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Isolation and characterization of a cDNA clone for bovine cytochrome *c* oxidase subunit IV

(NH_2-terminal presequence/oligonucleotide probes/nuclear gene/mitochondrial protein)

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ABSTRACT **We have isolated a cDNA clone for the precursor to subunit IV of bovine cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1). A cDNA library was constructed from poly(A)$^+$ RNA of adult beef liver by insertion of cDNA into the plasmid vector pBR322. Transformants were screened by colony hybridization with two mixtures of [^{32}P]-labeled synthetic oligodeoxyribonucleotides. We screened 20,000 transformants with a mixture of heptadecamers complementary to all 16 possible sequences encoding amino acids 98–103 and obtained two cDNA clones encoding subunit IV amino acid sequences. We determined the DNA sequence of the larger (416 base-pair) insert, which contains the coding sequence for amino acids 1–107 of the mature protein and an NH_2-terminal extension (presequence). The deduced amino acid sequence of the mature protein is identical with the previously determined protein sequence; the sequence of the NH_2-terminal extension contains a potential initiator methionine at amino acid −22 from the NH_2-terminus of the processed protein. The presequence is quite basic and contains several arginines, including one at the processing site. No hydrophobic region analogous to that found in bacterial and eukaryotic signal peptides is present, but there are homologies with other mitochondrial protein presequences, which may include a common signal for their destination and processing.**

Cytochrome *c* oxidase (cytochrome oxidase; ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1), a multisubunit enzyme complex located in the mitochondrial inner membrane, transfers electrons to oxygen in the terminal reaction of the electron transport chain. The enzyme isolated from beef heart is composed of at least seven subunits (reviewed in ref. 1). Like several other mitochondrial complexes, cytochrome oxidase is the product of two separate genomes. The three largest subunits (I–III) are encoded by mitochondrial DNA (2) and synthesized on mitochondrial ribosomes (3, 4), whereas the smaller subunits (IV–VII) are nuclear gene products. Subunits IV, V, and VI are synthesized on cytoplasmic ribosomes as precursor molecules containing NH_2-terminal extensions (presequences) (5–9), transported to mitochondria, and processed into mature subunits by a mitochondrial protease (reviewed in ref. 10; see also refs. 11–13). Little is known of either the signals that direct these proteins to mitochondria or the structural requirements for their correct processing.

To investigate both nuclear–cytoplasmic interactions and the rate of evolution of nuclear genes for mitochondrial proteins, we are cloning the nuclear genes for cytochrome *c* oxidase. Since the amino acid sequences of most of the nuclear-coded bovine cytochrome oxidase subunits are known (1), it was possible to design synthetic oligodeoxyribonucleotide mixtures to use as specific hybridization probes for identification of clones coding for cytochrome oxidase subunits.

In this paper we report the isolation and nucleotide sequence of a cDNA clone for the precursor to cytochrome oxidase subunit IV. This cDNA includes the coding region for a postulated NH_2-terminal extension that shows homology to the extensions of other nuclear-coded mitochondrial proteins.

MATERIALS AND METHODS

Enzymes and Reagents. 2′-Deoxyribonucleoside-5′-triphosphates, 2′,3′-dideoxyribonucleoside-5′-triphosphates, oligo(dT)-cellulose (type 7), M13mp11 replicative form, M13 primer, T4 polynucleotide kinase, and DNA polymerase I (Klenow fragment) were purchased from P-L Biochemicals. Restriction endonucleases were purchased from Bethesda Research Laboratories, New England Biolabs, and Boehringer-Mannheim and were used according to the manufacturers' recommendations. [α-^{32}P]dCTP and [α-^{32}P]dATP (both 800 Ci/mmol, 1 Ci = 37 GBq), and [α-^{32}P]dATP and [γ-^{32}P]ATP (both >5000 Ci/mmol) were purchased from Amersham.

