiment was performed by using the Weaver and Weissman modification of the Berk and Sharp procedure (23).

A single-stranded DNA fragment 556 nucleotides long, carrying 160 nucleotides of the upstream flanking region, was used. The results which are reported in Fig. 6b, show that a fragment of 485 ± 10 bases was protected from S1 digestion. This indicates that the 5' end of the putative messenger is localized 330 ± 10 nucleotides upstream of the initiator codon. A schematic drawing is shown in Fig. 6c.

DISCUSSION

The 1529-nucleotide sequence containing the gene coding for the cytochrome oxidase subunit 2 (COX2) of N. crassa mitochondrial DNA has a single reading frame, beginning with an AUG initiator codon and ending with a UAA terminator codon. Starting from codon 13 of this sequence, this
responding yeast (11) and human (1) proteins. Amino acid sequence identities are boxed. The deletions in the amino acid sequences were introduced to maximize the homologies.

**Table 1**

| Frequency of mitochondrial codons in the COX2 gene |
|-----------------------------------------------|
| Phe | UCU 6 | UAU 10 | UGG 2 |
| UUC 5 | Ser | UAC 2 | UGC 1 |
| UUA 20 | UCA 8 | UAA 3 | UGA 6 |
| UUG 2 | UCG 0 | UAG | UGG 0 |
| Leu | CCA 5 | Gln |  |
| CCG 1 | Arg |  |
| CUA 5 | Pro | CAC 4 | CGC 0 |
| CUC 1 | His | CAU 3 | CGU 0 |
| CUG 2 | OCG 0 | CAG 2 | CGG 0 |
| Ile | AUC 8 | ACC 0 | AAA 4 | ACA 3 | Arg |  |
| Met | AUG 8 | ACG 0 | AAG 1 | Arg |  |
| Thr | ACC 0 | AAA 4 | AAG 1 | Arg |  |
| Asp | AAC 1 | AAA 4 | AAG 1 | Arg |  |
| Gly | GCU 5 | GAA 15 | GGA 4 | GGC 0 |  |
| Ala | GCA 4 | GAA 15 | GGA 4 | GGC 0 |  |
| Val | GCG 0 | Glu | GAG 3 | GGA 4 |
| GGU 10 | GGU 5 | GAA 15 | GGA 4 | GGC 0 |  |
| reading frame matches the established NH2-terminal amino acid sequence of subunit 2 of cytochrome oxidase from N. crassa (18). The gene probably codes for a precursor protein as has been observed in S. cereuisiae (24). The mature protein exhibits a high degree of amino acid homology with the respective cytochrome oxidase subunits of yeast and mammals (see Fig. 4). The proline residues are the most conserved in number and position. Furthermore, the position of 70% of the charged amino acids is conserved. The amino acid sequence shown in Fig. 4 follows the general code with the exception of UGA, which has been shown in yeast (25), N. crassa (26), A. nidulans (20), and mammalian (1–3) mitochondrial DNA to code for tryptophan.

The regions flanking the COX2 gene contain several palindromic sequences. The longest transcript that we have detected certainly encompasses the entire sequenced region (22). A precursor RNA could be folded into several secondary structures, examples of which are shown in Fig. 5.

The putative messenger RNA is about 1100 bases long and contains a 330-nucleotide 5’ leader sequence, as shown by S1 analysis. Within the first 70 nucleotides of this leader sequence, there is a stretch of 11 nucleotides repeated six times. Since the length of the reading frame is 750 nucleotides, the 3’ tail can be calculated not to exceed 50 nucleotides. 10–50 nucleotides upstream and downstream from the two ends of this mRNA, the nucleotide sequence contains the long palindromic sequences that can form the stem structures diagrammed in Fig. 5. The structures are highly homologous to those described by Yin et al. (9), but some do not contain PstI restriction sites. This could mean that the number of such structures in the mitochondrial genome of N. crassa might be much higher than the figure of 100 calculated by Yin et al. (9).
Cytochrome Oxidase Subunit 2 Gene in N. crassa

FIG. 5. Homologous sequences in the G+C rich clusters in N. crassa mitochondrial DNA. A, homology between the PstI 3/4 boundary sequence taken from Yin et al. (9) and the sequence upstream from the COX2 gene, from nucleotide 1 to 70 (see Fig. 3). The two sequences occur in the same strand and are complementary to each other when the PstI 3/4 sequence is read from its 3' end. B, stem structures containing PstI or "PstI-like" sites. i, this sequence (from Yin et al. (9)) represents the boundary between the PstI fragments 5 and 8 of the N. crassa mitochondrial genome. ii, possible secondary structure of sequences downstream from the COX2 gene, from nucleotide 1290 to 1310 and from 1400 to 1440. The 18 nucleotides containing the PstI or "PstI-like" sites are boxed. The PstI sites are underlined. The arrows indicate the difference between the PstI and "PstI-like" sites.

FIG. 6. Analysis of the transcripts of the COX2 gene. a, agarose electrophoresis pattern of mitochondrial RNA denatured by glyoxal (track 1) and hybridization of nick-translated fragment pP16/34 to the same RNA blotted onto diazobenzyloxymethyl paper (track 2). The arrow indicates a transcript of 1100 bases that may represent the mature mRNA. b, localization of the 5' end of the postulated mature mRNA by S1 nuclease analysis. Track 1, specific cleavage at guanine of a 5' end-labeled single-stranded DNA fragment of known sequence (850 nucleotides long). Track 2, probe hybridized to mtRNA and then subjected to S1 nuclease treatment (see "Materials and Methods"). c, interpretation of the S1 experiment. The numbers above the lines indicate the lengths of the fragments.
Extensive transcript analysis of other regions of this genome is needed to investigate the role of these peculiar structures and their possible involvement in the processing of transcripts. The existence of long transcripts, spanning the entire region, does not suggest, at least in this case, a promoter function for these structures.

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G Macino and G Morelli

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