Conversion of a Mitochondrial Gene for Mammalian Cytochrome *c* Oxidase Subunit II into Its Universal Codon Equivalent and Expression in Vivo and in Vitro[†,‡]

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ABSTRACT: To begin to assess the independent structural and functional characteristics of the mitochondrially encoded subunits of mammalian cytochrome *c* oxidase, we have converted the cloned mitochondrial gene for rat subunit II (*cox*II) into its universal codon equivalent (*ucox*II) by oligonucleotide-directed, site-specific mutagenesis. This involved synthesizing 12 oligodeoxynucleotides to achieve the 13 ATA to ATG and the 5 TGA to TGG changes needed. To express *ucox*II in *Escherichia coli*, we used a number of different expression vectors in which the promoters and ribosome-binding sequences of the messenger RNA were varied. While *ucox*II alone was expressed at a low level, a striking increase in the level of expression resulted when the *ucox*II gene was fused to other *E. coli* genes. The COXII peptide was identified by proteolytic digestion, partial sequencing, and reaction with specific antisera. A cro-β-galactosidase–COXII fusion protein has been purified, characterized, and used to produce polyclonal antibodies to the COXII peptide. The *ucox*II gene was also expressed in a cell-free translation system and in *Xenopus* oocytes, yielding a nondenatured, membrane-associated peptide with the same apparent molecular weight as authentic subunit II. In oocytes and in a reticulocyte lysate in vitro system supplemented with microsomal membranes, the protein is glycosylated and coisolates with the washed membrane fraction. In both cases, the COXII peptide is soluble under mild conditions in a nonionic detergent and is precipitable by antibodies to subunit II. The production of subunit II in the in vitro translation system is stimulated as strongly by addition of soybean phospholipid vesicles as by microsomal membranes, providing further evidence of membrane insertion and stabilization. Artificial membranes are inexpensive, are relatively pure, and do not catalyze glycosylation. Their addition to the in vitro system has distinct advantages for expression and isolation of COXII and other membrane proteins. The results demonstrate that the converted gene for subunit II can be expressed as a nativelike, membrane-associated peptide in the absence of other oxidase subunits, making it feasible to pursue a study of its structural and functional properties.

Cytochrome *c* oxidase is a multisubunit enzyme that is capable of transporting electrons from cytochrome *c* to molecular oxygen and generating energy in the form of a transmembrane electrochemical gradient (Wikstrom, 1977). Mammalian cytochrome *c* oxidase consists of 13 different polypeptides (Kuhn-Nentwig & Kadenbach, 1985), with a total molecular weight of 204 000 (Buse et al., 1985). In addition to its complex subunit composition, cytochrome oxidase requires for activity at least two copper atoms (Cu_A and Cu_B) and two heme groups (heme *a* and a_3) (Wikstrom et al., 1981). Identification of evolutionarily conserved amino acid residues (Wikstrom et al., 1985) indicates that subunits I and II, encoded by the mitochondrial genome, provide the ligands for the redox-active metal centers and probably constitute the basic catalytic unit. However, neither the locations nor the ligands of any of the metal centers have been unambiguously assigned, and there is still conflicting evidence concerning the number of metal atoms that are true constituents of the enzyme (Müller et al., 1988; Steffens et al., 1987; Einarsdottir & Caughey, 1985). This and other issues regarding functions of the metal centers and the subunits might be resolved if individual subunits could be isolated and characterized, but so far separation has been achieved only under conditions that cause irreversible denaturation and loss of the prothestic groups.

Another approach to determining the properties and functional roles of the subunits would be to synthesize the peptides independently by cloning the genes and expressing them in alternative hosts. Although the expression of individual subunits of a multisubunit protein may pose unusual problems in terms of folding and stability, this type of expression, yielding functional peptides, has been accomplished in several instances, including the α-subunit of the *Torpedo californica* acetylcholine receptor in yeast (Fujita et al., 1986) and in *Xenopus* oocytes (White et al., 1985), and the β-subunit of rat liver ATP synthetase in *Escherichia coli* (Garboczi et al., 1988). In the case of mitochondrial genes, however, this approach is hampered by the unique features of the mitochondrial genetic machinery. First, the mitochondrial genetic code is somewhat different from the universal one: UGA codes for tryptophan, not for termination; AUA codes for methionine, not isoleucine; and the pair of codons AGA/AGG may be used for termination instead of arginine (Anderson et al., 1981). Thus, translation of mitochondrial polyadenylated mRNA in an *E. coli* cell-free system leads to only aberrant protein production (Pakmanaban et al., 1975; Scragg & Thomas, 1975; DeRonde et al., 1980). Furthermore, no one has yet succeeded in developing a mitochondrially derived translation system which would give the correct reading of a

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mitochondrial mRNA (Clarkson & Poyton, 1989). The conversion of a mitochondrial gene to its universal-codon equivalent is therefore required for expression in an alternative host.

In addition to an altered genetic code, a second complication arises from the fact that many of the commonly used codons in mitochondrial genes are rarely used in typical alternative hosts, such as *E. coli* and *Saccharomyces cerevisiae* (DeBoer & Kastelein, 1986). Thus, premature termination and/or slowed translation leading to increased proteolysis of the expressed polypeptide might be expected. A third potential problem is revealed by recent studies that indicate a requirement for initiation factors specific to mitochondria for efficient melting of secondary structure and translation of mitochondrial mRNA (Liao & Spremulli, 1989; Denslow et al., 1989). In spite of these difficulties, one report (Colleoux et al., 1986) shows that a yeast mitochondrial gene, encoding a soluble transposase, was successfully expressed in *E. coli* (although at low levels), after it was converted to its universal-codon equivalent by site-directed mutagenesis.

In this paper, we describe our success in addressing these problems in the expression of the mitochondrial gene for subunit II of rat liver cytochrome *c* oxidase (*cox*II).[1] This 26000 molecular weight subunit contains the postulated Cu_A-binding site as well as the domain where the substrate, cytochrome *c*, interacts with the oxidase. We document the conversion of subunit II to a universal-codon equivalent, the expression of the correct peptide in *E. coli* using a variety of vectors, and the production of a membrane-inserted, "native" form in an in vitro translation system and in *Xenopus laevis* oocytes.

Experimental Procedures

Materials. Rat liver mtDNA clones were generously provided by Drs. R. Grosskopf and H. Feldmann of the Federal Republic of Germany (Grosskopf & Feldmann, 1981). Expression vector pDR540 (Russell & Bennett, 1982) and *E. coli* strains N4830-1, N99CI$^+$, JM103, and JM105 were from Pharmacia P-L Biochemical. *E. coli* strain MCL22 was from Dr. J. Kaguni of Michigan State University. Plasmid pPLEX was from Dr. G. Sczakiel (Federal Republic of Germany) (Sczakiel et al., 1987). Plasmid pEX1 (Stanley & Luzio, 1984) was from P&S Biochemicals, Inc. M13mp18 vector, restriction enzymes, and synthetic linkers were purchased from Bethesda Research Laboratories or Boehringer Mannheim. Oligodeoxynucleotides were synthesized by the Macromolecular Structure and Synthesis Facility of Michigan State University. Goat anti-rabbit IgG (H+L) alkaline phosphatase conjugate, protein molecular weight markers, and nitrocellulose paper were from Bio-Rad. Complete and incomplete Freund's adjuvants were from Behring Diagnostic. *Xenopus laevis* females were purchased from Nasco, Fort Atkinson, WI. Plasmid pSP65 (Melton et al., 1984), *SP6* RNA polymerase, DNase I, RNasin, rabbit reticulocyte lysates, and microsomal membranes were from Promega. Poly(A) polymerase and mRNA cap analogue G(5′)ppp(5′)G were from Pharmacia P-L Biochemical. Protein A–agarose was obtained from Sigma. [^{35}S]Methionine (1200 Ci/mmol) was from New England Nuclear. Tran^{35}S-label was from ICN Biomedicals. ^{14}C-Labeled protein molecular weight standards were from Bethesda Research Laboratories. Asolectin was from Associates Concentrates, Woodside, NY. Lauryl maltoside was synthesized according to Rosevear et al. (1980). Monoclonal antibody to beef heart COXII peptide was prepared and characterized by Taha and Ferguson-Miller (personal communication). Other chemicals were from Sigma and were the highest grade available.