Construction of a Beef Liver cDNA Library. Total RNA was isolated from fresh beef liver by the guanidinium thiocyanate method (14) and enriched for poly(A)$^+$ RNA by chromatography on oligo(dT)-cellulose. Linear double-stranded cDNA was prepared by slight modifications of published procedures. The first strand of cDNA was synthesized using avian myeloblastosis virus reverse transcriptase (Life Sciences) (10 units/μg of RNA) by priming 25 μg of poly(A)$^+$ RNA with 25 μg of oligo(dT)$_{12-18}$ (15). The reaction included 0.5 unit of RNasin (Biotec)/μl to inhibit RNase. Synthesis of the second strand of cDNA was carried out in two steps. First, the cDNA (first strand) was used as template for second strand synthesis using DNA polymerase I (Boehringer-Mannheim, DNase-free) (15). Second, the double-stranded cDNA was extended by using reverse transcriptase. The final cDNA product was treated with a predetermined amount of nuclease S1 (Boehringer-Mannheim) (8 units/10^4 cpm in cDNA) at 37°C for 30 min (16) to remove single-stranded regions. The yield of duplex cDNA was 1.28 μg; its average length was 500 bp.

Oligo(dC) tails were added to cDNA with terminal deoxyribonucleotidyl transferase (Ratliff Biochemicals, Los Alamos, NM) as described (17). The oligo(dC)-cDNA was hybridized to oligo(dG)-pBR322 (New England Nuclear) at a 1:2 mass ratio of dC to dG in 0.15 M NaCl/20 mM Tris·HCl

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Abbreviation: bp, base pair(s).

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(pH 8.0)/1 mM EDTA (16). Transformation of competent (18) *Escherichia coli* RR1 with the hybridized cDNA yielded 2×10^6 transformants per μg of cDNA. The transformation mixture was plated onto sterile 82-mm-diameter nitrocellulose filters laid on LB agar plates (18) containing 12.5 μg of tetracycline/ml. Filters containing approximately 10^3 colonies were stored at −70°C (19).

Synthesis of Mixed Oligonucleotide Probes. A heterogeneous mixture of 16 heptadecamers [probe 1: A-C-C-C-A-(T/C)-T-C-(T/C)-T-C-(T/C)-T-C-(A/G)-A-A], one of which is complementary to cytochrome oxidase subunit IV mRNA, was synthesized using the solid-phase phosphite approach (20). A second mixture [probe 3: A-C-(A/G)-T-A-(A/G)-T-G-(T/C)-T-T-(T/C)-T-C-C-C-A] was synthesized with an Applied Biosystems DNA synthesizer. After synthesis, the heptadecamers were purified by reversed-phase high performance liquid chromatography using a μBondapak C_{18} column.

Screening cDNA Clones with Mixed Oligonucleotide Probes. Clones were screened by colony hybridization on duplicate nitrocellulose filters (19). The mixed oligonucleotide probes were labeled to a specific activity of $1–4 \times 10^8$ cpm/μg using polynucleotide 5′-hydroxyl-kinase and [γ-^{32}P]ATP. Filters were prehybridized in 0.9 M NaCl/0.09 M sodium citrate, pH 7/0.5% (vol/vol) sodium lauroyl sarcosine at 37°C for 3 hr. ^{32}P-labeled probes were added ($1–2 \times 10^5$ cpm/ml) and hybridization was continued for 16–24 hr at 37°C with gentle agitation. Filters were washed twice in 0.9 M NaCl/0.09 M sodium citrate, pH 7/0.05% sodium pyrophosphate for 30 min at room temperature, then twice in 0.45 M NaCl/0.045 M sodium citrate, pH 7/0.05% sodium pyrophosphate (once for 30 min at room temperature and once for 15 min at 37°C). Filters were dried and exposed to Kodak XAR-5 film for 4–12 hr at −70°C with intensifying screens. The melting temperatures for perfect hybrids were calculated (21) to be 46°C and 44°C for probes 1 and 3, respectively. Hybridization was performed 5–10°C below the estimated melting temperature. Colonies that hybridized on duplicate filters to either probe were rescreened.

Plasmid DNA was isolated from 1 liter of amplified culture and purified by centrifugation in CsCl gradients containing ethidium bromide. The purified DNA was passed through a Bio-Gel A-1.5m (Bio-Rad) agarose column to remove CsCl and RNA.

Hybridization of Probes to Plasmid DNA. Plasmid DNAs were digested with various restriction enzymes and the fragments were separated by electrophoresis on agarose gels made and run in 90 mM Tris borate (pH 8.3) containing 2.5 mM EDTA. The DNA was denatured in alkali, neutralized, and transferred to nitrocellulose filters, which were then baked for 2 hr *in vacuo* (22). The filters were hybridized with ^{32}P-labeled oligonucleotide probes under the same conditions used for screening colonies. Alternatively, filters were screened with the appropriate isolated cDNA insert labeled with ^{32}P by nick-translation.

