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Expression of Cytochrome *c* Oxidase Subunit II From a Nuclear Transgene

by

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Abstract

Cytochrome *c* oxidase (COX) is the terminal electron carrier in the electron transport chains of eucaryotes as well as many aerobic bacteria, and catalyzes the reduction of molecular oxygen to water. In mammals, COX consists of 13 polypeptide subunits and 4 redox centers. Subunit II of COX (COII) contains the Cu_A redox center, and is encoded in the mitochondrial genome. The long term goal of this research is to overexpress a mutant form of COII and create a dominant negative mutation in COX; transgenic mice containing the dominant negative mutation could be used to investigate the effects of the respiratory chain deficiencies which are characteristic of mitochondrial myopathies on mitochondrial biogenesis and gene expression. This thesis reports an attempt to express rat COII from a nuclear transgene. Using PCR strategies, the mitochondrial matrix targeting sequence from rat ornithine carbamyltransferase, was attached to a version of the rat COII gene which had been recoded into the universal genetic code. The transgene was cloned into the pLXSN retroviral vector and GP+E-86 ecotropic packaging cells were transfected with the construct. Infectious retrovirus particles were collected and used to infect C2C12 mouse myoblast and NIH3T3 mouse fibroblast cell lines. Expression of mRNA from the transgene was assessed in both cell lines using an RT-PCR strategy; expression levels of up to 6.7 times and 3.7 times the endogenous levels of COII mRNA were observed in infected NIH3T3 and C2C12 cells, respectively. For each cell type, protein expression from the transgene was assessed in the two clones showing the highest levels of mRNA expression. COII protein levels were determined by Western blot analysis using peptide-specific polyclonal antibodies directed to the C-terminus of rat COII; no significant protein expression was observed. The transgene was expressed in a rabbit reticulocyte lysate system in which transcription and translation are coupled; protein products of various lengths were produced. *In vitro* translation products were immunoprecipitated using the COII peptide-specific antibodies; no protein representing COII was specifically immunoprecipitated. The data suggest the

transgene cannot be efficiently translated on cytosolic ribosomes. Possible explanations for the data as well as suggestions for future research are discussed.

Résumé

La cytochrome oxydase c (COX) est le transporteur d'électrons terminal dans la chaîne de transport d'électrons des eucaryotes et plusieurs bactéries aérobes et elle catalyse la réduction de l'oxygène moléculaire en eau. Chez les mammifères, COX est constituée de 13 sousunités polypeptides et de 4 centres "redox". La sousunité II de COX (COII) comprend le centre Cu_A "redox" et est encodée dans le gènome mitochondrial. Une tentative pour exprimer COII de rat dans un noyau transgénique est rapportée dans ce mémoire. À l'aide des stratégies de PCR, la séquence cible de la matrice mitochondriale de l'ornithine carbamyltransférase de rat est fixée à une version COII de rat, lequel a été recodé dans le code génétique universal. Le transgène a été clôné dans un vecteur de rétrovirus, le pLXSN. Celui-ci a été utilisé pour transfecter l'ensemble des cellules écotropiques GP+E-86. Des particules infectieuses de rétrovirus ont été recueillies et utilisées de façon à infecter les lignées cellulaires de myoblastes de souris C2C12 et de fibroblastes de souris NIH3T3. L'expression de l'ARNm à partir de transgène a été évalué dans les 2 lignées cellulaires en utilisant le RT-PCR; des niveaux d'expression de l'ARNm de 6.7 fois et de 3.7 fois l'expression de COII endogène ont été observés chez les cellules infectés de NIH3T3 et C2C12, respectivements. Pour chaque type de cellules, l'expression des protéines du transgène est vérifiée par 2 clônes montrant des niveaux élevés d'expression d'ARNm. Les niveaux d'expression des protéines COII ont été déterminés par analyse de type Western à l'aide d'anticorps polyclonaux spécifiques au peptide dirigés contre l'extrémité C-terminale de COII de rat; aucune expression significative de protéines n'a été observé. Le transgène a été exprimé dans un lysat de réticulocytes de lapin lequel rassemble la transcription et la traduction; des protéines de différentes longueurs ont été produites. Les produits de traduction ont été immunoprécipités en utilisant les anticorps spécifiques au peptide de COII; aucune protéine représentant COII n'a été spécifiquement immunoprécipité. Les données suggèrent que le transgène ne peut être efficacement traduit par les ribosomes du cytosol.

Des explications possibles sur les résultats ainsi que des suggestions sur des recherches ultérieures sont élaborées dans ce mémmoire.

This thesis is dedicated to my parents who have always provided me with love, opportunity, encouragement and humour.

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I. Introduction

A. Mitochondria

1. Introduction

Mitochondria are organelles present in the cytoplasm of nearly all mammalian cells. They were first recognized as ubiquitous intracellular structures in 1890 by Altman who called them "bioblasts" because he believed they were 'elementary organisms' resembling bacteria living inside cells and carrying out vital functions (Ernster and Schatz, 1981). Altman's hypothesis has evolved into the endosymbiont theory, the most widely accepted explanation for the origin of mitochondria (reviewed in Reijnders, 1975). The name mitochondrion was introduced by C. Benda in 1898 (Benda, 1898); the term originates from the Greek words 'mitos' meaning thread, and 'chondros' meaning granule, which described the appearance of the organelles during Benda's observations of insect spermiogenesis. Mitochondria contain the cellular machinery for lipid metabolism, the citric acid cycle, the electron transport chain and oxidative phosphorylation; the primary function of mitochondria is aerobic energy generation.

2. Structure

Mitochondria are cylindrical in shape and have diameters of 0.5 to 1 μm. They are large enough to be visualized under the light microscope. In living cells, mitochondria are mobile and plastic, constantly changing shape, and are often associated with microtubules in the cytoplasm. Thus, when viewed under the microscope, the organelles appear as mobile networks in the cytoplasm rather than as individual cylindrical organelles.

Mitochondria are surrounded by two membranes, the outer and inner membranes, which create two distinct compartments called the intermembrane space and the matrix space. Structurally, mitochondria can be divided into 4 distinct compartments: the outer membrane, the intermembrane space, the inner membrane and the matrix space.

The outer membrane contains numerous gates and pumps. A major constituent of this membrane is the transport protein porin. Porin is capable of forming large channels that are permeable to molecules below 10 kDa, and thus, such molecules cross the outer membrane freely. Other protein components of the outer membrane include enzymes involved in lipid metabolism, as well as several components of the mitochondrial import machinery.

The intermembrane space is surrounded by the outer membrane on the cytosolic side and the inner membrane on the matrix side. It contains several enzymes including kinases that use the ATP passing out of the matrix to phosphorylate other molecules, various proteases, such as the mitochondrial intermediate peptidase which cleaves signal sequences of imported proteins, as well as the electron carriers ubiquinone and cytochrome *c*.

The structure of the inner membrane is highly specialized for its function in energy generation. The inner membrane has an extremely large surface area and is highly convoluted into foldings called cristae. This membrane consists of a high proportion of proteins; many inner membrane proteins including the enzyme complexes of the electron transport chain, and the enzyme complex ATP synthetase are involved in energy generation. In addition, the membrane contains specific transport proteins, which regulate the passage of metabolites into and out of the matrix, as well as components of the mitochondrial protein import machinery. Therefore, unlike the outer membrane, the inner membrane is highly specialized, allowing passage only to molecules specifically transported and required in the matrix.

The matrix space is enclosed by the inner membrane. It contains enzymes involved in oxidative energy generation, various chaperones and proteases as well as factors required for the expression of the mitochondrial genome.

