Short sequence-paper

Subunits I and II of *Dictyostelium* cytochrome *c* oxidase are specified by a single open reading frame transcribed into a large polycistronic RNA [1]

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Abstract

A single open reading frame (ORF) encoding cytochrome *c* oxidase subunit I and II (cox1/2) was identified in the mitochondrial genome of the slime mold *Dictyostelium discoideum*. The cox1 coding region shares intron positions with its counterparts in fungi and algae. Northern blot analysis, using exon and intron-specific probes, suggests that the cox1/2 gene is transcribed as part of a large, efficiently processed, polycistronic RNA.© 1997 Elsevier Science B.V. All rights reserved.

Cytochrome *c* oxidase (COX, EC 1.9.3.1), the terminal enzyme of the mitochondrial respiratory chain, is a large oligomeric complex, composed of up to ten different nuclear-encoded polypeptides assembled with three large subunits (identified by the roman numbers I, II and III) encoded in the mitochondrial genome [1]. Subunit I and II (COX1 and COX2) contain the three enzyme redox centers (cytochrome *a*, Cu_A, and the binuclear cytochrome a_3-Cu_B site) and constitute the highly conserved catalytic core of the complex [2,3]. With rare exceptions, the two polypeptides are present even in the structurally simplest forms of the enzyme found in prokaryotes, though not always encoded by the same operon [2]. The location of the genes is also variable in the mitochondrial DNA (mtDNA) where, mainly in the case of cox1, they can be split by introns. These observations and continuous new discoveries pose interesting questions concerning the evolution of the mechanisms that control the expression of functionally related genes. In this regard, an extreme example is represented by the gene encoding subunit II (cox2) that in the green alga *Chlamydomonas reinhardtii* was transferred to the nucleus, while in the ameboid protozoon *Acanthamoeba castellanii* it is fused with cox1 in a single continuous ORF termed cox1/2 [4]. The latter recent finding is intriguing because no evidence for the synthesis of a precursor polyprotein

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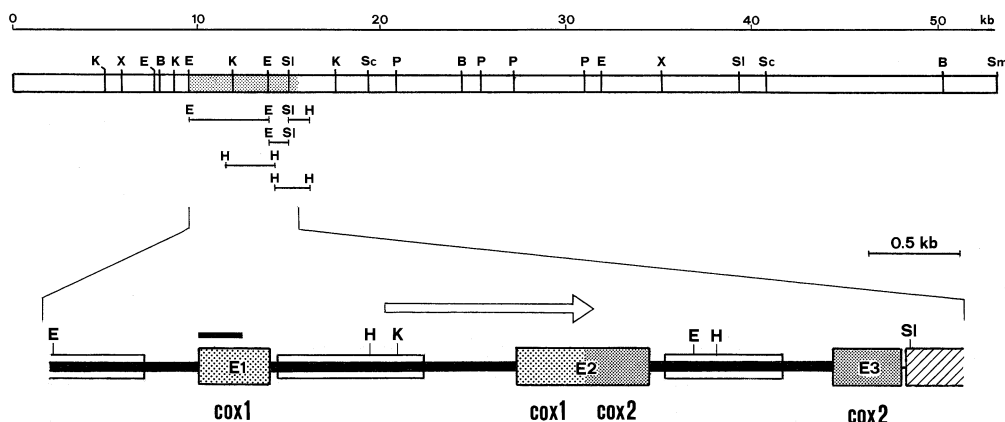
Fig. 1. Structural organization of the *Dictyostelium* mitochondrial DNA segment encoding the terminal part of subunit I and subunit II of cytochrome *c* oxidase. Its location in the restriction map of *D. discoideum* [5] is shown on the upper part of the figure, along with the positions of the cloned segments derived from the region. Exons (E) are indicated by boxes using a different shading for the cox1 and the cox2 coding regions. In the absence of a cox1 termination codon, the beginning of cox2 in exon 2 was defined by analysis of the sequence homologies (see text). Introns are shown by thick lines, while intronic ORFs are boxed. The striped box, downstream the cox1/2 gene, corresponds to a potentially functional open reading frame. The horizontal bar shows the extent and location of the 249 bp DNA probe used to map the gene. The direction of transcription is indicated by the arrow. The restriction enzyme sites are as follows: B, *Bam*HI, E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pvu*I; Sl, *Sal*I; Sc, *Sac*I; Sm, *Sma*I; X, *Xho*I.