DNA Sequence Analysis. The sequence of the cDNA insert was determined by the chemical method of Maxam and Gilbert (23), including an alternative cytidine reaction (24) and the adenosine and guanosine reaction described previously (25).

RESULTS

Design and Synthesis of Mixed Oligonucleotide Probes. Our cloning strategy depends on knowing the amino acid sequence of beef cytochrome oxidase subunit IV (28). Several regions in this subunit contain adjacent amino acids that are encoded by codons with minimum degeneracy; unambiguous mixed oligonucleotide probes corresponding to these regions may therefore be synthesized. We chose to design specific hybridization probes for sequences encoding two regions in the COOH-terminal half of the protein (Fig. 1*A*). Ideally, any cDNA clone that hybridizes to both probes 1 and 3 should contain the desired cDNA.

Construction of a Beef Liver cDNA Library and Screening with Oligonucleotide Probes. From 25 μg of beef liver poly(A)$^+$ RNA, we have synthesized 1.28 μg of linear double-stranded cDNA. Oligo(dC) tails were added to the cDNA, which was hybridized to oligo(dG)-tailed pBR322. *E. coli* cells that had been transformed with these recombinant plasmids were stored and screened at high density on nitrocellulose filters. Screening approximately 30,000 cDNA clones with probe 1 and 20,000 with probe 3 yielded five and three positive clones, respectively (Fig. 2*A*). None of these clones hybridized to both probes, however.

Characterization of cDNA Clones. We characterized each cDNA clone by Southern hybridization (22). Nitrocellulose filters containing purified plasmid DNA that had been digested with *Pst* I were first hybridized with each probe under the same conditions used for colony hybridization (Fig. 2*B*). Each cDNA insert hybridized to the probe used to isolate it but not to the other probe. Then each cDNA insert was isolated and itself used as a hybridization probe to detect homologous cDNAs. Only two cDNA clones, pCOX4.419 and pCOX4.501, cross-hybridized with each other (Fig. 2*C*). Both clones had been detected with probe 3.

Identification of a cDNA Clone for Beef Cytochrome Oxidase Subunit IV. We determined the DNA sequence of 83% of the insert in clone pCOX4.419, the larger of the two that cross-hybridized, by sequencing both strands (Fig. 3). (The structure of the clone and the chemical sequencing strategy for it are shown in Fig. 1*B*.) This 416-bp cDNA insert contains the coding region for the NH_2-terminal three-fourths of the mature protein (amino acids 1–107). The amino acid sequence derived from the DNA sequence (Fig. 3) agreed with the published amino acid sequence of beef cytochrome oxidase subunit IV (28), thus confirming the identity of the cDNA.

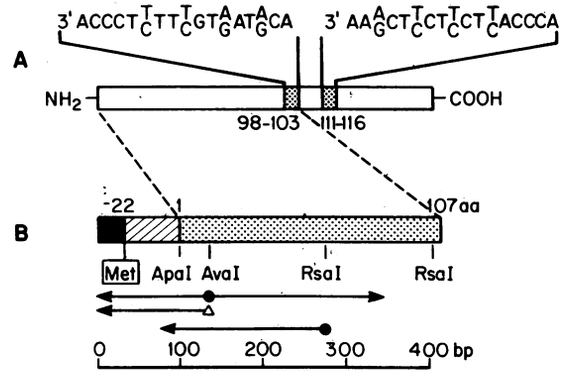

FIG. 1. Diagrammatic representation of cytochrome oxidase subunit IV protein and cDNA. (*A*) The mature protein. The mixed oligonucleotide sequences of probe 1 (amino acids 111–116) and probe 3 (amino acids 98–103) are shown above in the expansion of each amino acid block. The probes were made as the complements of messenger RNA and are thus shown 3′ to 5′ from left to right. (*B*) cDNA pCOX4.419. The stippling represents the sequences that correspond to the mature protein. The hatched region encodes the presumptive extension, starting with methionine at position −22. The reading frame is open, however, for at least 10 codons (shaded) that precede the methionine codon. The positions of several restriction sites are also shown. The sequencing strategy is summarized below the sites, with filled circles indicating positions of 5′ labeling, the open triangle indicating 3′ labeling, and horizontal arrows, the direction and extent of sequencing from a given labeled end. Sequences to the right of the Ava I site were also determined by dideoxy sequencing (26) of M13mp11 subclones (27).