3. Oxidative Energy Generation

(a) Production of Reducing Equivalents

Aerobic energy generation is fueled by fatty acids and pyruvate. Pyruvate is the end product of glycolysis, the anaerobic metabolism of glucose, which occurs in the cytosol. Fatty acids and pyruvate are transported from the cytosol into the matrix via specific transport pathways and proteins. In the matrix, they are both oxidized to acetyl coenzyme A (acetyl-CoA); fatty acids are oxidized by a series of reactions called β-oxidation, while pyruvate is oxidized by a matrix enzyme complex called pyruvate dehydrogenase. Acetyl-CoA is oxidized in a circular cycle of reactions in the matrix, called the citric acid cycle (CAC), which was proposed by Hans Krebs in 1937 (reviewed in Krebs, 1970). For every molecule of glucose oxidized, 2 molecules of actyl-CoA are produced, and for each molecule of acetyl-CoA entering the CAC, 1 molecule of carbon dioxide, 1 molecule of ATP, 1 molecule of reduced flavin adenine dinucleotide ($FADH_2$), and 3 molecules of reduced nicotinamide adenine dinucleotide (NADH) are produced. The carbon dioxide is a waste product of aerobic metabolism; however, the high energy electrons of NADH and $FADH_2$ are used as fuel for the electron transport chain and oxidative phosphorylation in the next stage of aerobic energy generation.

(b) Electron Transport Chain and Oxidative Phosphorylation

The electron transport chain (ETC) is a series of enzyme complexes embedded in the mitochondrial inner membrane. The ETC complexes were isolated and characterized in the early 1960's by solubilization of the mitochondrial inner membrane with detergents (summarized in Ernster and Schatz, 1981). There are four enzyme complexes in the

respiratory chain: NADH dehydrogenase (complex I), succinate dehydrogenase (complex II), cytochrome b-c_1 complex (complex III) and cytochrome *c* oxidase (complex IV). These enzymes consist of transmembrane proteins, which hold the complexes in the inner membrane, closely associated with electron carriers.

In 1961 Peter Mitchell proposed the chemiosmotic theory, which states that oxidative phosphorylation, the coupling of respiration to ATP synthesis, is via the vectorial translocation of protons across the inner membrane (Mitchell ,1961). Electrons from reduced NADH are transferred to complex I while electrons from $FADH_2$ are transferred to complex II. Electrons are then sequentially transferred to complexes III and IV. Electron transfer is possible because each complex in the chain has a greater electron affinity than its predecessor. Electrons are carried between the respiratory chain complexes by two shuttle molecules: ubiquinone and cytochrome *c*. These electron carriers diffuse rapidly in the inter membrane space, and collide with the complexes, thereby allowing their electrons to be transferred. Once electrons reach the final electron carrier in the chain, cytochrome *c* oxidase (COX), they are transferred to molecular oxygen which is reduced to water. As electrons are transferred from complex to complex, they fall to successively lower energy levels and free energy is released. Part of the free energy is harnessed by proton pumps associated with complexes I, III and IV to pump protons from the matrix, across the inner membrane, into the intermembrane space. The movement of protons generates an electrochemical proton gradient across the inner membrane. Protons then flow down this gradient through a proton channel in ATP synthetase (complex V), which phosphorylates adenosine diphosphate (ADP) to adenosine triphosphate (ATP) using the energy released as protons move down the electrochemical gradient.

Each pair of electrons in reduced NADH provides energy for the synthesis of 3 molecules of ATP via oxidative phosphorylation. For each molecule of glucose oxidized, approximately 36 molecules of ATP are produced; since there is a net gain of only 2

molecules of ATP when glucose is anaerobically broken down by glycolysis, oxidative phosphorylation is the most efficient means of energy generation for the cell.

ATP synthesis is not the only process driven by the electrochemical proton gradient produced by the ETC; import of many charged substrates across the inner membrane, for example pyruvate and fatty acids, is accomplished by carrier proteins which couple the transport of specific substrates with the flow of protons down their electrochemical gradient.

B. Mitochondrial DNA

The hypothesis that mitochondria contain their own genetic material was first proposed in the 1940's when Boris Ephrussi and his colleagues discovered the 'petite' mutation in yeast (Ephrussi et al., 1949). Mitochondrial DNA (mtDNA) was first identified in 1963 when fibers resembling DNA were found inside mitochondria (Nass and Nass, 1963). The first evidence that mtDNA is expressed in animal cells came from studies by Guiseppe Attardi who found that RNA that hybridized with mtDNA was present in mitochondrial fractions of HeLa cells (Attardi and Attardi, 1967). In 1981, the complete sequence of the human mitochondrial genome was published (Anderson et al., 1981).

Human mtDNA is a closed circular molecule of double-stranded DNA consisting of 16 569 base pairs. It contains 37 genes which code for 2 ribosomal RNAs (12S and 16S), 22 transfer RNAs and 13 proteins. The 13 proteins are all subunits of enzyme complexes in the respiratory chain, including 7 subunits of complex I (ND1, ND2, ND3, ND4, ND4L, ND5 and ND6), 1 subunit of complex III (CYT b), 3 subunits of complex IV (COI, COII and COIII), and 2 subunits of complex V (ATPase 6 and ATPase 8).

mtDNA is unique both structurally and functionally for several reasons. First, mammalian mtDNA is extremely compact in structure containing virtually no noncoding stretches, introns, or flanking regions. In fact, most mitochondrial genes lack a complete

termination codon; completion of the termination codon occurs at the time of RNA processing by polyadenylation of mRNAs. Second, all mitochondria contain multiple copies of mtDNA. In mammalian cells, each mitochondrion contains between two and ten copies of mtDNA (Satoh and Kuroiwa, 1991). Therefore, cells such as liver cells that contain thousands of mitochondria, contain several thousand molecules of mtDNA. Third, mtDNA can exist in a heteroplasmic state where not all mtDNAs in a specific cell, tissue, organ or individual are identical. This is significant for expression of a disease phenotype, as will be discussed below. Fourth, mtDNA utilizes a unique codon recognition pattern distinct from the universal genetic code. In mammalian mtDNA, UGA codes for tryptophan, AUA for methionine, AGA and AGG are stop codons, while in the universal genetic code UGA is a stop codon, AUA codes for isoleucine and AGA and AGG code for arginine. Fifth, unlike nuclear DNA, mammalian mtDNA is maternally inherited, (Giles et al., 1980). Sixth, mtDNA replication, transcription, and protein synthesis occur in the mitochondrial matrix and use unique DNA and RNA polymerases, the 22 tRNAs encoded in the mitochondrial genome and a mitochondrial specific 55S ribosome. Seventh, there appears to be no recombination between mammalian mtDNA molecules. Last, mitochondrial genes are transcibed as polycistronic mRNA molecules rather than as single gene transcripts. Processing of the polycistronic transcripts occurs posttranscriptionally.

C. Protein Import Into Mitocohondria

Since the mitochondrial genome encodes only 13 polypeptides, the other hundreds of proteins required by mitochondria are encoded in the nuclear genome, synthesized on cytosolic ribosomes and imported into the organelle. These proteins must be targeted to the mitochondrion, translocated through the mitochondrial membranes and sorted to the different mitochondrial subcompartments. Significant advances in understanding mitochondrial protein import have been made in recent years. Achievements have come mainly from the study of *Saccharomyces cerevisiae* and *Neurospora crassa*. Recent

evidence suggests that protein import has been conserved throughout eucaryotes (Goping et al., 1995; Iwahashi et al., 1997).

1. Presequences

Most nuclear encoded mitochondrial proteins contain targeting sequences that are both necessary and sufficient to direct proteins to mitochondria. In most cases, the targeting sequences are at the N-terminus of proteins and are called presequences. Most presequences are cleaved upon import into mitochondria. Several experiments demonstrating import of heterologous non-mitochondrial proteins fused to various leader sequences have suggested that presequences are sufficient for targeting and import into mitochondria (Hurt et al., 1984; Horwich et al.,1985).

Although presequences do not have extensive sequence identity, they do share a number of characteristics; they are usually between 20 and 60 amino acids in length, have an abundance of positively-charged residues, frequently hydroxylated and hydrophobic residues and very few negatively-charged residues. Thus, presequences are amphipathic in nature, and tend to form α-helices.