could be provided, possibly as a consequence of an unusual mode of expression of the two subunits. We now show that this peculiar gene organization is shared by the slime mold *Dictyostelium discoideum*, in spite of features suggesting a large evolutionary distance from *Acanthamoeba*. In *Dictyostelium* the cox1/2 gene is interrupted by introns and transcribed to give a single large 8.5 kb polycistronic RNA, that is not edited.

In order to locate the cytochrome *c* oxidase genes on the available restriction map of the *Dictyostelium* mitochondrial genome [5], a 249 bp DNA fragment corresponding to a highly conserved region of cox1 was used as a probe (Fig. 1). The fragment was previously isolated as a by-product of a polymerase chain reaction (PCR) cloning and characterized by sequencing (EMBL data library accession number: X95896). It specifically hybridizes to a 3.6 kb *Eco*RI

mtDNA segment that, after purification by gel fractionation, was ligated into the pUC19 vector (New England Biolabs). To obtain further information, additional overlapping fragments, from the same genomic region, were purified and cloned (Fig. 1). Mitochondrial DNA was isolated as previously described [6], with minor modifications. Sequencing was performed by the chain termination method [7] on sets of deleted subclones, obtained by the exonuclease III/mung bean nuclease degradation system [8], and directly on the mitochondrial genome using suitable primers.

Fig. 1 shows the structural organization of a 5 kb mtDNA segment that encodes the terminal part of subunit I and the whole subunit II of cytochrome *c* oxidase. Unlike previously characterized protein coding genes, identified by sequencing approximately 50% of the *Dictyostelium* mitochondrial genome [9],

Fig. 2. Nucleotide sequence of the *Dictyostelium* mitochondrial DNA region encoding the subunit I C-terminus and subunit II of cytochrome *c* oxidase. Introns are shown by lower case letters. The amino acid sequences derived from exons and intronic putative ORFs are reported below the nucleotide sequences by the single-letter amino acid code. Closed squares indicate termination codons. Positions, extent and direction of the primers used for RT-PCR analysis (see text) are shown by arrows above the correspondent sequence. The key methionine that precedes the COX2-homologous region is underlined. The numbering of aminoacids is indicated on the right. The sequence is available from the EMBL Data Library under the accession number X81884.

```
ctgtaagagtgaaaaaagtaaaagaagtatgtgaaaagaataataatagaaataaagtatccagacaaggaattaagtttagacactgcttggttatctggatttttttgatggagtaggtt   120
 V  R  V  K  K  K  V  K  E  V  C  E  K  N  N  I  E  I  K  Y  P  D  K  E  L  S  L  D  T  A  W  L  S  G  F  F  D  G  V  G
orf1
ccatcgtaattaaccaaacaactttagaaaataaaaaacaattagtattgacatttaatcaaaagaataatcaaatattaataaaaattcaacgaattgtgggcggtaattttttatataa   240
 S  I  V  I  N  Q  T  T  T  L  E  N  K  K  Q  L  V  L  T  F  N  Q  K  N  N  Q  I  L  I  K  I  Q  R  I  V  G  G  N  F  Y  I
atacaaatacacaaagtggaaagtataaatatgctctatatttacaagtaaagaggagattttacagttatatgaatattttaaaaagtatccctcaagagcatataaaataaaagaa   360