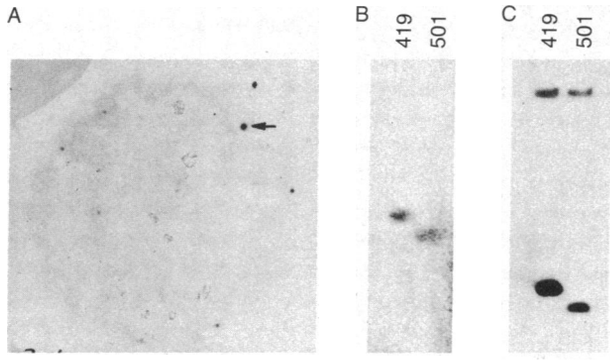

FIG. 2. Identification of cDNA clones for cytochrome oxidase subunit IV. (*A*) Colony hybridization. Filters were hybridized with ^{32}P-labeled probe 3. The arrow indicates the colony containing pCOX4.419. The dark spot above the colony is a keying mark. (*B* and *C*) Hybridization of *Pst* I digests of plasmid DNA from pCOX4.419 (419) and pCOX4.501 (501). Plasmid DNA was digested with *Pst* I to separate the cDNA insert from pBR322, subjected to electrophoresis on 1.4% agarose gels, and transferred to either GeneScreen (*B*) or nitrocellulose (*C*) filters. The filters were hybridized with ^{32}P-labeled probe 3 (*B*) or nick-translated cDNA insert from pCOX4.419 (*C*).

The cDNA insert also contains 98 bp preceding the sequence of the mature protein. These 98 bp contain an open reading frame in phase with the sequence for the mature protein, with methionine as amino acid −22. The absence of a methionine initiation codon immediately adjacent to the sequence for the mature protein and the presence of a methionine codon upstream from and in the same reading frame as the mature protein confirms the supposition (5–9) that the precursor contains an NH_2-terminal polypeptide extension. The calculated mass of the 22-amino acid extension, 2789 Da, is in good agreement with estimates that in both yeast (7–9) and rat liver (5, 6) the precursor to subunit IV is about 3000 Da larger than the mature form. However, since the reading frame in pCOX4.419 remains open upstream for ten additional codons to the end of the cDNA insert, and none of the ten encodes methionine, we cannot rule out a larger extension. Estimates of the presequence length of another cytochrome oxidase component, subunit V, in yeast (11) and rat (6) provide similar values of about 20–25 residues.

The cDNA insert in pCOX4.501, which we presume to be a cytochrome oxidase subunit IV clone because of its homology with the pCOX4.419 cDNA insert, has been characterized only by restriction analysis. It contains the *Ava* I site at nucleotide 45 and the two *Rsa* I sites at nucleotides 177 and 309. Since the cDNA insert is only 360 bp long [including oligo(dG·dC) tails], it must consist mostly of the coding sequence for the mature subunit and contain little, if any, of the presequence. It is thus clear that both cDNA inserts failed to hybridize with probe 1 because the cDNAs do not contain sequences complementary to that probe.

The sequence that hybridized with probe 1 was determined in another cDNA clone, pBL734, prior to our identifying clones whose inserts cross-hybridize with both probes. Interestingly, pBL734 does not have perfect homology to the probe mixture. The region of greatest complementarity in pBL734 matches at 15 of 17 positions:

734 A-G-G-G-T-A-A-G-A-A-G-A G-T-T-T 5′

probe 1 A/C-C-C-A-T/C-T-C-T/C-T-C-T/C-T-C-A/G-A-A 3′

The longest uninterrupted match is 11 bp with base composition 45% G+C. We note, however, that the probe was not recovered from the hybrid and characterized. Another clone was partially characterized by sequencing and shown not to be related to cytochrome oxidase subunit IV DNA. The other four positive clones identified were not characterized further.