General Recombinant DNA Techniques. All restriction enzyme digestion reactions were performed according to the supplier's specifications. For DNA fragment isolation, the restriction digest was electrophoresed through a 5% polyacrylamide gel in TBE buffer (89 mM Tris, 89 mM boric acid, and 2 mM EDTA, pH 8.3) or a 0.8–1% agarose gel in TEAC buffer (40 mM Tris, 20 mM sodium acetate, 18 mM NaCl, and 2 mM EDTA, pH 8.1). Gels were stained with ethidium bromide (0.5 μg/mL) in TBE or TEAC and visualized by UV illumination. The desired fragments were excised, electroeluted, and concentrated by ethanol precipitation. The fragment was ligated to the cloning vector by overnight incubation with T4 DNA ligase at 16 °C. For blunt-end ligation, the sticky ends of the inserts and vectors were first filled in by using the Klenow fragment of DNA polymerase I and then ligated according to Maniatis et al. (1982). A ligation mixture was used to transform competent cells according to the procedure of Hanahan and Meselson (1980). Aliquots of cells were spread on Luria–Bertani plates containing 50 μg/mL ampicillin, and plasmids from ampicillin-resistant colonies were prepared by the alkaline–SDS method (Maniatis et al., 1982). The replicative and single-stranded forms of M13 recombinant DNA were purified according to Messing (1983).

Site-Directed Mutagenesis of Multiple Sites in the Gene for Subunit II. In order to change the unique mitochondrial codons to universal codons in the *cox*II gene, 12 oligodeoxynucleotides that cover the 18 necessary changes were synthesized. A *Hin*dIII–*Hin*dIII fragment containing the *cox*II gene was isolated from plasmid pMD (Grosskopf & Feldmann, 1981) and inserted into M13mp18 at the *Hin*dIII site. There are three unique restriction sites, *Eco*RI, *Hin*fI, and *Hap*II (from 5′ to 3′), within the *cox*II gene (Figure 1). The 18 nucleotides to be changed in the *cox*II gene were altered in parallel by using four M13 clones. Clones I, II, III, and IV covered the mutations in the sequence between the 5′ *Hin*dIII and *Eco*RI sites, *Eco*RI and *Hin*fI sites, *Hin*fI and *Hap*II sites, and *Hap*II and 3′ *Hin*dIII sites, respectively. The corresponding single-stranded recombinant M13 DNAs were isolated and used as templates for site-directed mutagenesis based on the method of Zoller and Smith (1983). To distinguish phage containing wild-type inserts from those with the desired mutant sequences, phage were probed with the corresponding radioactively labeled oligodeoxynucleotides. Filters containing the hybrid DNA molecules were washed at elevated temperatures chosen by calculations using an empirical formula (Meinkoth & Wahl, 1984). The altered *cox*II gene segment within the M13 DNA was verified by sequencing using the M13 dideoxy chain termination method (Sanger et al., 1977) before proceeding to the next cycle of site-directed mutagenesis. After all the necessary changes were made, the altered fragments in the four clones (I–IV) were excised by digestion with the appropriate restriction enzymes, isolated, and ligated in proper order into M13mp18, yielding the complete *ucox*II gene. To facilitate the cloning of *ucox*II into different ex-

[1] Abbreviations: *cox*II, mitochondrial gene of cytochrome oxidase subunit II; COXII, cytochrome oxidase subunit II peptide; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; *ucox*II, universal form of gene for cytochrome oxidase subunit II; *gal*K, gene for galactokinase; β-gal, β-galactosidase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBS, phosphate-buffered saline; MBS, modified Barth's saline; PMSF, phenylmethanesulfonyl fluoride; *lac*Z, gene for β-galactosidase.

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                                10                                  20
  M  A  Y  P  F  Q  L  G  L  Q  D  A  T  S  P  I  M  E  E  L
ACATGGCTTACCCATTTCAACTTGGCTTACAAGACGCCACATCACCAATCATAGAAGAACTT   60
C NcoI                                                  G

  T  N  F  H  D  H  T  L  M  I  V  F  L  I  S  S  L  V  L  Y
ACAAACTTTCATGACCACACCCTAATAATTGTATTCCTCATCAGCTCCCTAGTACTTTAT  120
                        G

  I  I  S  L  M  L  T  T  K  L  T  H  T  S  T  M  D  A  Q  E
ATTATTTCACTAATACTAACAACAAAACTAACACACACAAGCACAATAGACGCCCATGAA  180
         G                                       G

  V  E  T  I  W  T  I  L  P  A  V  I  L  I  L  I  A  L  P  S
GTAGAAACAATTTGAACAATTCTCCCAGCTGTCATTCTTATTCTAATCGCCCTTCCCTCC  240
         G

  L  R  I  L  Y  M  M  D  E  I  N  N  P  V  L  T  V  K  T  M
CTACGAATTCTATACATAATAGACGAGATTAATAACCCAGTTCTAACAGTAAAAACTATA  300
   C EcoRI    G  G                                       G

  G  H  Q  W  Y  W  S  Y  E  Y  T  D  Y  E  D  L  C  F  D  S
GGACACCAATGATACTGAAGCTATGAATATACTGACTATGAAGACCTATGCTTTGACTCC  360
         G  G                                       HinfI

  Y  M  I  P  T  N  D  L  K  P  G  E  L  R  L  L  E  V  D  N
TACATAATCCCAACCAATGACCTAAAACTAGGTGAACTTCGCTTATTAGAAGTTGATAAT  420
   G                          C

  R  V  V  L  P  M  E  L  P  I  R  M  L  I  S  S  E  D  V  L
CGGGTAGTCTTACCAATAGAACTTCCAATTCGTATACTAATCTCATCCGAAGACGTCCTG  480
 C          G           G

  H  S  W  A  I  P  S  L  G  L  K  T  D  A  I  P  G  R  P  N
CACTCATGACCCATCCCTTCACTAGGGTTAAAAACCGACGCAATCCCCGGCCGCCCGAAC  540
      GG                                    HapII

  Q  A  T  V  T  S  N  R  P  G  L  F  Y  G  Q  C  S  E  I  C
CAAGCTACAGTCACATCAAACCGACTAGGTCTATTCTATGGCCAATGCTCTGAAATTTGC  600
            C C

  G  S  N  H  S  F  M  P  I  V  L  E  M  V  P  L  K  Y  F  E
GGCTCAAATCACAGCTTCATACTCATTGTACTAGAAATAGTGCCTCTAAAATATTTCGAA  660
            G C                  G

  N  W  S  A  S  M  I  *
AACTGATCAGCTTCTATAATTTAA                                      684
   G        G
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FIGURE 1: Complete nucleotide and deduced amino acid sequence of rat liver *cox*II. Bases changed to convert the mitochondrial gene to a universal form are indicated below the mitochondrial codons; in all cases, the conversion required replacing an A with a G (ATA and TGA to ATG and TGG, respectively). Three underlined bases below the sequence represent the changes made to convert rare codons for arginine to codons more commonly used in *E. coli*. Four boldbase bases indicate errors in the original published sequence, the correct bases and amino acids are underlined. The restriction sites used in experimental constructions are also underlined. The numbering system is that corresponding to the bovine subunit II gene (Steffens & Buse, 1979).

pression vectors, a *Nco*I site (CCATGG) was introduced at the initiation codon region of the gene by site-directed mutagenesis, in which the first A of the original sequence, ACATGG, was replaced by a C (Figure 1). The *Nco*I site allows cloning of *ucox*II into expression vectors such as pDR540 in such a way that translation begins at the proper AUG codon of the messenger RNA.