 N  T  N  T  Q  S  G  K  Y  K  Y  A  L  Y  F  T  S  K  E  E  I  L  Q  L  Y  E  Y  F  K  K  Y  P  S  R  A  Y  K  N  K  R
taggtttaatcccacaatattatgaactgcaaaccttgatgacttctattcatgataatcagttacaagaaactctatggaaaaaattatgatacaatgggattataaggaaatagatg   480
 I  G  L  I  P  Q  Y  Y  E  L  Q  T  L  M  T  S  I  H  D  N  Q  L  Q  E  T  L  W  K  K  F  M  I  Q  W  D  Y  K  E  I  D
aaaatacagagtgggggagattttaaaacagaaaggagtaattcgttagagaggagagtaaaatcgtgaccgaaaaataaagtcattaaactatggctaatatactatctatgttaaagatt   600
 E  I  Q  S  G  E  I  L  K  Q  K  G  V  I  R  ●
aaaaagagaagtatgttatgtataattttttgtaagaggaaagggtagcaaaaattataatgaaaagttgtaaaaagatactaaataatcctaaaaaaatgaaaaagattcctataaaaa   720
cacgaaaaagataatattattacaaacgaaactcaaaatcaaaaaaaagcccccacagcttttttaataaaaataaaagaaagattgtaataaagatagagtccacaatgCATCCAGAAGT   840
                                                                                                               H  P  E  V
                                                                                                                 cox1
ATATATTTTAATATTACCAGGATTTGGATTAGTAAGTATAATATTATCTAAATATAGTAATAAAGGAATCTTTGGAGTAAAAGGAATGATCTCAGCAATGAGTGCAATTGGATTTTTAGG   960
 Y  I  L  I  L  P  G  F  G  L  V  S  I  I  L  S  K  Y  S  N  K  G  I  F  G  V  K  G  M  I  S  A  M  S  A  I  G  F  L  G
ATTCTTAGTATGGGCTCATCACATGTACACTGTAGGATTAGACGTGGATACACGAGCATATTTTACAGCAGCAACAATGATAATTGCAATCCCAACAGGAATAAAAATTTTTAGTTGGTT   1080
 F  L  V  W  A  H  H  M  Y  T  V  G  L  D  V  D  T  R  A  Y  F  T  A  A  T  M  I  I  A  I  P  T  G  I  K  I  F  S  W  L
AGCAACATTATGGGGAGGAGTAATAAAAATTACAACACCAATGTTATTCGTAATAGGATTTTTAGTTCTATTTACAATAGGAGGATTAACAGGAGTAGTATTAGCAAATGGAGGATTAGA   1200
 A  T  L  W  G  G  V  I  K  I  T  P  M  L  F  V  I  G  F  L  V  L  F  T  I  G  G  L  T  G  V  V  L  A  N  G  G  L  D
TATTAGTTTACATGATAgacaaaaataaattattaaaaaattttgatttagatatatgtatgtgtatattaattataattttatatataatcacaatgacaataaacccacttataaatg   1320
 I  S  L  H  D                                                                 M  C  I  L  I  I  I  L  Y  I  I  T  M  T  I  N  P  L  I  N
                                                                              orf2
gtataatgtggggatttaacttcataagtgtacaagaaaaatatgatcaaatatttatgaattaacacttttaacggatttatcaaaaaaagatttacaattcatatttccgtataagc   1440
 G  I  M  W  D  F  N  F  I  S  V  Q  E  K  Y  D  Q  I  Y  E  L  T  L  T  D  L  S  K  K  D  L  Q  F  I  F  P  Y  K
atattgaattagaacttaaggaattaaaatttaaaaataaagatgcatatttagatcaacttatagcaagaatatttgagtatagtaaaagtgagctatataaattaattttataagatat   1560
 H  I  E  L  E  L  K  E  L  K  F  K  N  K  D  A  Y  L  D  Q  L  I  A  R  I  F  E  Y  S  K  S  E  L  Y  K  L  I  Y  K  I
ataaaataagttcaaaaaaatcaatgaaagtagaagaaagagagtatataaaaaagtattgggtaggacttatggatggagttggtagtatagaagtaaatcattatcgtatgaaaaatt   1680
 Y  K  I  S  S  K  S  M  K  V  E  E  R  E  Y  I  K  K  Y  W  V  G  L  M  D  G  V  G  S  I  E  V  N  H  Y  R  M  K  N
tacaatatagattagtattaaatgtaaatgatagtagtgaaaatatggctctatgttactaaagattcaacagtaattggaggttatcttattaaaagaaaagagaaagctttaatagcct   1800
 L  Q  Y  R  L  V  L  N  V  N  D  S  S  E  N  M  A  M  L  L  K  I  Q  Q  V  I  G  G  Y  L  I  K  R  K  E  K  A  L  I  A
ggacgattaataataaaatgcaaatagaagatgtgataaaaattttcgaagattataatttagtaacagttcgaaaaagaaatcaattgcaattttttaaaagaaaatttaaaaagaaatg   1920