DISCUSSION

Using synthetic oligonucleotide mixtures as specific hybridization probes, we have isolated two cDNA clones for the precursor to cytochrome *c* oxidase subunit IV. Both cDNA clones contain the NH_2-terminal three-quarters, but not the COOH-terminal one-quarter, of the coding region. In addition, the larger of the clones contains the sequence encoding the NH_2-terminal extension not found in the mature protein (Fig. 1*A*). The cloning of the yeast gene for cytochrome oxidase subunit V was recently reported (29).

Several studies have suggested tissue-specific differences in some of the smaller nuclear-coded cytochrome oxidase subunits from beef (30) and from rat and chicken (31). We note that no differences were found between the amino acid

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                                                    -90
                                   AC GGT GGG CAG CGG CTG GTC GGT GGC ATC AGA

     -60                                            -30                                  -1
ATG TTG GCA ACC AGA GTA TTT AGC CTG ATT GGT AGG CGT GCA ATC TCC ACC TCG GTG TGT GTT CGG
Met Leu Ala Thr Arg Val Phe Ser Leu Ile Gly Arg Arg Ala Ile Ser Thr Ser Val Cys Val Arg
      -20                                           -10                                  -1
1                                  30                                  60
GCC CAT GGA AGT GTT GTA AAG AGC GAA GAT TAT GCA CTC CCG AGT TAT GTG GAC CGG CGT GAC TAC
Ala His Gly Ser Val Val Lys Ser Glu Asp Tyr Ala Leu Pro Ser Tyr Val Asp Arg Arg Asp Tyr
  1                                10                                  20
                               90                                  120
CCC TTG CCC GAC GTG GCC CAT GTC AAG AAC CTG TCT GCC AGC CAG AAG GCC TTG AAG GAG AAG GAG
Pro Leu Pro Asp Val Ala His Val Lys Asn Leu Ser Ala Ser Gln Lys Ala Leu Lys Glu Lys Glu
                               30                                  40
                     150                                 180
AAG GCT TCC TGG AGC AGC CTC TCC ATT GAT GAG AAA GTT GAA CTG TAC CGC CTT AAG TTC AAG GAG
Lys Ala Ser Trp Ser Ser Leu Ser Ile Asp Glu Lys Val Glu Leu Tyr Arg Leu Lys Phe Lys Glu
                     50                                  60
            210                                 240
AGC TTC GCC GAG ATG AAC AGG AGC ACA AAT GAG TGG AAG ACG GTG GTG
Ser Phe Ala Glu Met Asn Arg Ser Thr Asn Glu Trp Lys Thr Val Val
             70                                  80
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FIG. 3. Nucleotide sequence of the cDNA insert in pCOX4.419 corresponding to the presumptive presequence and amino acids 1–107 of the mature protein. The nontranscribed strand is shown, along with the derived amino acid sequence. For both the amino acid and nucleotide positions, positive numbers start at the first residue of the mature protein. Amino acids are given starting with the putative initiator methionine of the presequence.

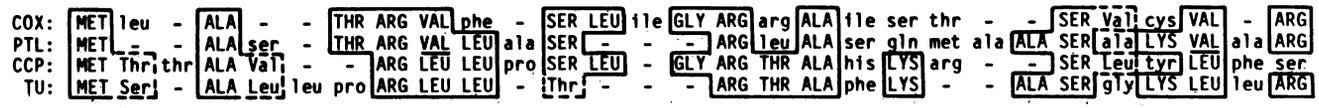

FIG. 4. Homology among the predicted amino acid presequences of beef cytochrome oxidase subunit IV and three other nuclear-coded mitochondrial proteins. COX, beef cytochrome oxidase subunit IV; PTL, *Neurospora* ATPase proteolipid (39); CCP, yeast cytochrome *c* peroxidase (38); TU, yeast elongation factor Tu (40). Total reported presequence sizes for the fungal proteins are 66, 68, and 37 amino acids, respectively. The amino acids in all capital letters correspond in at least two of the four sequences; those with an initial capital letter are chemically homologous but not identical. Dashes represent arbitrary gaps inserted to maximize homology.

sequence derived from our liver cDNA clone and the amino acid sequence determined (28) for subunit IV from heart.

Most mitochondrial proteins are nuclear gene products that are translated in the cytoplasm on free ribosomes and then imported into mitochondria (10). In general, proteins destined for the matrix space, the inner membrane, or the intermembrane space are synthesized as larger precursors containing an NH_2-terminal extension. Precursors can be taken up by mitochondria, processed to the mature form, and inserted into their correct mitochondrial location, whereas the mature proteins cannot. Thus, these presequences, when present, are necessary for the import process.