Construction of Various Expression Vectors: pDROXII, pPLEXOXII, and pEXOXII. As shown in Figure 2, the *ucox*II gene was obtained by digestion of recombinant M13mp18 with *Nco*I and *Sma*I; it was inserted by blunt-end ligation into plasmid pDR540 (Russell & Bennett, 1982) at the *Bam*HI site as described above. The new construct, plasmid pDROXII, contains a *tac* (*trp/lac* hybrid) promoter that can be induced by IPTG, followed by the *ucox*II gene with *Nco*I and *Bam*HI sites at its 5′ end and a *Bam*HI site at its 3′ end. Another expression vector, pPLEXOXII, was made as follows. The pDROXII construct was digested with *Bam*HI, and the *ucox*II gene fragment was isolated and blunt-end-ligated into the *gal*K gene at the *Mlu*I site of plasmid pPLEX (Debouck et al., 1985). The resulting plasmid, pPLEXOXII, contains the *ucox*II gene located within the reading frame of the truncated *gal*K gene, under control of the λ P_L promoter. The third expression vector was constructed by digesting pDROXII with *Bam*HI and ligating the *ucox*II gene into plasmid pEXI at the *Bam*HI site in the polylinker region; this places the *ucox*II gene under control of the temperature-inducible λ P_R promoter and results in a fused gene in which *ucox*II is preceded by partial sequences

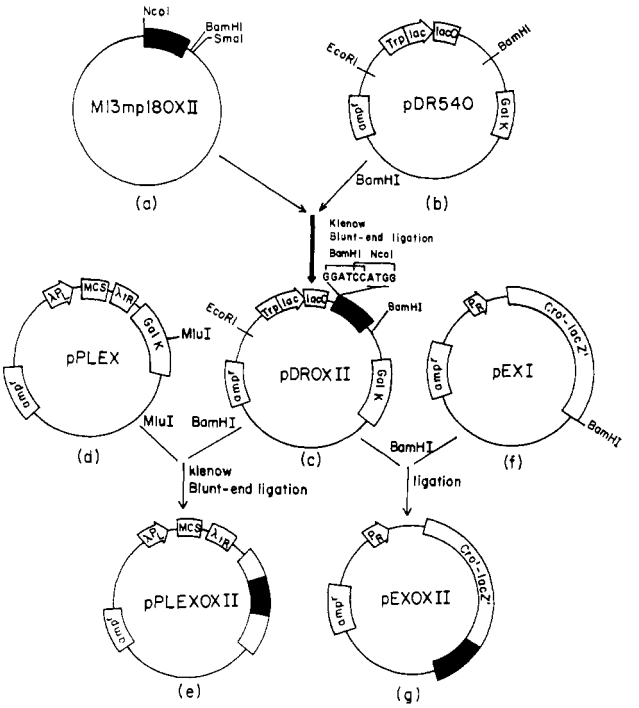

FIGURE 2: Construction of expression vectors: pDROXII, pPLEXOXII, pEXOXII. The *ucox*II gene was obtained by digestion of recombinant M13mp18 DNA (a) with *Nco*I and *Sma*I, and was blunt-end-ligated into pDR540 (b) at the *Bam*HI site. The new construct, pDROXII (c), was digested with *Bam*HI and the *ucox*II gene blunt-end-ligated into plasmid pPLEX (d) at the *Mul*I site within the *gal*K gene, resulting in the new expression vector, pPLEXOXII (e). The vector pEXOXII (g) was constructed by digestion of pDROXII (c) with *Bam*HI, followed by ligation of the *ucox*II gene into pEX1 (f) at the *Bam*HI site located at the 3′ end of the *cro-lacZ* gene. The dark bars (a, c, e, and g) represent the *ucox*II gene; promoters, restriction sites, multiple cloning sites (MCS), and other genes are also indicated in this figure.

from the λ cro protein and from the β-galactosidase gene of *E. coli*.

Expression of the ucoxII Gene. The expression vector pDROXII was transformed into *E. coli* strain MCL22, and the presence of COXII was detected by the "maxicell" technique as described by Sancar et al. (1979). Expression from pPLEXOXII and from pEXOXII was achieved as follows. Cells (strain N4830-1) harboring plasmid were diluted 100-fold with fresh Luria–Bertani medium (Maniatis et al., 1982) and grown to OD_{600} = 0.5 at 30 °C. The culture was shifted from 30 to 42 °C and grown for 1.5 h (Stanley & Luzio, 1984). Aliquots of cells were lysed with SDS loading buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 3% SDS, and 5% β-mercaptoethanol), run in SDS–PAGE, and analyzed by Coomassie blue staining or immunoblotting as described below.

Purification of the cro-β-gal-COXII Fusion Protein. Cells containing pEXOXII were induced at 42 °C and then lysed with lysozyme (400 μg/mL in 50 mM Tris-HCl, pH 8.0, 25% sucrose, and 1 mM EDTA), and the cro-β-gal–COXII inclusion bodies were obtained by detergent wash and centrifugation of the lysate (Nagai & Thogerson, 1987). The crude fusion protein was run on 7.5% SDS–PAGE (4 mm thick gel). The gel was briefly washed with water, and the bands were visualized by immersing the gel in 1 M cold potassium acetate for 15 min. The discrete fusion protein band was excised, washed with water for 30 min, mashed by passing through a 5-mL syringe with a 18.5-gauge needle, and soaked in 0.05% SDS/0.05% ammonium bicarbonate buffer at room temperature for 24 h. The mixture was filtered through Whatman 3MM filter paper, and the fusion protein in the solution was

lyophilized and dissolved in 5 mL of water (yielding a final concentration of SDS of ~0.2%).

Partial Sequencing of the COXII Peptide. The fusion protein was digested with the proteinase endolysin-C (about 1 μg of enzyme/100 μg of protein at 0.2% SDS) according to the manufacturer's instructions. The digestion was analyzed by SDS–PAGE and immunoblotting (see below). Fragments from the endolysin digest were electroeluted from the gel, precipitated with ethanol to separate them from SDS, and subjected to sequence analysis by automated Edman degradation on an Applied Biosystems 477A protein sequencer. Phenylthiohydantoin derivatives were identified by high-performance liquid chromatography.

Preparation and Purification of Anti-COXII IgG. Antisera were raised in New Zealand female rabbits as described by Carroll and Laughon (1987). To purify COXII-specific antibody that is free of anti-cro-β-gal IgG and any other nonspecific IgG, the anti-cro-β-gal–COXII fusion protein antiserum was first applied to a β-gal affinity column that was made by the method of Burton et al. (1988). A cro-β-gal–COXII column was made by using the purified fusion protein in the same procedure. Serum from a rabbit inoculated with cro-β-gal–COXII was passed 5 times through the β-gal column and then 5 times through the cro-β-gal–COXII column. The glycine/HCl (pH 2.5) eluate of the β-gal column was the affinity-selected anti-β-gal antibody, and the glycine/HCl eluate of the cro-β-gal–COXII column was the affinity-selected anti-COXII antibody. These eluates were neutralized with 1 M HEPES (pH 7.9) and immediately dialyzed against PBS (10 mM sodium phosphate, pH 8.0, and 150 mM NaCl) at 4 °C with three changes. Control IgG was the whole IgG fraction obtained from the rabbit before injection with any protein and was purified by using protein A–agarose according to the procedure described by Ey et al. (1978). Antibody concentrations were calculated by assuming an A_{280} of 1.4 = 1 mg/mL IgG (Carroll & Laughon, 1987).