Some nuclear encoded mitochondrial proteins, such as the ADP/ATP translocase of the inner mitochondrial membrane, contain targeting sequences that reside internally in the protein sequence (Liu et al., 1988).

2. Mitochondrial Protein Import Machinery

Each of the two mitochondrial membranes possesses a unique translocation machinery for preproteins. The various components of the import machineries have been studied by several groups and, in many cases, different names were assigned to identical components. For simplification, a uniform nomenclature was adopted in 1996 (Pfanner et

al., 1996). The complexes are now called translocase of the outer membrane (TOM) and translocase of the inner membrane (TIM).

(a) TOM Complex

In *S. cerevisiae* and *N. crassa*, the TOM complex is composed of at least 9 proteins, including Tom20, Tom22, Tom70, Tom71, Tom37, Tom40, Tom6 Tom7 and Tom5. Recently, the TOM complex from *N. crassa* was purified and reconstituted into liposomes (Künkele et al., 1998). Both human and rat proteins with homology to Tom20 from *S. cerevisiae* and *N. crassa* have been identified and shown to be components of the mammalian protein import apparatus (Goping et al., 1995; Iwahashi et al., 1997).

Cross-linking and coimmunoprecipitation experiments have suggested the Tom proteins are in close proximity to each other and form a complex in the outer membrane (Haucke et al., 1996; Schlossmann and Neupert, 1995; Mayer et al., 1995). The complex functions both as a receptor to recognize preproteins destined for the mitochondrion, and as a general insertion pore (GIP) to transfer preproteins through the outer membrane. There are two receptor systems in the TOM complex. The first is composed of the Tom20 and Tom22 proteins. The second receptor system consists of the proteins Tom70, Tom71 and Tom37. Tom5, Tom6, Tom7, and Tom40 form the GIP.

(b) TIM Complex

Protein components of the TIM complex include Tim17, Tim23 and Tim44. Both Tim17 and Tim23 are integral membrane proteins and form a stable complex that is proposed to form a proteinaceous channel (Berthold et al., 1995). Tim44 is a peripheral membrane protein that is associated with the Tim23/Tim17 complex. Tim44 is associated with the complex on the matrix side and can be coimmunoprecipitated with mitochondrial Hsp70 and a co-chaperone known as MGE1 (Schneider et al., 1994; Kronidou et al., 1994; Rassow et al., 1994). In yeast, additional proteins have been implicated in TIM

function including 33kDa and 14 kDa proteins (Berthold et al., 1995) and 55 kDa and 20 kDa proteins (Blom et al., 1995). The function of these components has not yet been characterized.

3. Protein Import Into the Mitochondrial Matrix

Newly synthesized precursor proteins released from cytosolic ribosomes must be maintained in an unfolded conformation for efficient import into mitochondria. This is achieved by binding of cytosolic chaperones to the precursor protein. Several cytosolic chaperones of mammalian origin have been identified and shown to stimulate protein import into mitochondria including cytosolic Hsp70 (Deshaies et al., 1988), mitochondrial import stimulation factor, MSF (Hachiya et al., 1993), a presequence binding factor (Murakami and Mori, 1990), as well as a targeting factor (Ono and Tuboi, 1988). The function of MSF in protein import has been studied in detail (Hachiya et al., 1995), and two import pathways have been identified: an MSF-dependent pathway, and an MSF-independent pathway.

MSF recognizes and binds precursor proteins both with and without cleavable targeting sequences in an ATP-dependent reaction. The MSF-preprotein complex is recognized by the Tom70/Tom71/Tom37 receptor complex which is selective for preproteins bound to MSF. After recognition by the Tom70/Tom71/Tom37 receptor complex, MSF is released and the precursor is transferred to the Tom20/Tom22 receptor complex; this step requires further ATP hydrolysis.

Precursor proteins that are not recognized by MSF but are able to maintain an unfolded conformation are directly targeted to the Tom20/Tom22 receptor complex. Binding of precursors to this receptor complex appears to be mediated by cytosolically exposed acidic domains of the receptor subunits which are thought to bind the positively-charged presequence (Bollinger et al., 1995). Unlike the MSF-dependent pathway, import

of these proteins does not require extramitochondrial ATP. Once bound to the Tom20/Tom22 receptor complex, the import pathways of both subsets of precursors are identical.

Translocation of precursor proteins across the outer membrane occurs through the GIP. Tom6 was shown to stabilize the interaction between the receptors and the GIP so the precursor can be transferred (Alconada et al., 1995), while Tom7 exerts a destabilizing effect on the interaction between the Tom20/Tom22 receptor complex and the GIP (Honlinger et al., 1996). Protein translocation across the outer membrane does not require ATP, but is driven by the sequential binding of the presequence to acidic receptor domains on both sides of the membrane (Bollinger et al., 1995).

From the GIP, the N-terminus of the protein is transferred to the TIM complex. Tim23 acts as a binding site on the inner membrane for preproteins and, in association with Tim17 and Tim44, likely forms a translocation channel which is opened upon binding of the preprotein to Tim23 (Bauer et al., 1996). The N-terminus is translocated into the matrix where it is bound by Tim 44 and mt-Hsp70. Both a membrane potential and mt-Hsp70 binding are required for the initial translocation reaction, and the binding of mt-Hsp70 to the preprotein requires matrix ATP hydrolysis. Import of the rest of the preprotein is mediated by the Tim 44 mt-Hsp 70 complex along with the co-chaperone GrpEp. The mechanism by which mt-Hsp 70 and ATP hydrolysis drive the vectorial movement of preproteins remains controversial; both molecular ratchet (Neupert et al., 1990) and translocation motor (Glick, 1995) models have been proposed.

The TOM and TIM complexes can function independently of one another. However, antibodies against a constituent of one system can co-immunoprecipitate constituents of the other system when a matrix-targeted protein is arrested during import; no association is seen in the absence of an arrested precursor (Horst et al., 1995). These observations suggest the two translocases can interact and cooperate during protein import.

Once translocated into the matrix, presequences are cleaved by the matrix processing peptidase (MPP). MPP consists of two components,α- and β-MPP, both of which are required for activity (Braun and Schmitz, 1995). The interaction between the two components varies among organisms; in mammals and *S. cerevisiae*, α- and β-MPP form a tight complex which can be isolated as a heterodimer while in *N.crassa* the MPP components are present as individual proteins in the matrix and in plants, MPP is an integral part of the cytochrome bc_1 complex of the respiratory chain (Braun and Schmitz, 1995).

There are several factors in the matrix that are essential for folding of imported proteins. mt-HSP 70, MDJ1 and MGE1 function together in import, folding and assembly of imported proteins, as well as in the degradation of some proteins (reviewed in Neupert, 1997). The chaperones Hsp 60 and Hsp 10 form a stable complex in the matrix, and also assist in the folding of certain imported proteins (Rospert et al., 1993). In addition, the mitochondrial cyclophilin Crp3p is thought to act as a folding catalyst in the matrix (Matouschek et al., 1995).

4. Subcompartmental Localization

Once translocated into the matrix, intermembrane space and inner membrane proteins must be directed to their appropriate locations. The majority of these proteins contain bipartite signal sequences. The first portion of such sequences targets the preprotein to the matrix and is cleaved by MPP. The second part of the presequence directs the protein from the matrix to the inner membrane or intermembrane space; this portion is cleaved by a metal dependent peptidase in the intermembrane space, called the mitochondrial intermediate peptidase (MIP).

Certain inner membrane and intermembrane space proteins, such as the ADP/ATP translocator, cytochrome *c*, cytochrome *c* heme lyase and several components of the TIM

complex, do not contain signal sequences. These proteins usually have unique import pathways.

Proteins destined for the mitochondrial outer membrane follow the same import pathway as matrix proteins. However, during translocation through the outer membrane, the proteins are released from the TOM import machinery and embedded in the outer membrane. Direction of proteins to the outer membrane is often achieved by specific domains such as signal-anchor sequences. Import and insertion into the outer membrane of Tom70, which contains a signal-anchor sequence, has been studied in detail by Gordon Shore and his colleagues and the group has reviewed the mechanism of protein import and insertion into the outer membrane (Shore et al., 1995).