 W  T  I  N  N  K  M  Q  I  E  D  V  I  K  I  F  E  D  Y  N  L  V  T  V  R  K  R  N  Q  L  Q  F  L  K  E  N  L  K  R  N
atgtgaactggtacctttctgaaagaaagaataaatataaaaaatcacttatagtttcgaatattgaagaataagctatttcaatgaatggtttagtggctttgtagaagcaacaggtt   2040
 D  V  N  W  Y  L  S  E  R  K  N  K  Y  K  K  S  L  I  V  S  N  I  E  E  I  S  Y  F  N  E  W  F  S  G  F  V  E  A  T  G
catttttgtataagagcacaaaagagaagtattttagaatagcacatcgatatgatatgcctttattattaaatttaataataaaatttaatataaccaacaaaattaagagagcaaaaga   2160
 S  F  C  I  R  A  Q  K  R  S  I  L  E  ●
atgagcaatatgccattgaaatttataaaaaaaacgttattagaagtttttaattacacattttgagaaatacccattattaggagataaaaaaaattctttagaaactttcaaaacccgat   2280
aaaattaagaaaagatatgtgtcatgtggtcacgttgctatgaaaaataaaaaataacaatatttttcggtatttttttaagagtcatttgcaattaaaaaaatgctaacggtgaaggctttc   2400
aagctgatgcagaaacaacattagaaataaaattgaaaaagttaataccgtggaaacccaaacgagaaattcggagtaggatctgtagagactatacgcaacgaatagaaaattaattaag   2520
tttaatttataattaagatatagtccgaacccttaagtgattgaggaaaaagaaatagACATATTATGTAGTAGCTCATTTTCATTATGTATTATCAATGGGAGCAATATTTGCAATATT   2640
                                                             T  Y  Y  V  V  A  H  F  H  Y  V  L  S  M  G  A  I  F  A  I  F
                                                             cox1
TGCAGGATATTATTATTATTCAATTATGAATAGTACAAGATTATTCGGAGTCCGATATAATGAACAATTAGGAAGAATACATTTCTGGACAATGTTTATTGGAGTAAATGTTAC   2760
 A  G  Y  Y  Y  Y  Y  S  I  M  N  S  T  R  L  F  G  V  V  R  Y  N  E  Q  L  G  R  I  H  F  W  T  M  F  I  G  V  N  V  T
ATTTTTTTCCAATGCATTTCTTAGGATTGGCAGGTATGCCACGTAGAATAGGAGATTATCCAGATGCATATATAGGATGGAATTTAATAGCATCATATGGATCATTAATAACAGCATTTGG   2880
 F  F  P  M  H  F  L  G  L  A  G  M  P  R  R  I  G  D  Y  P  D  A  Y  I  G  W  N  L  I  A  S  Y  G  S  L  I  T  A  F  G
ATTATTATTTTTCGTTGTAAATATTTTACACCATATAATAAGAAGAAGTGTAAATAAAAAAACGGAGCAATAATTTTAATGGGATTAGACTTTGCCCGAGACTGGCAAATAGGATTTCA   3000
 L  L  F  F  V  V  N  I  F  T  P  Y  I  R  R  S  V  N  I  K  N  G  A  I  I  L  M  G  L  D  F  A  R  D  W  Q  I  G  F  Q
                                                                                                   cox2
AGATCCAGCAACACCAATAATGGAAGGAATAATTGATTTACATAATTATATATTCTTTTATTTAATAGTAGTTGCAGTATTTATCGGATGGGTAATGGGACGAATTTTATGGAGATTTTC   3120
 D  P  A  T  P  I  M  E  G  I  I  D  L  H  N  Y  I  F  F  Y  L  I  V  V  A  V  F  I  G  W  V  M  G  R  I  L  W  R  F  S
TTATAAATGGAGTTATCCTACAATAGGAGATATTGAAATATTTAAAAATTTTACAGCATATAATCAAATAATACATGGACAGTTATTGAAATCGTTTGGACATTAATTCCAACAGTAAT   3240
 Y  K  W  S  Y  P  T  I  G  D  I  E  I  F  K  N  F  T  A  Y  N  Q  I  I  H  G  T  V  I  E  I  V  W  T  L  I  P  T  V  I
ATTATATTTAATAGCAATTCCATCCTTTACATTATTATATGCGATGGATGAAATTATTAATCCAACAGTAACAATCAAAATTATAGGTtatttgatggcctaaaattatatataataaaa   3360
 L  Y  L  I  A  I  P  S  F  T  L  L  Y  A  M  D  E  I  I  N  P  T  V  T  I  K  I  G
taaaatagaaattgccaataataattggaaaaaaaatcaatgatgtgtttatattcctaaaataagagcccaaacagaatcggaccacataaaggcgagataattagtatattaatag   3480
                                                                             M  M  F  Y  I  P  K  I  R  A  Q  N  R  I  G  P  H  K  G  E  I  I  S  I  L  I
                                                                            orf3