SDS–PAGE and Immunoblotting. Routine SDS–PAGE analysis, in either 7.5 or 18.5% gels, was performed according to Laemmli (1970) or Kadenbach et al. (1983), respectively. After electrophoresis, the proteins were transferred onto a nitrocellulose filter at 150 mA (4 °C) for 1–2 h in transfer buffer (12.5 mM Tris, 96 mM glycine, pH 8.2, 0.02% SDS, and 20% methanol). Immunoblotting analysis of the bound protein followed the protocol of Bio-Rad in which alkaline phosphatase conjugated goat anti-rabbit IgG was used as the color developing system.

Construction of the pSPOXII Plasmid and RNA Synthesis. The *ucox*II gene containing universal codons was excised from the plasmid pDROXII and cloned into pSP65 at the *Bam*HI site. This plasmid was linearized by *Hin*dIII digestion and used for in vitro transcription according to the supplier's protocol. The transcription reaction was extracted with phenol and chloroform followed by precipitation by adding of 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethanol. The RNA was adenylated by using poly(A) polymerase as described by Gething et al. (1980) and was further purified with a Sephadex G-50 spin column (Maniatis et al., 1982). The length of the mRNA product was checked by agarose gel electrophoresis, and its concentration was determined spectrophotometrically.

Preparation of Phospholipid Vesicles. Phospholipid vesicles were prepared as described by Casey et al. (1979). Briefly, a mixture of 40 mg/mL asolectin in 2% potassium cholate/100 mM HEPES, pH 7.2, was sonicated to clarity with a point sonicator. The solution was centrifuged at 7000*g* for 15 min to remove titanium particles and dialyzed against 100 mM HEPES for 4 h and then against 10 mM HEPES, 40 mM KCl, and 45 mM sucrose overnight with one change of the same buffer. The dialyzed lipid vesicle solution was centrifuged in a microfuge at 12000*g* for 15 min, and the pellet containing larger vesicles was saved and resuspended in 20 mM Tris, pH 7.5, to a final concentration of approximately 200 mg/mL. The lipid vesicles were aliquoted and stored at −70 °C. All the procedures were performed at 4 °C.

In Vitro Translation. In vitro translation of COXII mRNA in rabbit reticulocyte lysates was performed according to the supplier's instructions (Promega) in the presence or absence of canine pancreatic microsomal membranes. In vitro translations were also performed in the presence of phospholipid vesicles at various concentrations (10, 15, and 20 mg/mL) with other components remaining constant as above. In all translation reactions, [^{35}S]methionine was used to label the synthesized peptide. Aliquots of reaction mixtures were directly analyzed for protein by SDS–PAGE; gels were fluorographed as described by Banner and Laskey (1974). To isolate the microsomal membranes and lipid vesicles from the translation mixtures, about 50 μL of each reaction was centrifuged in a Beckman Airfuge at 120000*g* for 1 h. The pellet was washed with PBS, and both pellet and supernatant were analyzed by SDS–PAGE as above.

Extraction and Immunoprecipitation of COXII. Membrane-associated COXII synthesized in vitro was solubilized with 0.5 mL of PBS containing 2% lauryl maltoside and proteinase inhibitors (0.1 mM PMSF and 10 μg/mL aprotinin) followed by centrifugation for 15 min at 12000*g* at 4 °C. The supernatant was saved. Five microliters (5 mg of protein/mL) of anti-COXII IgG or the COXII monoclonal antibody was added to the supernatant and incubated at 4 °C overnight. About 40 μL of protein A–agarose slurry (preincubated with 5% bovine serum albumin in PBS) was added to the above mixture and incubated for 2 h at room temperature and centrifuged. The immunoadsorbed proteins were eluted from protein A–agarose with SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 3% SDS, and 5% β-mercaptoethanol) and analyzed by SDS–PAGE.

Analysis of the COXII–Membrane Preparation by Trypsin and Proteinase K Digestion. COXII synthesized by in vitro translation was analyzed by digestion with trypsin or proteinase K. Lipid vesicles or microsomal membranes containing the COXII were collected from 50 μL of translation reaction by centrifugation in the Airfuge as above. For trypsin digestion, the pellet was dissolved in 10 μL of PBS containing 2% lauryl maltoside, and 1 μg of trypsin was added. The digestion reaction was incubated at 4 °C for 90 min, stopped by heating at 95 °C for 5 min, and analyzed by 18.5% urea–SDS–PAGE. For proteinase K digestion, the pellet was dissolved in 100 μL of PBS without lauryl maltoside; 10 μL of this COXII–membrane solution was mixed with 1 μL of a solution of proteinase K dissolved in water (0.1 mg/mL), and samples were incubated at 4 °C for 60 min. Proteinase K digestion was also performed in the presence of 0.5% sodium deoxycholate. Following incubation in a boiling water bath for 2 min, aliquots were immediately loaded onto an 18% urea–SDS gel and electrophoresed.

Expression of ucoxII in Xenopus Oocytes. Mature *Xenopus laevis* females were anesthetized by immersing in ice water for 30 min. An incision was made in the lower part of the abdomen, and a few loops of ovary were removed and washed with sterile Barth's solution (Gurdon, 1968). Individual oocytes were obtained by digestion of the ovary with collagenase

at 2 mg/mL for 1–2 h at room temperature. The oocytes were washed with sterile MBS (Khorana et al., 1988) supplemented with 5% fetal calf serum, and left at 4 °C overnight. The next morning, healthy oocytes at stage V–VI were selected for injection with 50 nL of mRNA solution (0.02–0.8 mg/mL in water). Control oocytes were injected with water. The injected oocytes were incubated in MBS supplemented with 5% fetal calf serum for 24–72 h at 18 °C. Tran^{35}S-label was added to the oocyte incubation medium to 0.1 mCi/mL 10 h after injection. To immunoprecipitate the expressed COXII, a pool of four injected oocytes was homogenized with 0.5 mL of PBS buffer containing 2% lauryl maltoside, 0.1 mM PMSF, and 10 μg/mL aprotinin. Homogenates were incubated at room temperature for 15 min, followed by centrifugation at 12000*g* at 4 °C for 15 min. COXII was immunoprecipitated from the supernatant as described above.

Oocyte cytoplasmic membranes were prepared by the method of Kobilka et al. (1987). Briefly, about 4000 oocytes which had been injected with 20 ng of RNA/oocyte were homogenized in 20 mL of PBS containing 30% sucrose/0.1 mM PMSF using a Dounce homogenizer. The homogenate was centrifuged at 3000*g* for 10 min to remove cell debris and pigment granules. The supernatant was centrifuged at 10000*g* for 15 min, and the resulting supernatant was centrifuged at 200000*g* for 2 h. The pellet from this high-speed spin was the membrane preparation and was solubilized in 10 mL of PBS containing 2% lauryl maltoside, 5% sucrose, 20 mM potassium phosphate, pH 7.4, and 0.1 mM PMSF. After being stirred at 4 °C for 30 min, the detergent-solubilized membranes were centrifuged at 200000*g* for 30 min. Aliquots of supernatant and pellet were analyzed by SDS–PAGE.