D. Mitochondrial Diseases

1. Introduction

Mitochondrial myopathies form a heterogeneous group of diseases. Biochemically, these diseases result in a reduced ability to carry out oxidative phosphorylation. Mitochondrial diseases primarily affect tissues with high energy demands, specifically skeletal muscle and central nervous system tissue. These diseases are usually maternally-inherited and late-onset in nature.

In 1988, Holt et al. found large-scale rearrangements of mtDNA in muscle biopsies from patients with mitochondrial myopathies (Holt et al., 1988), and, in the same year, Wallace et al. associated a mtDNA mutation involving the ND4 gene with Leber's hereditary optic neuropathy, LHON (Wallace et al., 1988). These were the initial reports linking mtDNA mutations with pathological conditions. Since then, many other mtDNA mutations have been identified in patients with different clinical phenotypes. The mtDNA mutations can be grouped into four categories: large-scale rearrangements of mtDNA, point mutations in tRNA or rRNA genes, point mutations in protein coding genes and

nuclear gene defects resulting in mtDNA defects. The clinical and biochemical phenotypes of specific mitochondrial diseases and the mtDNA mutations associated with them is reviewed in Grossman and Shoubridge (1996).

2. mtDNA Heteroplasmy and Threshold Effect

As previously mentioned, each mitochondrion contains multiple copies of mtDNA. In the case of mtDNA deletions and duplications, point mutations in tRNA genes, mutations associated with NARP and mtDNA mutations caused by nuclear gene defects, both wild type and mutant mtDNA exist within a tissue. This is called mtDNA heteroplasmy and leads to a threshold effect for the biochemical and clinical phenotypic expression of the specific mutations. A certain proportion of mutant mtDNAs is required for phenotypic expression of the disease, and conversely, a certain percentage of wild type mtDNAs is able to rescue the disease phenotype. For example, in the case of the common 5 kb deletion associated with KSS, 75% of mutant mtDNA molecules is required for expression of the mitochondrial protein translation defect (Hayashi et al., 1991). The mitochondrial protein translation defects associated with tRNA point mutations in MERRF and MELAS can be completely rescued by the presence of 10 to 15% of wild type mtDNA molecules (Boulet et al., 1992; King et al., 1992).

Different proportions of the same mutation can also cause different clinical phenotypes. This is the case for point mutations in the ATPase6 gene, which, at levels below 90% cause NARP, while levels above 90%, result in Leigh's syndrome (Tatuch et al., 1992). Further, the proportion of mutant mtDNA varies amongst different tissues. For example, deleted mtDNA is present in highest proportions in post mitotic cells such as skeletal muscle and central nervous system tissue (Shanske et al., 1990).

3. Cytochrome *c* Oxidase Activity and Ragged-Red Fibers

Although mitochondrial myopathies form a heterogeneous group of diseases and each disease has a distinct clinical phenotype, there are several molecular characteristics common to many mitochondrial diseases. The skeletal muscle of patients with mtDNA deletions, duplications, tRNA point mutations and mutations in the COXIII gene, contains fibers which are negative for COX activity when stained with diaminobenzidine, as described by Arnold Seligman (1968). The muscle fibers consist of normal segments interspersed with COX negative fiber segments. Of the COX negative fibers, some appear ragged-red when stained with a modified Gomori trichrome stain which stains mitochondria red (Johnson et al., 1983). Ragged-red fibers (RRFs) reflect large subsarcolemmal accumulations of morphologically abnormal mitochondria. Since only a subset of the COX negative fibers are ragged-red, the enzyme defect most likely precedes the development of morphological mitochondrial changes and proliferation of these abnormal mitochondria. As previously mentioned, the skeletal muscle of patients with these specific mtDNA mutations is always heteroplasmic, containing a mixture of both wild type and mutant mtDNAs.

4. The Study of Mitochondrial Diseases

The relationship between the proportion of mutant mtDNAs and an abnormal biochemical phenotype can be assessed using techniques such as *in situ* hybridization and polymerase chain reaction (PCR) on single muscle fibers (Shoubridge et al., 1990). However, such experiments provide little indication of the biochemical basis of mitochondrial diseases. Most of our knowledge concerning the molecular basis of mitochondrial myopathies comes from studies using muscle cell culture systems. Myoblasts from patients with mitochondrial diseases can be grown in culture directly and studied on a clonal basis. However, such primary cells have limited growth potential. To obviate this problem, cytoplasts from patient cells can be fused to immortalized cells such

as 143B osteosarcoma cells, which have been depleted of their mtDNA by treatment with ethiduim bromide (ρ^0 cells). The resulting cybrid cells contain mitochondria, and therefore mtDNA, from the patient cells, but have the nuclear background of the 143B cells, and are therefore immortal. Cybrid cells have been used extensively to demonstrate the segregation of a biochemical phenotype with mtDNAs carrying putative pathogenic mutations, as well as to investigate the molecular basis of such biochemical defects (Boulet et al., 1992; King et al., 1992).

The knowledge available from cell culture studies is limited for several reasons. First, although myoblasts can be induced to differentiate into myotubes in culture by serum deprivation, myotubes do not fully differentiate into mature muscle fibers in culture. In addition, is has not been possible to reproduce RRFs in culture. Therefore, it is not possible to investigate the mechanisms controlling mitochondrial biogenesis, and, more specifically, the relationship between the proliferation of dysfunctional mitochondria and the severity of a respiratory chain defect.

Theoretically, an animal model of mitochondrial myopathies would allow such mechanisms to be investigated. Other aspects of mitochondrial diseases, including the role of neuronal input in mitochondrial biogenesis and gene expression in skeletal muscle could also be investigated. Further, such a model would be useful for the evaluation of potential drug therapies for respiratory chain diseases.

As previously mentioned, a common characteristic of skeletal muscle from patients with most mitochondrial myopathies is a deficiency in COX. Therefore, a mouse model of COX deficiency would be representative of this group of diseases.

E. COX

1. Introduction

The cytochrome oxidase superfamily of enzymes has two major branches. The first consists of cytochrome *bo* quinol oxidases, which use ubiquinol as a primary electron donor. The second consists of cytochrome *c* oxidases (COXs), which use cytochrome *c* as their primary electron donor. COX is the terminal electron carrier in the respiratory chains of eucaryotes as well as many aerobic bacteria. COX catalyzes the reduction of molecular oxygen to water.

Using X-ray crystallography, high resolution structures of COX from the soil bacterium *Paracoccus denitrificans* and from bovine heart mitochondria have been determined (Iwata et al., 1995; Tsukihara et al., 1995; Tsukihara et al., 1996; Yoshikawa et al., 1998). The bacterial enzyme consists of only 4 subunits, while mammalian COX, consists of 13 polypeptide subunits and has a molecular mass of 200 kilodaltons. The subunits of mammalian COX are called I, II, III, IV, Va, Vb, VIa, VIb, VIc, VIIa, VIIb, VIIc and VIII. In the bovine heart enzyme, the 13 different polypeptides are each present in one copy in the monomer. The functional form of the enzyme is a dimer consisting of 2 monomeric subunits (Tsukihara et al., 1996).

COX can be divided into three domains. The upper part of the molecule is the cytosolic domain and is calculated to extend approximately 35 Å above the membrane bilayer surface. The central part of the enzyme is an α-helical transmembrane domain of 45-50 Å. At the opposite side of the bilayer from the cytosol, the COX complex extends for approximately 30-35 Å into the matrix space.

The COX complex contains 4 redox metal centers, Cu_A ,Cu_B , heme *a* and heme a_3. In addition, X-ray cyrstallography data indicate that beef heart COX is associated with

magnesium and zinc ions as well as 8 phospholipid molecules which are bound to the protein by salt bridges or hydrogen bonds (Tsukihara et al., 1996)

2. mtDNA Encoded Subunits

The three largest subunits of mammalian COX, COI, COII and COIII are encoded by mtDNA and synthesized in the mitochondrial matrix. These subunits are highly homologous to the 3 largest subunits of the bacterial enzyme (Capaldi, 1990).