ggtgtttattaggagatgcatatggtagtaatagatctagagaaggtgtcgtttagtttaaacaaagtagtaaaaataaagaatatttatttatgatctatgaattcttaataaa   3600
 G  C  L  L  G  D  A  Y  G  S  N  R  S  R  E  G  V  R  F  S  F  K  Q  S  S  K  N  K  E  Y  L  F  M  I  Y  E  F  F  N  K
atggttattgtaccaataataattccaagaaaatatatagtaaaattaaaacataacgtgacgtaaaaagactttgaacgttatgaatttgtgacctatacgtttagaagctttaaatata   3720
 N  G  Y  C  T  N  N  I  P  R  K  Y  I  V  K  L  K  H  N  D  D  V  K  D  F  E  R  Y  E  F  V  T  Y  T  F  R  S  F  K  Y
tttatgaaatgttctataaaaagcaaaaaaaagtattacatttagaaaaataaaagaatctagatgaacgagcattagcccattggattatggatgatggcgggtatactggagcag   3840
 I  Y  E  M  F  Y  K  K  Q  K  K  V  L  H  L  E  K  I  K  E  Y  L  D  E  R  A  L  W  I  M  D  D  G  G  Y  T  G  A
GtattaGaataGcaacaaatGcctttactttaaaaGaaGtaatGttttGaaaGaGGttattaGaGacaaatttaaattaaaatGtacaattcaaGaGatctcatataaaaGataaatGGt   3960
 G  I  R  I  A  T  N  A  F  T  L  K  E  V  M  F  L  K  E  L  L  E  D  K  F  K  L  K  C  T  I  Q  E  I  Y  I  K  D  K  W
caatctatattacaaaagactcgatgactcatttaatttctataattaaaccatatatgcatcctagcatgtattataaattggaataaaataattataaagaattttttaagtcaaaa   4080
 S  I  Y  I  T  K  D  S  M  T  H  L  I  S  I  I  K  P  Y  M  H  P  S  M  Y  Y  K  L  E  ●
aatattatataatagtcgtgaggcattagacaaataaaaggataatgcttctaaaaacccgacggggtgtttaaaggaggataatatatactctttttggcgacatgagtgaaaacggtta   4200
aaatagttagtggattaccataaacaactacaagaccgtcagtaaagacagttgattttttgaactttattgcgacagactgggtcacttgtgggtatctgaaaaggatgcttaatgtaca   4320
gtcgaacagCATCAATGGTATTGGAGTTATGAATATGGAGATAATGCAAGTAATTTAATAGAATTTGATAGTTATATGGTTTATGAACGAGATTTAGCAGAAGGACAACTAAGATTATTA   4440
         H  Q  W  Y  W  S  Y  E  Y  G  D  N  A  S  N  L  I  E  F  D  S  Y  M  V  Y  E  R  D  L  A  E  G  Q  L  R  L  L
         cox2
GAAGTAGATAATGCAATGGTAGTTCCAGTAAAAACACATATTAGATTAATAATAACATCAGGAGATGTTTTACATAGTTGGGCGATTCCATCATTTGGAATAAAAGTAGATGCCGTACCA   4560
 E  V  D  N  A  M  V  V  P  V  K  T  H  I  R  L  I  I  T  S  G  D  V  L  H  S  W  A  I  P  S  F  G  I  K  V  D  A  V  P
GGACGATTAAATCAAATTGGATTGTATGTGTAAAAAAGGAAAGGTACATTCTATGGACAATGTAGTGAATTATGTGGGAGTAGATCATGGATTTATGCCAATTAAAGTTCAAGCTGTTAAATTA   4680
 G  R  L  N  Q  I  Y  G  Q  C  E  L  C  G  V  D  H  G  F  M  P  I  K  V  Q  A  V  K  L
GGAGAGTATTTTTCAAAACTAAATGAAAAATAACAATCAATAATAATAAAGTCCTAAAGG
 G  E  Y  F  S  K  L  N  E  K  ●
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this region contains three introns. All of them have a long ORF exhibiting consistent similarities to putative mRNA maturases and DNA endonucleases found in mitochondrial introns of different organisms, including the yeast *Saccharomyces cerevisiae* [10], the ancestral fungus *Allomyces macrogynus* [11] and the primitive plant *Marchantia polymorpha* [12]. Their sequences are shown in Fig. 2 and they will not be further discussed here. The exon-intron boundaries were determined by comparison of the deduced COX1 and COX2 amino acid sequences with the homologous polypeptides of other eukaryotes, an approach that was facilitated by the highly conserved structure of the proteins at the exon borders (shown by arrowheads in Fig. 3). Noteworthy, the two cox1 introns are located at positions that are considered as preferred insertion sites of group I introns in the same gene of fungi and algae [13,14].