Results

Sequencing the coxII Gene. The wild-type and mutated rat *cox*II genes were completely sequenced by both the chemical (Maxam & Gilbert, 1980) and dideoxy chain termination (Sanger et al., 1977) methods. Our results (Figure 1) confirm those of Brown and Simpson (1982), which show four discrepancies with the original published version (Grosskopf & Feldmann, 1981) of the COXII sequence. In particular, the correct codons at positions 130, 164, 189, and 208 are CCA, GCC, CCA, and CCC, which correspond to Pro, Ala, Pro, and Pro, respectively. Further support for these corrections to the sequence of Grosskopf and Feldmann derives from comparison of the sequence of rat liver COXII peptide with those of other known mammalian COXII subunits (human, bovine, and mouse) (Wikstrom et al., 1981); the residues in question are conserved in all the mammalian proteins.

Expression of the ucoxII Gene in E. coli. The *ucox*II gene was cloned into the expression vector pDR540, under control of the hybrid *tac* promoter; this allows protein synthesis from the natural AUG initiation codon using the ribosome-binding site of the vector. Analysis of the maxicells revealed a 26-kDa band, equal in size to the COXII peptide, in cells in which pDROXII was present, but which was not seen in the strain containing control plasmid, indicating that the COXII peptide encoded on the vector was synthesized in *E. coli* (data not shown). The *ucox*II gene was also expressed from pPLEXOXII as a galK–COXII fusion protein, as detected by immunoblotting with antibodies to beef heart cytochrome oxidase (data not shown). In both these systems, the nonfusion and fusion proteins were present at very low levels. The *ucox*II gene was expressed much more efficiently when linked to a *cro–lac*Z sequence in pEXOXII, as a cro-β-gal–COXII fusion protein. As seen in Figure 3, a band of 141 kDa appears in the recombinant plasmid lane at 42 °C, which equals the sum

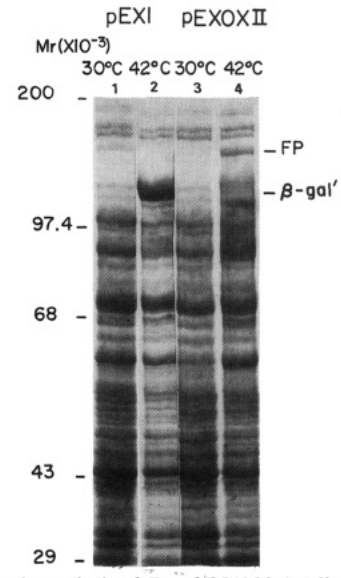

FIGURE 3: Protein analysis of *E. coli* N4830-1 cells transformed with pEXOXII. Cells were boiled and electrophoresed on a 7.5% SDS gel, which was stained with Coomassie blue. FP represents the cro-β-gal–COXII fusion protein; β-gal′ indicates the cro-β-gal fusion sequence without COXII; the locations of other molecular weight markers are indicated at the left of the figure. The plasmid pEXI does not contain the *ucox*II gene, while pEXOXII does. The gene sequences of interest are under control of the λP_R promoter, which is derepressed at 42 °C.

of the molecular weights of the cro-β-gal (115K) and COXII (26K) peptides. When not fused to the *ucox*II gene, the *cro–lac*Z gene product accumulated to about 30% of the total protein. When the *ucox*II gene was fused to the 3′ end of the *cro–lac*Z sequence, the cro-β-gal–COXII product accumulated to only about 0.5% of the total cell protein; there was also a small amount of the cro-β-gal peptide without COXII still made. Thus, the presence of the *ucox*II gene or peptide sequences appears to inhibit expression or promote degradation of the fusion protein.

Partial Sequencing of the COXII Peptide. To establish further that the COXII synthesized in *E. coli* is the correct peptide, amino acid sequence information on the 15-kDa endolysin-C fragment of the COXII fusion protein, cro-β-gal–COXII, was obtained. The first five amino acid residues are N-Thr-Met-Gly-His-Gln-C, corresponding to residues 99–103 of the encoded gene product. Methionine-100 is one of the residues whose original codon, AUA, was converted by site-directed mutagenesis to AUG.

Inhibition of Cytochrome Oxidase Activity by Anti-COXII IgG. The specificity of the anti-COXII IgG was determined by immunoblotting of cro-β-gal, cro-β-gal–COXII, and holocytochrome oxidase as described under Experimental Procedures. The affinity-purified anti-COXII IgG reacted with the cro-β-gal–COXII fusion protein and COXII, but not with any other subunits of the enzyme, and as expected shows only very weak cross-reaction with the cro-β-gal peptide (data not shown). Addition of anti-COXII IgG to a rat liver cytochrome oxidase preparation resulted in a dose-dependent inhibition of the cytochrome oxidase activity (Figure 4). Almost 90% of the cytochrome oxidase activity of 14 pmol of enzyme was inhibited by the addition of 50 μg of affinity-purified anti-COXII IgG. As seen in Figure 4, the activity of the enzyme was not significantly inhibited by the addition of the same amount of control IgG or anti-β-gal IgG.

In Vitro Translation. Figure 5 shows the products obtained from translation of COXII mRNA derived from pSPOXII in a rabbit reticulocyte lysate system. A peptide migrating

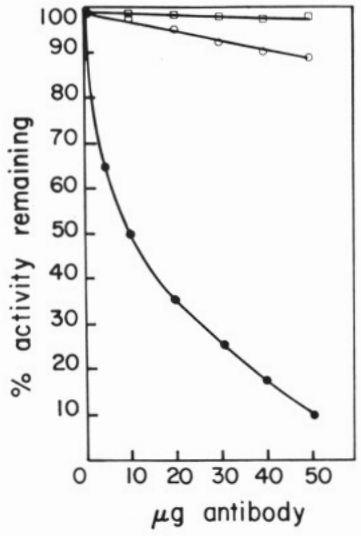

FIGURE 4: Inhibition of purified rat liver cytochrome oxidase activity by monospecific polyclonal COXII antibody. Rat liver cytochrome oxidase (0.014 nmol) was incubated with various amounts of antibody in PBS containing 0.05% lauryl maltoside at room temperature for 1 h. Steady-state kinetic measurements were performed in 50 mM potassium phosphate, pH 6.5, in the presence of 0.05% lauryl maltoside, 0.5 mM TMPD (*N,N,N',N'*-tetramethyl-*p*-phenylenediamine), 2.5 mM ascorbate, and cytochrome *c* (concentration range of 0.01–60 μM). Rates of oxygen consumption were measured polarographically as described by Ferguson-Miller et al. (1976, 1978): black circles represent results with anti-COXII IgG; open circles represent anti-cro-β-gal IgG control; open boxes represent the control IgG from preimmune serum.

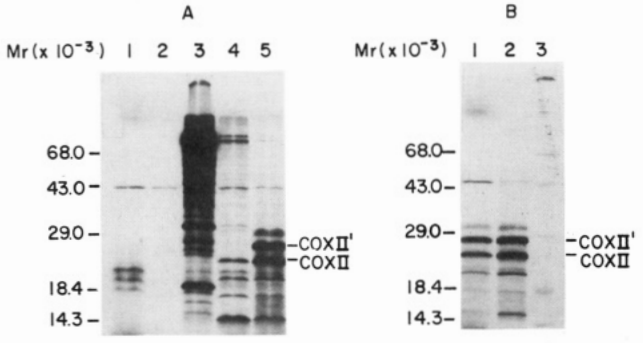

FIGURE 5: In vitro translation of COXII mRNA in rabbit reticulocyte lysates. Panel A is a fluorograph of a 12.5% SDS gel: lane 1, lysate only; lane 2, lysate plus microsomal membranes; lane 3, lysate plus brome mosaic virus RNA; lane 4, lysate plus COXII mRNA (10 μg/mL); lane 5, lysate plus COXII mRNA plus 2 μL of microsomal membranes. Panel B is a fluorograph of a 12.5% SDS gel showing fractionation of the in vitro translation mixture shown in lane 5, panel A. Lane 1, supernatant; lane 2, pellet; lane 3, molecular weight markers. COXII' represents the glycosylated form of COXII (see text).