COI and COII bind the 4 metal centers and form the functional core of COX. COI contains heme *a* and the heme a_3-Cu_B center. COII contains Cu_A and the cytochrome *c* binding site.

COIII was previously thought to be involved in proton translocation, although recent results suggest this subunit is involved in assembly of the COX complex. Mutation of the residues in COIII thought to be involved in proton pumping, does not affect proton translocation activities of the enzyme (Haltia et al., 1991), and absence of the COIII gene affects assembly of the enzyme complex (Haltia et al., 1989). In addition,Western blot analysis and immunocytochemistry on skeletal muscle from a patient with a microdeletion in a highly conserved region of COIII showed a lack of assembly of the COX complex (Keightley et al., 1996). COIII may affect proton translocation indirectly by affecting the conformation of the rest of the enzyme.

3. Nuclear Encoded Subunits

The remaining ten subunits of COX are encoded by nuclear DNA, made on cytosolic ribosomes and imported into the mitochondrial matrix where they are assembled with the three mitochondrially encoded polypeptides to form the functional COX complex. These additional subunits are only found in the eucaryotic enzyme.

The specific function of the nuclear encoded subunits is unclear. Structural data provide no indication of either electron transfer or proton pumping activities of these subunits (Tsukihara et al., 1996). In 1986, Kadenbach proposed that the nuclear encoded subunits may act as regulators of COX activity by providing binding sites for effectors of enzyme activity, such as nucleotides, hormones and fatty acids (Kadenbach et al., 1986). Genetic studies in yeast have provided direct evidence that many of the nuclear encoded subunits are required for assembly of functional COX (Poyton et al., 1988).

Several nuclear encoded subunits exist in tissue-specific isoforms. The expression of tissue-specific isoforms is very complex and shows a loose degree of evolutionary conservation; different isoform patterns are present in different mammals. For example, in bovine heart COX, subunits VIa, VIIa, and VIII are present in two isoforms, a heart (H) and skeletal muscle form, and a liver (L) form that is also found in brain and kidney (Yanamura et al., 1988). Humans also have H and L isoforms of subunits VIa and VIIa, but only the L form of subunitVIII (van Kuilenburg et al., 1988). Rats, on the other hand, have 2 forms of VIa and VIII, but only the L form of VIIa (Capaldi, 1990). In addition, developmental switching from the L to H forms, has been reported in human skeletal muscle (Taanman et al., 1992), mouse (Kim et al., 1995) and bovine fetal heart and skeletal muscle (Ewart et al., 1991). Co-expression of the H and L forms of VIIa in human skeletal muscle and cultured myoblasts has also been observed (Taanman et al., 1992).

4. Electron Pathway and Proton Pumping in COX

It is now generally accepted that Cu_A is the primary acceptor of electrons from cytochrome *c* in COX (Winkler et al., 1995). Electrons flow from cytochrome *c* to Cu_A and then to heme *a*, before entering the heme a_3-Cu_B oxygen binding site of COI. It has been suggested that the rate limiting step in the reaction is the electron transfer from heme *a* to heme a_3 (Winkler et al., 1995). The crystal structure of COX allowed the identification

of hydrogen-bonded systems that may act as electron transfer pathways between the various prosthetic groups. For example, both Iwata et al. (1995) and Tsukihara et al. (1996) propose an electron pathway between Cu_A and heme *a* involving one of the histidine ligands of the Cu_A center, the peptide bond between two closely-positioned arginine residues and the propionate of heme *a*. This is thought to be the most-likely pathway between Cu_A and heme *a*, although both groups also describe direct electron transfer pathways between Cu_A and the heme a_3-Cu_B centers. A pathway between heme *a* and heme a_3 involving a phenylalanine that is equidistant from the two centers was also proposed.

In 1977, Wikstrom showed that electron transfer by COX is coupled to proton translocation within the enzyme complex (Wikstrom, 1977). For each electron transferred, 2 protons are taken up from the matrix space; one proton is consumed in the reduction of oxygen, and the other is translocated across the inner membrane and released into the IMS to generate the electrochemical gradient. It is therefore likely there are two proton channels in COX, one for 'chemical' protons used in water formation and the other for 'pumped' protons translocated across the inner membrane. Evidence for 2 proton channels came from results of experiments by Wikstrom's group who identified a mutation in COI that blocks proton pumping but not water formation (Thomas et al., 1993). In agreement with these results, Iwata et al. (1995), and Tsukihara et al. (1996), identified two possible proton transfer pathways in the high-resolution crystal structure. The groups also discuss mechanisms of proton pumping.

The reduction of one molecule of molecular oxygen requires the serial binding and release of 4 electrons which are donated by cytochrome *c* on the outer surface of the membrane and transferred via the two metal centers to the active site, and the pumping of 4 protons across the inner membrane.

5. COII

(a) Structure

In mammals, COII is a 227 amino acid peptide with a molecular mass of 25-30 kDa. Subunit II of both quinol and cytochrome *c* oxidase complexes has the same membrane topology. Structurally, COII can be divided into three domains. The N-terminus of the protein sits on the cytosolic side of the inner membrane. The N-terminus is followed by a transmembrane domain which is arranged as 2 α-helices; the transmembrane domain anchors COII in the inner membrane. The C-terminus of COII is also on the cytosolic side of the inner membrane and is arranged as a 10-stranded β-barrel. This domain contains the Cu_A and cytochrome *c* binding sites and protrudes approximately 8 Å above the membrane surface (Tsukihara et al., 1995).

(b) Cu_A Site

The first suggestion that COX contains copper came in the 1930's when David Keilin and C.A. Elvehjem noticed that copper-deficient yeast showed weak respiration (Elvehjem, 1931), and Keilin and Hartree consistently found copper associated with COX in heart muscle preparations (Keilin and Hartree, 1939). In the 1960's results of chemical analyses, reductive titrations and EPR experiments suggested there are at least 2 significant copper ions per monomer of oxidized COX (Beinert et al., 1962). Between 1987 and 1993, results from three different groups suggested there are not 2, but 3 intrinsic copper atoms in COX (Bombelka et al., 1986; Oblad et al., 1989; Steffens et al., 1993). In 1989, Kroneck et al. reported that Cu_A is a mixed-valence binuclear site (Kroneck et al.,1989). The third copper atom associated with COX forms the Cu_B site in subunit I.

Molecular biological approaches have provided a wealth of information concerning the structure of the Cu_A site. Unambiguous sequence alignment of subunit II from quinol

and cytochrome *c* oxidases showed that the C-terminus of both proteins contains a Greek-key β-barrel subdomain characteristic of cupredoxins, a family of blue copper proteins in plants and bacteria (Saraste, 1990), and that COII contains 9 totally conserved residues that are not conserved in quinol oxidases (Farrar et al.,1995). Four of these residues, 2 histidines and 2 cysteines, are present in a homologous stretch of sequence between COXs and nitric oxide reductases (Zumft et al., 1992). These data suggest that the Cu_A site is located in COII and also suggest the 2 cysteine and 2 histidine residues likely coordinate Cu_A.