As shown by Fig. 1 and in more detail by Fig. 2, the COX1 C-terminus and the COX2 N-terminus are encoded by the same exon (exon 2), on a single reading frame that does not contain standard termination codons. Nevertheless, information on the boundary of the two coding regions can be obtained by the analysis of sequence homologies (Fig. 3) and of the three-dimensional structure of the bovine enzyme [3] recently made available (PDB code: 1OCC). These data show that in beef the last transmembrane peptide segment of subunit I (termed helix XII) ends with a triplet of charged residues (KRE in Fig. 3). The equivalent position in *Dictyostelium* is occupied by a couple of arginines (Fig. 3), encoded by codons located for nucleotide positions 2921–2926 (Fig. 2). The polypeptide chain then continues on the matrix side of the inner mitochondrial membrane [3] with its poorly conserved C terminus, usually constituted by an hydrophilic stretch of 30–40 residues (Fig. 3). The correspondent *Dictyostelium* peptide appears to be much shorter since only 11 codons are found in the
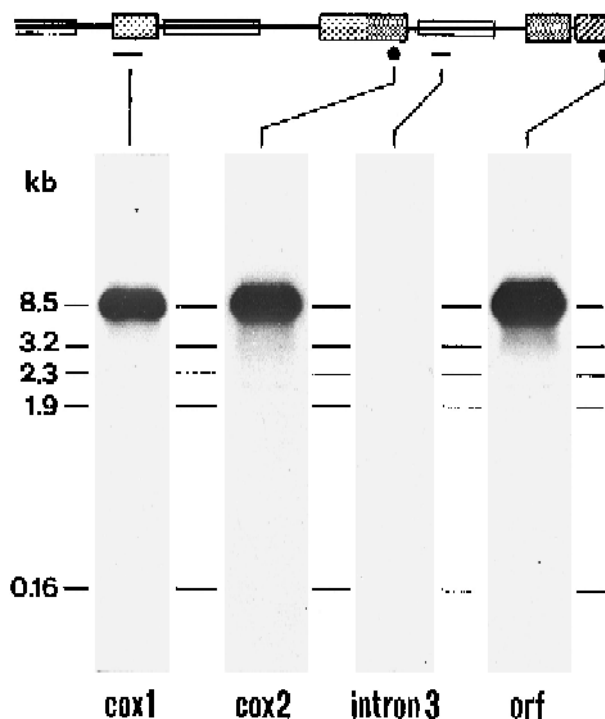

Fig. 4. Nothern blot analysis. Total *Dictyostelium* RNA was hybridized with probes specific for selected regions of the cox1/2 gene. Their origin (●, oligonucleotide; –, mtDNA fragment) and position in the sequence are shown by the scheme on the upper part of the figure. The size of the 8.5 kb transcript was calculated from the migration distance of *Dictyostelium* cytoplasmic and mitochondrial rRNAs, indicated by the horizontal lines.

DNA that precedes the beginning of the fused cox2-homologous region, marked by a methionine codon at nucleotide position 2961 (Fig. 2). All of them are sense codons at conserved sites of other characterized *Dictyostelium* mitochondrial protein-coding genes, ruling out the presence of a non-standard translation termination codon in the sequence.