at the same position as the authentic COXII appears in Figure 5A, lane 4, which reflects a reaction containing COXII mRNA at 10 μg/mL; this band is not present in the control lanes 1–3. When COXII mRNA is translated in the presence of canine pancreatic microsomal membranes, protein synthesis is stimulated significantly. In addition to the COXII, at least two peptides with larger apparent molecular weight are seen (Figure 5A, lane 5). Treatment of the mixture with glycopeptidase F (Tarentino et al., 1985) leads to conversion of the most prominent larger band into a form that migrates with COXII, indicating that it is a glycosylated form of COXII (data not shown). All of these bands appear primarily in the membrane fraction after centrifugation (Figure 5B) and can be immunoprecipitated by COXII-specific antibodies (Figure 6). The anti-COXII IgG precipitates both forms of COXII,

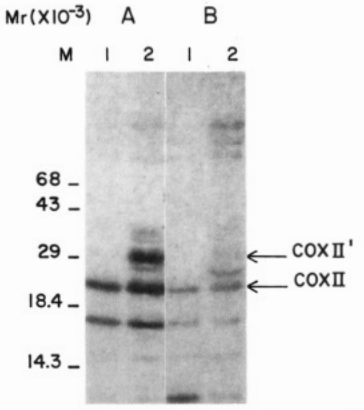

FIGURE 6: Immunoprecipitation of in vitro synthesized COXII. Translation reactions were performed in the absence (lane 1) or presence (lane 2) of microsomal membranes. (Panel A) immunoprecipitation using anti-COXII IgG; (panel B) immunoprecipitation using monoclonal antibody to the beef heart COXII.

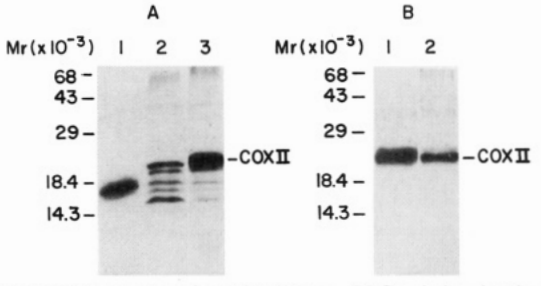

FIGURE 7: Fluorograph of an 18.5% urea–SDS gel showing in vitro translation of COXII mRNA in the presence of artificial membrane vesicles. (Panel A) Lane 1, lysate plus lipid vesicles alone (15 mg/mL); lane 2, lysate plus COXII mRNA alone (10 μg/mL); lane 3, lysate plus COXII mRNA (10 μg/mL) and lipid vesicles (15 mg/mL). (Panel B) Fractionation of the in vitro translation mixture in the presence of lipid vesicles: lane 1, pellet; lane 2, supernatant.

glycosylated and nonglycosylated, as well as a lower molecular weight peptide that may represent a premature termination fragment. The beef heart COXII monoclonal antibody, raised to native cytochrome oxidase as immunogen and inhibitory to native enzyme, precipitates mainly nonglycosylated COXII. A weak band that migrates between the major glycosylated and nonglycosylated forms of COXII is also seen in Figure 6; this is likely a partially glycosylated form that can be precipitated by both antibodies. The probable glycosylation sites for COXII are near the C-terminal (Asn-His-Ser^{205}, Asn-Trp-Ser^{223}) (Kornfeld & Kornfeld, 1985). Since the monoclonal antibody is specific to the C-terminal of beef heart COXII (Taha and Ferguson-Miller, personal communication), glycosylation at both these sites apparently inhibits binding of the COXII to this antibody.

In vitro translation of COXII RNA in a rabbit reticulocyte lysate was also strongly stimulated by the addition of phospholipid vesicles (Figure 7A, lane 3 compared to lane 2), with maximum stimulation achieved at 15 mg/mL vesicles. In this system, only the nonglycosylated form was produced. The peptide sediments with the lipid vesicles upon centrifugation (Figure 7B). Thus, the artificial membranes appear to accomplish the same stimulation of protein synthesis observed with microsomal membranes, but avoid the complication of glycosylation. Breakdown of the newly made protein in the absence of added membranes (Figure 5, lane 4; Figure 7A, lane 2) clearly demonstrates the stabilizing effect of the membranes. Trypsin digestion of the newly made COXII leads to two major bands, 15 and 6.5 kDa, as well as a minor band of 12.5 kDa (data not shown). The digestion fragments are

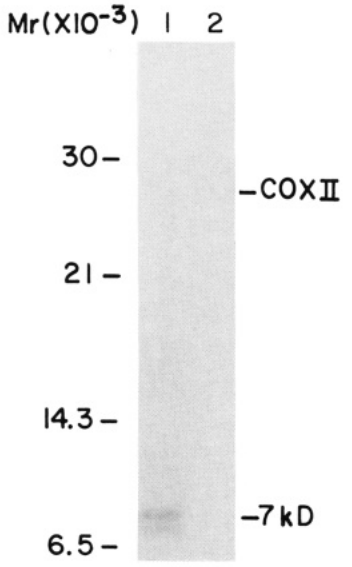

FIGURE 8: Proteinase K digestion of microsomal membrane associated COXII. Lanes 1 and 2 represent digestion of microsomal membrane associated COXII in the absence and presence of 0.5% sodium deoxycholate, respectively. The microsomal-associated COXII was produced by in vitro translation and subject to proteinase K digestion as described under Experimental Procedures.

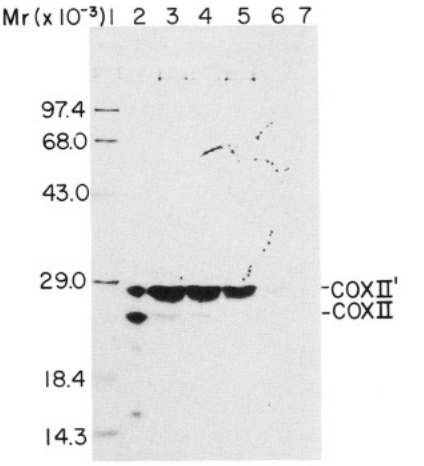

FIGURE 9: Fluorograph of a 12.5% SDS gel showing the expression of COXII mRNA in *Xenopus laevis* oocytes. The ^{35}S-labeled COXII was immunoprecipitated with anti-COXII IgG. Lane 1, molecular weight markers; lane 2, in vitro translation in rabbit reticulocyte lysate of COXII mRNA in the presence of microsomal membranes. Lanes 3–7 represent data from injected oocytes: lane 3, 20 ng of mRNA/oocyte; lane 4, 10 ng/oocyte; lane 5, 5 ng/oocyte; lane 6, 1 ng/oocyte; lane 7, control oocytes injected with water. COXII' represents the glycosylated form of COXII.

consistent with those predicted from the sequence of rat subunit II and are similar to those found for digestion of beef subunit II which has the same trypsin cleavage sites.

Evidence that COXII synthesized in vitro is inserted into, not just associated with, membranes derives from studies using proteinase K. If COXII is inserted in the predicted manner (Holm et al., 1987), residues 27–82 that form the hairpin across the membrane should be protected, and a residual peptide of about 7 kDa should be detected in SDS–PAGE. Indeed, a peptide with a molecular weight of about 7K is protected when membrane-associated COXII is digested with proteinase K. This band disappears when membranes are solubilized in the presence of sodium deoxycholate prior to addition of the proteinase (Figure 8). Thus, these experiments provide support for the idea that the COXII synthesized in vitro is inserted into membranes in the expected manner.