Using recombinant DNA technology, van der Oost et al. (1992) expressed the C-terminal fragment of the CyoA subunit of E. coli cytochrome *o* quinol oxidase in a water-soluble form. This protein is homologous to COII but does not contain a Cu_A site. The group was able to engineer a Cu_A-like site into this peptide by introducing 6 amino acid residues that are conserved in COII: the 2 histidine and 2 cysteine residues thought to bind copper, as well as isoleucine and glutamic acid residues. Molecular mass determinations showed the engineered protein contained 2 copper ions, as expected. Using site-directed mutagenesis, the same group individually substituted, with different amino acids, the 2 histidine and 2 cysteine residues as well as a methionine residue that is conserved among quinol oxidases, COII and nitric oxide reductases (Kelly et al., 1993). Biochemical analysis and electrospray mass spectrometry were used to study the metal binding capacity of the mutated proteins. These experiments confirmed that all 5 residues are major ligands of the binuclear copper center, since substitution of these residues resulted in either a complete loss of colour or dramatic changes in the absorbance spectrum of the protein. In addition, substitution of the conserved glutamic acid residue located betweeen the Cu-binding cysteines, lead to minor perturbation of the optical spectrum of the fragment. Farrar et al. (1995) then performed similar SDM studies on the isolated Cu_A domain from *P. denitrificans* and confirmed that the 2 histidine, 2 cysteine, and methionine residues are

sufficient for assembly of an intact Cu_A center. Their results further suggest that the methionine residue has an important structural role for the protein rather than acting as a Cu_A ligand. This is not surprising since the methionine residue is conserved in quinol oxidases which do not contain a Cu_A site.

The putative structure of the Cu_A site in COX was confirmed by X-ray crystallography. A few months after the structures of *P. denitrificans* and beef heart COX were resolved by crystallography in 1995, Matthias Wilmanns and his colleagues published the crystal structure of the soluble domain from subunit II of E. coli cytochrome *bo* ubiquinol oxidase with the engineered dinuclear copper centre (Wilmanns et al., 1995). The X-ray data show that the Cu_A site is a highly symmetrical binuclear site with the 2 copper ions 2.6 A apart. The copper atoms are bridged by 2 cysteines, 2 histidines, a methionine, and the carbonyl oxygen of a glutamic acid residue.

(c) Cytochrome *c* Binding Site

Results of direct binding experiments and enzyme activity measurements indicate there is one high affinity binding site for cytochrome *c* and at least one lower affinity substrate site on the COX complex (Discussed in Capaldi, 1996). Cross-linking studies established the high-affinity site is located in COII (Bisson et al., 1980). In 1995, Lappalainen et al. expressed a fragment of subunit II from *P. denitrificans* COX in a soluble form and studied its interaction with cytochrome *c* by stopped-flow spectroscopy. Using site-directed mutagenesis, the group identified 5 conserved residues including a glutamine, 2 glutamates and 2 aspartate residues in the C-terminus of COII that are involved in cytochrome *c* binding. Substitution of these residues led to a 35-85% decrease in the rate of cytochrome *c* oxidation, and simultaneous substitution of the 2 aspartates and one of the glutamate residues led to a 95% decrease in the reaction rate. These residues are therefore believed to be involved in cytochrome *c* binding to COII.

F. Research proposal

1. Towards a Mouse Model

As discussed above, conserved amino acid residues in COII that are required for functional Cu_A and cytochrome *c* binding sites have been identified (Kelly et al., 1993; Lappalainen et al., 1995). Theoretically, if overexpressed, a COII peptide mutated at any of these essential residues should outcompete the endogenous wildtype COII peptide for assembly into the COX complex and significantly decrease or abolish activity of the enzyme. However, it is not possible to manipulate the mitochondrial genome directly. Therefore, the COII gene would have to be recoded into the universal genetic code, expressed from the nucleus and targeted to the mitochondrial matrix where it could assemble with the other subunits into the COX complex. This could be achieved by attaching a mitochondrial targeting sequence to the N-terminus of the COII protein. If the mutant protein were expressable from the nucleus and the dominant negative phenotype observed, expression of the transgene could be placed under the control of a muscle specific promoter, such as the muscle creatine kinase promoter; transgenic mice made with this construct should have little or no COX activity in their skeletal muscle. Such mice would be models for the respiratory chain deficiency characteristic of mitochondrial myopathies.

2. Present Work

The first step in creating a dominant negative mutation in COX is to determine whether it is possible to express mammalian COII from a nuclear transgene. This was the purpose of the work to be presented in this thesis. The mitochondrial targeting sequence from rat ornithine carbamyltransferase was attached to a rat COII gene that had been recoded into the universal genetic code. The transgene was introduced into the C2C12 mouse myoblast and NIH3T3 mouse fibroblast cell lines via retroviral infection.

Expression of the transgene in the infected cells was assessed at the levels of both mRNA and protein. Expression of the transgene was also assessed *in vitro* in a rabbit reticulocyte lysate system.

II. Materials and Methods

A. Construction of OCTuCOII Transgene

The pOCT plasmid, which contains the entire coding sequence for rat ornithine carbamyltransferase (Nguyen et al., 1986), was a gift from Dr. Gordon Shore. The pDR540 plasmid which contains the sequence for rat COII recoded into the universal genetic code (uCOII), was a gift from Shelagh Ferguson-Miller (Cao et al., 1991).

The OCT leader and rat uCOII sequences were joined using gene splicing by overlap extension, (gene SOEing) as described by Horton (1993). Primers were synthesized at the Sheldon Biotechnology Center at McGill University; their sequences are shown in Figure 1. The specific PCR reactions performed are described below. All PCR reactions were performed in a GeneAmp PCR System 2400 DNA thermal cycler (Perkin Elmer, Norwalk, CT).

1. Amplification of the OCT Leader Sequence From pOCT

50 µL reactions contained 1X *Pfu* reaction buffer (Stratagene, La Jolla,CA), 200 µM each dNTP, 20 pmol each OCT fwd and OCT rev primers, 100 ng pOCT template DNA, 2.5 units *Pfu* polymerase (Stratagene). For each cycle, DNA was denatured at 94°C for 30 seconds, annealed at 55°C for 30 seconds, and elongated at 72°C for 30 seconds; a total of 25 cycles was performed.

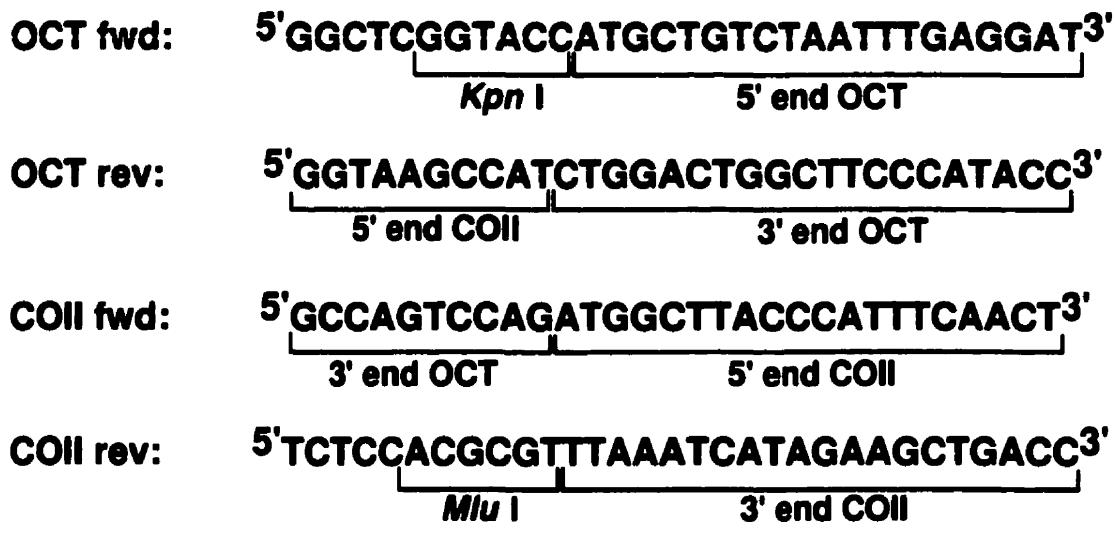

Figure 1: Sequences of primers used for splicing of OCT leader and COII sequences.

2. Amplification of uCOII From pDR540

50 μL reactions contained 1X *Pfu* reaction buffer, 200 μM each dNTP, 20 pmol each COII fwd and COII rev primers, 100 ng pDR540 template DNA, 2.5 units *Pfu* polymerase. *Pfu* polymerase and dNTP's were added to the reaction after an initial denaturation at 99.9 °C for 10 minutes. For each cycle, DNA was denatured at 94 °C for 30 seconds, annealed at 65°C for 30 seconds, and elongated at 72°C for 1 minute. A total of 25 cycles was performed.