The metal-requiring mitochondrial protease involved in processing is located in the matrix space and has been partially purified from yeast (11, 12, 32) and rat liver (13). It appears to be neither tissue-specific (32, 33) nor species-specific (34, 35). Its action in the import of cytochrome oxidase subunit V in yeast has been particularly well studied: the polypeptide has been shown to be synthesized as a precursor about 2-kDa larger than the mature form (8, 9). The precursor can be taken up by isolated mitochondria and directed to the correct intramitochondrial location, a process accompanied by cleavage of the extension to generate the correct mature NH_2-terminus (11).

Our sequence data include the beef subunit IV extension. The predicted amino acid sequence of the 22-residue NH_2-terminal polypeptide is basic, containing several arginines including one at the processing site. Furthermore, the presequence lacks an extended hydrophobic region that could span the membrane and thus does not correspond to the structures of secreted bacterial or eukaryotic proteins (reviewed in refs. 36 and 37). An additional difference between this mitochondrial presequence and bacterial signal sequences lies in the nature of the residue at the cleavage site. In secreted proteins this residue is usually an amino acid with a small side chain, such as glycine or alanine, whereas the presequence in pCOX4.419 encodes arginine at this site.

Sequence data for other known or probable NH_2-terminal extensions are available for only a few nuclear-coded mitochondrial proteins, all from fungi. These are yeast cytochrome *c* peroxidase (38), *Neurospora* ATPase proteolipid (39), and yeast elongation factor Tu (40). Although these proteins are localized in three different mitochondrial compartments, we compared the NH_2-terminal regions of their presequences with the sequence of cytochrome oxidase subunit IV from positions -22 to -1 to determine whether there were common features for mitochondrial presequences. Despite the evolutionary distance between these lower eukaryotes and mammals, all four sequences can be aligned, allowing arbitrary insertions and deletions, to provide apparent homology (Fig. 4). For instance, the comparison of the extension of cytochrome oxidase subunit IV with the extension of the *Neurospora* proteolipid, the only one of the three other proteins to be directed to the inner membrane, shows 11 identities out of 22 or 23 residues. The proposed homology among presequences for mitochondrial proteins from both fungi and mammals suggests the possibility of using the corresponding region of the cDNA insert as a hybridization probe for other nuclear genes encoding mitochondrial proteins.

The apparent homology of the cytochrome *c* oxidase subunit IV and cytochrome *c* peroxidase extensions is interesting since the peroxidase is directed to the intermembrane space. Its transport into mitochondria appears more complicated than that of inner membrane proteins because it is processed in two steps (38, 41), as are other proteins of the intermembrane space (41, 42). A model has been proposed (38) for cytochrome *c* peroxidase in which the precursor is transiently bound to the inner membrane, processed by the matrix protease, and then released into the intermembrane space by the action of a putative second protease. The similarity of the cytochrome *c* peroxidase NH_2-terminal sequence to those of other substrates of the matrix protease lends support to the first part of the model.

A presequence for mitochondrial elongation factor Tu was proposed (40) on the basis that the nuclear gene for the yeast mitochondrial protein codes for a product 37 amino acids longer at the NH_2-terminus than the *E. coli* elongation factor, with which yeast mitochondrial Tu can be aligned. The homology of the predicted Tu presequence to the proteolipid and cytochrome *c* peroxidase presequences supports the proposal. We also note that a basic residue precedes the proposed NH_2-terminus of the mature protein, as is the case with cytochrome oxidase subunit IV.

It has been suggested (39) in the case of the hydrophobic *Neurospora* proteolipid that the function of the long basic presequence is to solubilize it. The polarity index (43) of the proteolipid is below the range for soluble proteins in the absence of its polar presequence. Thus, it was interesting to evaluate whether presequences might play a similar role in other cases. For cytochrome oxidase subunit IV, however, the situation is reversed: the polarity index of the presequence, 40.9%, puts it near the lower limit for typical soluble proteins, whereas the mature protein has the higher value of 49%. For the other two proteins in Fig. 4, the extensions are more polar than the mature proteins, but the mature proteins are themselves within the polarity-index range of typical soluble proteins (43).

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