Expression in Xenopus Oocytes. Injection of mRNA derived from the pSPOXII construct into *Xenopus* oocytes led to expression of full-length glycosylated COXII. Oocytes that were injected with COXII mRNA showed a major new immunoprecipitation band compared to the control oocytes. As shown in Figure 9, there is no detectable difference in gel mobility between COXII made in oocytes and the glycosylated peptide made in rabbit reticulocyte lysates in the presence of microsomal membranes. The maximum expression of COXII was achieved at about 20 ng of mRNA/oocyte; injection of more than 40 ng of mRNA led to decreased expression of COXII peptide. The highest level of expression was achieved about 24–48 h after injection. Under optimal conditions, the yield of COXII was approximately 10 ng/oocyte, estimated on the basis of intensity of the ^{35}S-labeled band detected by autoradiography. Figure 10A shows that the newly synthesized COXII was found in oocyte cytoplasmic membranes, which were isolated by differential centrifugation as described under Experimental Procedures. When these membranes were extracted with the nonionic detergent lauryl maltoside, all the COXII was solubilized into the supernatant fraction (Figure 10B, lane 2). Similar to its behavior in the in vitro system, it appears that the COXII peptide undergoes glycosylation presumably at Asn-X-Ser sites (Kornfeld & Kornfeld, 1985), in the endoplasmic reticulum of oocytes.

Discussion

When the vector pDR540 was used, the COXII peptide was expressed in *E. coli* without fusion to any other protein. The COXII peptide was sufficiently stable to be detected by the maxicell technique as a radioactive band in the same position as a subunit II standard, and by cross-reaction with antibodies to beef heart cytochrome oxidase. However, COXII was made at very low level compared to the amount of the galactokinase protein produced by a control plasmid under the same conditions. When *ucox*II was fused to the 3′ end of a truncated *gal*K sequence, the level of expression was improved, but the fusion protein was still produced in much smaller amounts than galactokinase alone. In experiments with pPLEXOXII, the level of expression of the fusion protein, cro-β-gal–COXII, was higher, amounting to about 0.5% of the total cell protein. Both the *gal*K–COXII and cro-β-gal–COXII fusion proteins are found in the cell in inclusion bodies that require SDS or urea for solubilization. Other expression vectors such as pKK223-3, pPL-λ, pCQV2, and pUC18 were also tried, but no detectable level of COXII was found.

Gene expression in *E. coli* (or any other system) is a trial and error procedure, especially in the case of a mitochondrially coded polypeptide. The poor expression of the *ucox*II gene may be due to a number of factors, such as the structure of the ribosome-binding site, inefficient transcription, instability of the COXII mRNA, or differences between *E. coli* and mitochondrial gene codon usage. To address the latter issue, we changed three arginine codons in the *ucox*II gene to codons that are used more often in *E. coli* (see Figure 1). This did not lead to improved expression, perhaps because of a number of other codons in the *ucox*II sequence that also are rarely used in *E. coli* cells.

Recent studies show that mitochondrial initiation factors are required for the proper recognition and melting of secondary structure in the 5′-terminal region of mitochondrial mRNAs, as a prerequisite for initiation of protein synthesis in mammalian mitochondria (Liao & Spremulli, 1989). It has also been found that initiation factors and initiator tRNA from *E. coli* neither promote binding of mitochondrial mRNA

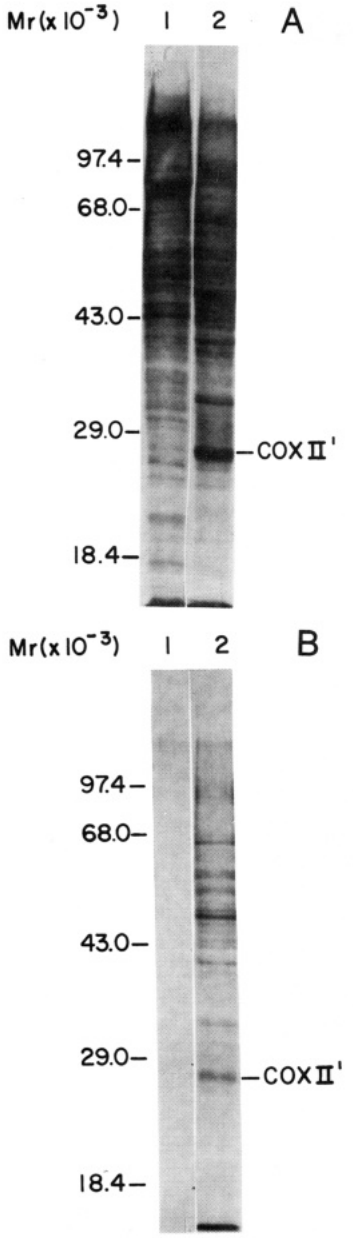

FIGURE 10: Fractionation and solubilization of oocyte membranes after synthesis of COXII from injected mRNA. (Panel A) The ^{35}S-protein content of oocyte membranes and cytosol fractions as analyzed on a 12.5% SDS gel. The gel was fluorographed as described under Experimental Procedures. Lane 1, oocyte cytosol (supernatant from centrifugation); lane 2, total oocyte membranes (pelleted by centrifugation). (Panel B) Solubilization of COXII from oocyte membrane. Lane 1, pellet of lauryl maltoside solubilized membranes; lane 2, supernatant of lauryl maltoside solubilized membranes (membrane extracts). Approximately equal amounts of protein were applied to each lane. The pellet showed no radioactive bands but had a considerable amount of unlabeled protein as determined by Coomassie blue staining (data not shown).

to its ribosome nor facilitate the melting of the 5′-end proximal mRNA stem structure (Denslow et al., 1989). The factors involved in initiation of mitochondrial protein synthesis appear to be specific for the mitochondrial system. Therefore, in our case, the poor expression of the *ucox*II gene in *E. coli* might be due to the absence of those initiation factors.

In general, it is apparent that *E. coli* is not the ideal system for expression of mitochondrial proteins in a native form. However, a denatured fusion protein was obtained at reasonable levels and proved useful for making COXII antibodies and for making affinity columns with which to purify these antibodies. The high molecular weight cro-β-gal–COXII fusion protein is a very good antigen, eliciting a strong, specific polyclonal response. The purified monospecific COXII antibody provides a valuable tool for detection of COXII peptide produced in other systems.

On the other hand, our results demonstrate that COXII can be produced in a nondenatured form in an in vitro translation system and in vivo by using *Xenopus laevis* oocytes. We identified the newly synthesized peptide as COXII by the following criteria: (1) the synthesized peptide has the same molecular weight as that of the authentic COXII peptide on SDS–PAGE; (2) the peptide can be immunoprecipitated by beef heart cytochrome oxidase antibodies; (3) proteinase K digestion shows that the COXII peptide is inserted into membranes in the predicted manner (Figure 8); (4) the peptide made in both systems (in vitro and in vivo) coisolates with membranes and is extractable with a nonionic detergent, lauryl maltoside, as would be expected for a nondenatured membrane protein. Although enough material for accurate spectral and copper analysis has not been isolated, the existence of a readily soluble form of the peptide suggests that its prosthetic group is likely to be present.

Canine pancreatic microsomal membranes (Shields & Blobel, 1977; Katz et al., 1977), mitochondrial membranes (Zhuang et al., 1977), and *Xenopus* oocyte membranes (Ohlsson et al., 1981) have been used previously in in vitro translation of membrane proteins. The finding that soybean phospholipid vesicles can replace natural membranes for stimulating the synthesis of the COXII peptide is of great interest and provides a number of potential advantages for expression of membrane proteins. Artificial membrane vesicles are easy to make and are much less expensive than the commercial microsomal membranes. They are also free of other proteins, providing an easy route for purification of the inserted peptide.