3. Splicing of OCT Leader and uCOII Sequences

OCT presequence and uCOII PCR products of the reactions described above were purified using WIZARD PCR Preps (Promega, Madison, WI), and the purified genes were spliced together in a subsequent PCR reaction. The 100 μL reaction contained 1X *Pfu* reaction buffer, 200 μM each dNTP, 20 pmol each OCT3.fwd and uCOII2.rev primers, 40 ng of each purified PCR product, 2.5 units *Pfu* polymerase. *Pfu* polymerase and dNTP's were added to the reaction after an initial denaturation at 99.9°C for 10 minutes. For each cycle, DNA was denatured at 94°C for 30 seconds, annealed at 55°C for 30 seconds, and elongated at 72°C for 30 seconds; a total of 25 cycles was performed.

B. Subcloning of OCTuCOII Transgene

1. Cloning into PCRScript™ Amp SK(+) Vector

The recombinant 800bp PCR product was purified by ethanol precipitation in the presence of sodium acetate. The purified OCTuCOII PCR product was cloned into the

PCRScript™ Amp SK(+) cloning vector using the PCRScript™ Amp SK(+) Cloning Kit (Stratagene), following manufacturer's instructions. Insert:vector DNA ratios of 80:1 were used. Transformation of Epicurian Coli XL1-Blue MRF'Kan supercompetent cells (included with cloning kit) was performed following manufacturer's instructions. White colonies were picked and grown overnight in 5mL Luria-Bertani (LB) Medium containing 50 μg/mL ampicillin. Plasmid DNA was isolated from LB cultures using Wizard Minipreps (Promega). To test for the presence of the 800 bp OCTuCOII insert, miniprep DNA was digested with *Kpn* I and *Mlu* I (Promega) in 1X RE buffer 4 (Promega) for 4 hours at 37°C, and digestion products visualized on 1% agarose gels. For large-scale preparation of cloned DNA, colonies containing the insert were picked and grown overnight at 37°C in 200 mL LB medium containing 50 μg/mL ampicillin. Plasmid DNA was isolated using the QIAfilter Plasmid Maxi Kit (QIAGEN Inc., Mississauga, Ontario), quantitated using a UV/Vis Spectrometer (1 OD at 260 nm=50 μg/mL double-stranded DNA), digested and analyzed as above.

2. Subcloning into pLXSN

The transgene was then subcloned into the retroviral vector pLXSN which was used to obtain infective virus stock for infection of mouse cell lines with the construct. 4 μg of the PCRScript™ vector containing the OCTuCOII insert was digested with 10 units of *Kpn* I (GibcoBRL, Burlington, Ontario) in 1X React 4 buffer, overnight at 37°C. The digested DNA was run on a 1% agarose gel and the 800 bp band corresponding to the OCTuCOII insert was cut from the gel and purified using Prep-A-Gene®DNA Purification System (Bio-Rad Laboratories Ltd., Mississauga, Ontario). Sticky ends were filled with the Klenow fragment of T_4 DNA polymerase (Pharmacia Biotech, Baie D'Urfe, Quebec);

the 40 μL reaction contained 4 μL SuRECut Buffer M (Boehringer Mannheim, Montréal, Canada), 50 μM dNTP's, 12 units Klenow enzyme and incubation was at 37°C, overnight.

4 μg of pLXSN DNA was digested with 5 units *Hpa* I restriction enzyme (New England Biolabs Inc., Mississauga, Ontario) according to manufacturer's conditions; digestion was overnight at 37°C.

Digested DNA was run on a 1% agarose gel. The appropriate vector and insert bands were cut from the gel, purified and quantitated as above.

The 10 μL ligation reaction contained 1X T_4 DNA ligase buffer (GibcoBRL), 1 unit T_4 DNA Ligase (GibcoBRL), and insert: vector ratios of 20:1. To linearize any religated vector DNA, after ligation, potassium chloride was added to a final concentration of 50 mM, and ligation reactions were digested with 5 units of *Hpa* I enzyme for 1 hour at 37°C

2 μL of digested ligation reactions were used to transform Max Efficiency DH5α cells (GibcoBRL), according to protocol provided with PCRScript™ Amp SK(+) Cloning Kit. Colonies were picked and analyzed as above.

C. Cell Culture

1. Cell Lines and Growth Conditions

C2C12 mouse myoblast, and GP+E-86 retroviral packaging cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5 g/L glucose and L-glutamine (BioWhittaker,Walkersville, MD) supplemented with 10 % (vol/vol) fetal bovine serum.

NIH3T3 fibroblasts were grown in DMEM containing 4.5 g/L glucose and L-glutamine supplemented with 10 % calf serum.

2. Transfection of GP+E-86 Cell Line

GP+E-86 cells were grown to 50 % confluence in 6-well tissue culture dishes. Transfection of the pLXSN retroviral vector containing the OCTuCOII insert was accomplished using LipofectAMINE™ Reagent (GibcoBRL), according to the manufacturer's protocol. 6 μL of LipofectAMINE™ were used per transfection. Stable transfectants were selected by addition of 800 μg/mL of G418 (GibcoBRL) to growth medium; this G418 concentration is required for selection of NIH-3T3 cells (dose-response experiments) and GP+E-86 cells are NIH3T3 derived. Colonies were visible after 7 days in the antibiotic-containing medium.

3. Retroviral Infection of C2C12 and NIH3T3 Cell Lines

Infectious virus stock was obtained from GP+E-86 packaging cells transfected with the pLXSN retroviral vector containing the OCTuCOII insert as follows: once colonies were visible, cells were grown to near confluence on 100 mm tissue culture plates, at which point growth medium was replaced. 24 hours later, medium was collected, filtered through 0.45 μM Cellulose Acetate Membrane Filters (Corning Costar, MA, USA), and used immediately for infection of cells.

For infection of C2C12 and NIH3T3 cell lines, cells were grown to 30-50 % confluence on 100 mm tissue culture plates. Growth medium was removed, and 1 mL of the virus stock plus 3 mL of growth medium containing 4 μg/mL polybrene (Sigma, Oakville, Ontario) was added, followed by incubation at 37°C for 2 hours. 5 mL of

growth medium containing 4 μg/mL polybrene were then added and cells were incubated overnight at 37°C. The following day, growth medium was replaced with fresh medium, and cells were moved to 150 mm tissue culture plates at dilutions of 1:1000 and 1:10 000. 48 hours after the beginning of infection, G418 was added to the medium for selection of infected cells at concentrations of 700 μg/mL and 800 μg/mL for C2C12 and NIH3T3 cells, respectively; these G418 concentrations were determined by previous dose-response expermiments in the lab. After 10 days, colonies were picked using a pipette tip under a light microscope, and grown in regular growth medium in 24-well tissue culture dishes.

4. Collection of Cells for Analysis of Transgene Expression

Cells were grown to 50-80 % confluence on 100 mm tissue culture dishes, and were scraped into ice-cold phosphate-buffered saline (PBS). The cell suspension was centrifuged at 500 x g for 15 minutes to pellet cells. PBS was then aspirated, and pellets frozen at -80°C until analyzed.

5. Treatment with Ethidium Bromide

C2C12 and NIH3T3 clones infected with the transgene, as well as uninfected control cells, were grown in the presence of ethidium bromide (EtBr). 200 ng/mL EtBr as well as 50 μg/mL uridine and 110 mg/L sodium pyruvate were added to appropriate growth media. Regular medium was replaced with EtBr-containing medium and cells were grown in this medium for 1 week. Every 24 hours, cells were harvested, as described above, and analyzed on Western blots.