The alternative possibilities that the cox1/2 precursor transcript might be endonucleolytically processed, to give two separate mature mRNAs, or

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Fig. 3. Alignments of amino acid sequences. The predicted aminoacid sequences of the C-terminus of subunit I (A) and subunit II (B) of *Dictyostelium* cytochrome *c* oxidase (Ddi) are aligned with their homologous conterparts from the red alga *Chondrus crispus* (Ccr), the ameboid protozoon *Acanthamoeba castellanii* (Aca), the yeast *Saccharomyces cerevisiae* (Sce) and *Bos taurus* (Bta). For *Dictyostelium* (this work) and *Acanthamoeba* [4], the beginning of subunit II was arbitrarily defined on the basis of the sequence homologies (see text). Sequences were aligned using the Clustal W Multiple Sequence Alignment Program [19]. Residue numbers for *Dictyostelium* subunit I are omitted since the sequence of the polypeptide N-terminus was not determined. Arrowheads mark the sites of intron insertion in the slime mold. The protein sequences are available from the SwissProt data base under the following accession numbers for COX1 and COX2, respectively: *C. crispus* (P48866, P48869), *S. cerevisiae* (P00401, P00410) and *B. taurus* (P00396, P00404).

edited, to introduce a termination codon, were also analyzed. As shown by the Northern blots of Fig. 4, both the 249 bp cox1-specific probe and an oligonucleotide (5′-ATTACTGTTGGAATTAATGTCC), specific for the cox2 transcript derived from exon 2, hybridize to a 8.5 kb RNA indicating that the two coding regions are co-transcribed. The absence of additional signals at lower molecular weight, unexpected in the light of the estimated size of the cox1/2 mRNA (approximately 2.5 kb), was further investigated. Two additional probe, a 122bp *Eco*RI-*Hin*dIII DNA fragment obtained from intron 3 and an oligonucleotide (5′-AGTAAAGCCTTTAATGCCGT-TACCTGCTG) complementary to the RNA-like strand of the partially characterized ORF downstream the cox1/2 gene, were used (Fig. 4). While no hybridization was detectable with the intronic probe, a strong signal overlapping the 8.5 kb band was obtained with the downstream probe. These data suggest that the transcript containing the cox1/2 coding region is efficiently processed and is polycistronic.

Analysis by reverse transcription-polymerase chain reaction (RT-PCR) demonstrated the absence of editing between the subunit I and II coding regions and confirmed the predicted location of the intron 2 insertion site. Total *Dictyostelium* RNA, isolated as previously described [15,16], was primed with the oligonucleotide 5′-ATTACTGTTGGAATTAATGTCC followed by reverse transcription [8]. The resulting cDNA was amplified by PCR using the same primer and the oligonucleotide 5′-TTAACAGGAGTAG-TATTAGC. These primers and the oligonucleotide 5′-GGCAGGTATGCCACGTAGAA were then used for direct sequencing of the isolated PCR fragment (see Fig. 2 for position and orientation of the primers).

This work demonstrates that the presence of a single mitochondrial ORF encoding the two catalytic subunits of cytochrome *c* oxidase is not restricted to *Acanthamoeba* [4], but involves other distantly related eukaryotes [9]. This structural organization is likely the result of limited DNA rearrangements, favored by the proximity of the genes that still is detectable in the mitochondrial genome of different organisms. As an example, in the red alga *Chondrus crispus* only 2 bp separate the regions encoding the two COX subunits [17]. Noteworthy, when different organisms are considered, the same polypeptides ex-

hibit the highest degree of similarity with their counterparts in *Dictyostelium.* The above observations may also suggest that the gene fusion event happened more than once during evolution. If this is the case, the mechanisms that in *Dictyostelium* and *Acanthamoeba* control the synthesis of the two oxidase subunits could be different. In this regard, the analysis of the mRNA secondary structure offers an intriguing result. In the slime mold transcript, in fact, a potential stem loop is predicted in a position only few nucleotides upstream the region encoding subunit II. Similar structures on the 5′ and 3′ of untranslated regions are involved in the control of mRNA expression and stability in land plants and *Clamydomonas* [18]. Alternative possibilities, such as a post-translational processing of a hybrid protein precursor, will be also investigated. It is clear, however, that the partial sequencing of the two enzyme subunits is now as an essential step in these studies.

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