Thus, in two different expression systems we have shown that COXII can be produced independently of other subunits of the oxidase complex in a membrane-bound, nondenatured form. Although neither expression system is ideal for producing large amounts of protein, our results are highly significant since they indicate the feasibility of pursuing a study of the independent structural and functional characteristics of COXII, and ultimately of its interaction with other oxidase subunits of both nuclear and mitochondrial origin.

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REFERENCES

Anderson, S., Bankier, A. T., Barrell, B. G., deBruijn, M. H. L., Coulson, A. R., Drouin, J., Eperon, I. C., Nierlich, D. P., Roe, B. A., Sanger, F., Schreier, P. H., Smith, A. J. H., Staden, R., & Young, I. G. (1981) *Nature 290*, 465–475.

Banner, W. M., & Laskey, R. A. (1974) *Eur. J. Biochem. 46*, 83–88.

Brown, G. G., & Simpson, M. V. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3246–3250.
Burton, Z. F., Killeen, M., Sopta, M., Ortolan, L. G., & Greenblatt, J. (1988) *Mol. Cell. Biol. 8*, 1602–1613.
Buse, G., Meinecke, L., & Bruch, B. (1985) *J. Inorg. Biochem. 23*, 149–153.
Carroll, S. B., & Laughon, A. (1987) in *DNA Cloning: a practical approach* (Glover, D. M., Ed.) Vol. III, pp 89–111, IRL Press, Oxford and Washington.
Casey, R. P., Chappell, J. B., & Azzi, A. (1979) *Biochem. J. 182*, 149–156.
Clarkson, G. H. D., & Poyton, R. O. (1989) *J. Biol. Chem. 264*, 10114–10118.
Colleoux, R. A., D'Auriol, L., Betermier, M., Cottarel, G., Jacquier, A., Galibert, F., & Dujon, B. (1986) *Cell 44*, 521–533.
DeBoer, H., & Kastelein, R. A. (1986) in *Maximizing Gene Expression* (Reznikoff, W., & Gold, L., Eds.) pp 227–228 and 253–255, Butterworths, Boston.
Debouck, C., Riccio, A., Schumperli, D., McKenney, K., Jeffers, J., Hughes, C., & Rosenberg, M. (1985) *Nucleic Acids Res. 13*, 1841–1853.
Denslow, N. D., Michaels, G. S., Montoya, J., Attardi, G., & O'Brien, T. (1989) *J. Biol. Chem. 264*, 8328–8338.
DeRonde, A., VanLoon, A. P. G. M., & Grivell, L. A. (1980) *Nature 287*, 361–363.
Einarsdottir, O., & Caughey, W. S. (1985) *Biochem. Biophys. Res. Commun. 129*, 840–847.
Ey, P. L., Prowers, C. R., & Jenkin, C. R. (1978) *Immunochemistry 15*, 429–436.
Ferguson-Miller, S., Brautigan, D. L., & Margoliash, E. (1976) *J. Biol. Chem. 251*, 1104–1115.
Ferguson-Miller, S., Brautigan, D. L., & Margoliash, E. (1978) *J. Biol. Chem. 253*, 149–159.
Fujita, N., Nelson, N., Claudio, F. T., Lindstrom, J., Riezman, H., & Hess, G. P. (1986) *Science 231*, 1284–1287.
Garboczi, D. N., Hullihen, J. H., & Pedersen, P. L. (1988) *J. Biol. Chem. 263*, 15694–15698.
Gething, M. J., Bye, J., & Waterfield, M. (1980) *Nature 287*, 301–306.
Grosskopf, R., & Feldmann, H. (1981) *Curr. Genet. 4*, 151–158.
Gurdon, J. B. (1968) *J. Embryol. Exp. Morphol. 20*, 401–414.
Hanahan, D., & Meselson, M. (1980) *Gene 10*, 63–69.
Holm, L., Saraste, M., & Wikstrom, M. (1987) *EMBO J. 6*, 2819–2823.
Kadenbach, B., Jarausch, J., Hartmann, R., & Merle, P. (1983) *Anal. Biochem. 129*, 517–521.
Katz, F. N., Rothman, J. E., Lingappa, V. R., Blobel, G., & Lodish, H. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3278–3282.
Khorana, H. G., Knox, B. E., Nasi, E., Swanson, R., & Thompson, D. A. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7917–7921.
Koblika, B. K., MacGregor, C., Daniel, K., Koblika, T. S., Caron, M. G., & Lefkowitz, R. J. (1987) *J. Biol. Chem. 262*, 15796–15802.
Kornfeld, R., & Kornfeld, S. (1985) *Annu. Rev. Biochem. 54*, 631–664.
Kuhn-Nentwig, L., & Kadenbach, B. (1985) *Eur. J. Biochem. 148*, 147–156.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Liao, H.-X., & Spremulli, L. L. (1989) *J. Biol. Chem. 264*, 7518–7522.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor, NY.
Maxam, A. M., & Gilbert, W. (1980) *Methods Enzymol. 65*, 499–560.
Meinkoth, J., & Wahl, G. (1984) *Anal. Biochem. 138*, 267–284.
Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K., & Green, M. R. (1984) *Nucleic Acids Res. 12*, 7035–7056.
Messing, J. (1983) *Methods Enzymol. 101*, 20–78.
Müller, M., Schläpfer, B., & Azzi, A. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 6647–6651.
Nagai, K., & Thogerson, H. C. (1987) *Methods Enzymol. 153*, 461–481.
Ohlsson, R., Lane, C. D., & Guengerich, F. P. (1981) *Eur. J. Biochem. 115*, 367–373.
Pakmanaban, G., Hendler, F., Patzer, J., Ryan, R., & Rabinowitz, M. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4293–4297.
Rosevear, P., Van Aken, T., Baxter, J., & Ferguson-Miller, S. (1980) *Biochemistry 19*, 4108–4115.
Russell, D. R., & Bennett, G. N. (1982) *Gene 20*, 231–242.
Sancar, A., Hack, A. M., & Rupp, W. D. (1979) *J. Bacteriol. 137*, 692–693.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.
Scragg, A. H., & Thomas, D. Y. (1975) *Eur. J. Biochem. 46*, 183–192.
Sczakiel, G., Wittinghofer, A., & Tucker, J. (1987) *Nucleic Acids Res. 15*, 1878.
Shields, D., & Blobel, G. (1977) *Proc. Natl. Acad. Sci. U.S.A 74*, 2059–2063.
Stanley, K. K., & Luzio, J. P. (1984) *EMBO J. 3*, 1429–1434.
Steffens, G. J., & Buse, G. (1979) in *Cytochrome Oxidase* (King, T. E., Orii, Y., Chance, B., & Okunuki, K., Eds.) Vol. 5, pp 79–90, Elsevier/North-Holland, New York.
Steffens, G. C. M., Biewald, R., & Buse, G. (1987) *Eur. J. Biochem. 164*, 295–300.
Tarentino, A. L., Gomez, C. M., & Plummer, T. H., Jr. (1985) *Biochemistry 24*, 4665–4671.
White, M. M., Mayne, K. M., Lester, H. A., & Davidson, N. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4852–4856.
Wikstrom, M. (1977) *Nature 266*, 271–273.
Wikstrom, M., Krab, K., & Saraste, M. (1981) *Annu. Rev. Biochem. 50*, 623–655.
Wikstrom, M., Saraste, M., & Penttila, T. (1985) in *The Enzymes of Biological Membranes*, (Martonosi, A. N., Ed.) Vol. 4, pp 111–148, Plenum, New York.
Zhuang, Z., Chau, V., & McCauley, R. (1989) *J. Cell Biol. 107(6)*, 344a.
Zoller, M. J., & Smith, M. (1983) *Methods Enzymol. 100*, 269–500.