D. RT-PCR Analysis

1. RNA Isolation

Cells were lysed using the QIAShredder Kit (QIAGEN), and total RNA isolated using the RNeasy Minikit (QIAGEN), following manufacturer's instructions. To remove any DNA contamination, isolated RNA was digested with 5 units of RQ1RNase-free DNase (Promega) for 2 hours at 37°C. RNA was then repurified using the RNeasy MiniKit. Concentrations of isolated RNA were determined by measuring absorbance at 260 nm (1OD at 260 nm=40 μg/mL RNA). To assess the purity of isolated RNA, ratios of absorbances at 260 nm over absorbances at 280 nm were calculated; samples were not used if ratios were less than 1.7.

2. cDNA Synthesis

For reverse transcription, 1 μg of total cellular RNA, 10 uM random hexamers (Pharmacia), 1 X RT buffer (Promega), 20 units RNasin®Ribonuclease Inhibitor (Promega) in a total volume of 16.5 μL, was heated to 65°C for 5 minutes, and quickly transferred to ice. 200 units of M-MLV Reverse Transcriptase (Promega) and 0.5 mM of each dNTP were added, and the reaction incubated at 37°C for 45 minutes. cDNA products were used immediately in PCR reactions or stored at -80°C.

3. Primer Design

Synthetic oligonucleotides (COII337 fwd and COII572 rev)were designed to amplify a region of rat and mouse cDNA between base pairs 337 and 572. Primer sequences are shown in Figure 2.

COII337 fwd: $^{5'}$TATGAAGACCTATGCTTTGA$^{3'}$

COII572 rev: $^{5'}$AGCATTGGCCATAGAATA$^{3'}$

Figure 2: Sequences of primers used for RT-PCR reactions.

4. PCR Amplification of cDNA

50 μL amplification reactions contained 2 μL synthesized cDNA, 1X PCR buffer (GibcoBRL), 1.5 mM $MgCl_2$, 20 pmol each COII337 fwd and COII572 rev primers, 200 μM dNTP's and 2.5 units *Taq* DNA Polymerase (Gibco). Denaturation was at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and elongation at 72°C for 1 minute. 25 cycles were performed. 1.5 uCi of [α-^{32}P]-dCTP were added to each tube immediately before the last cycle. 100 ng each of mouse and rat COII DNA were used as control templates .

5. *Hinf* I Digestion of PCR Products

10 units of *Hinf* I restriction enzyme (Promega) was added to 15 μL of each PCR product. Digestion was at 37°C, overnight.

6. Acrylamide Gel Electrophoresis

Samples were electrophoresed through 15 % acrylamide gels in 1X TBE buffer. Dried gels were exposed to a phosphor screen, the screen was scanned on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA), and the results analyzed using ImageQuant software (Molecular Dynamics).

7. Verification of Method

The reliability of this method for distinguishing between mouse and rat COII genes, and in quantitating the relative levels of each, was evaluated using various ratios of rat and mouse COII DNA as template in PCR reactions. 100 ng of total template were used in each PCR reaction. Rat:mouse DNA ratios of 100:0, 90:10, 60:40, 50:50, 40:60, 10:90, and

0:100 were used and PCR reactions, digestions, and electrophoresis were performed as described above.

E. Preparation of Polyclonal Antibodies Against Rat COII

1. Coupling of Synthetic COII Peptide to KLH

A synthetic peptide corresponding to the last 11 amino acids at the carboxy terminus of rat COII was made at the Sheldon Biotechnology Center at McGill University. The synthetic peptide was coupled to keyhole limpet hemacyanin (Pierce, Rockford, IL) using glutaraldehyde, as follows. KLH was dissolved in PBS at a concentration of 1 mg/mL (total volume 5 mL). The KLH solution was dialysed against 1 L of PBS for 2 hours at room temperature. 5 mg of the synthetic peptide were then added to the dialysate. Glutaraldehyde was added to a final concentration of 0.25 %, and the solution incubated at room temperature, in the dark for 1 hour with vortexing every 15 minutes. Solid glycine was added to a final concentration of 0.1 M, followed by further incubation in the dark for 1 hour. The peptide-KLH conjugate was separated from free peptide by dialysis against PBS overnight at room temperature. The peptide-KLH conjugate was stored at -80°C.

2. Injection of Rabbits

All animal procedures were performed by technicians at the Animal Resources Centre at McGill University. The peptide-KLH conjugate was emulsified in TiterMax® research adjuvant (CytRx® Corporation, GA, USA) according to manufacturer's protocol. 3 female rabbits were immunized. For primary injections, each rabbit was injected at 4 subcutaneous sites with 0.1 mL of the peptide-KLH/TiterMax® emulsion. Two booster injections, identical to the primary injection, were given to each rabbit, 4 weeks and 9 weeks following initial injections.

3. Collection of Preimmune and Immune Sera

Prior to injection, rabbits were bled for collection of preimmune serum. Rabbits were bled 6 weeks after primary injections and exsanguinated 11 weeks following primary injections. Serum was separated from whole blood by centrifugation at 5000 x g for 10 minutes and was stored at -80°C.

F. Isolation of Mouse Liver Mitochondria

One female BALB/C mouse was sacrificed by cervical dislocation and its liver dissected. All subsequent procedures were performed at 4°C. The liver was minced with scissors and homogenized in 10 volumes of 0.25 M sucrose using a Dounce glass homogenizer with a tight-fitting pestle. The homogenate was centrifuged at 600 x g for 15 minutes. The supernatant was retained and subsequently centrifuged at 7700 x g for 15 minutes. The pellet, containing mitochondria, was resuspended in 10 mL of 0.25 M sucrose, and the suspension centrifuged at 7700 x g for 15 minutes. Pelleted mitochondria were then washed in 150 mM potassium chloride and centrifuged as above. Isolated mitochondria were finally resuspended in 2 mL of 10 mM potassium phosphate-DTT buffer and stored at -80°C until used for Western blots.

G. Western Analysis

Protein concentrations of harvested cells (resuspended in 200 uL PBS), and isolated mouse liver mitochondria were determined using the Pierce Micro BCA Protein Assay Kit (Pierce) according to manufacturer's instructions.

For characterization of rat COII polyclonal antibodies, 25, 50 and 75 μg mouse liver mitochondrial protein were used. For harvested cells, 50 μg protein were used.

Protein samples along with protein molecular weight markers were electrophoresed through Laemmli gels consisting of 4 % stacking and 12 % resolving gels (Laemmli 1970). Following electrophoresis, gels were equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 20 % methanol) for 15 minutes. Proteins were transferred onto nitrocellulose (Protran) membranes (Zymotech Biosystems, Montreal, Quebec) at 4°C in transfer buffer.

Non-specific binding sites were blocked by incubating membranes overnight in 10 % skim milk powder in Tris-buffered saline with 0.5 % Tween20 (TBS-T). Incubation of membrane with primary antibody (rabbit immune serum) was for 1 hour at room temperature and immune serum was diluted 1/500 in TBS-T containing 1 % skim milk powder. To test the specificity of the COII polyclonal antibodies, primary antibody was incubated with either 20 mg/mL of the COII peptide, or 20 mg/mL of a non-specific peptide (C-PNVPSRPQA-N, gift from Dr. Paul Holland), for 1 hour at room temperature prior to incubation with blot. Blots were washed 4 times for 5 minutes in 50 mL TBS-T at room temperature. Incubation of membrane with secondary antibody (horseradish peroxidase-linked anti-rabbit IgG (Amersham,Baie D'Urfe, Quebec)) was for 1 hour at room temperature. When biotinylated protein molecular weight standards were used, blots were also incubated with anti-biotin antibody (NEB). Both antibodies were diluted 1/1000 in TBS-T with 1 % skim milk powder. Blots were washed as above, and antibody binding detected with either NEB Phototype-HRP Kit (NEB), or the ECL detection kit (Amersham) according to company protocols. Blots were exposed to Kodak BioMax MS system film (Intersciences, Markham, Ontario) and films run through an automatic processor.

H. *In Vitro* Transcription and Translation

In vitro transcription and translation of the transgene were accomplished using Promega's TNT®Coupled Reticulocyte Lysate System (Promega), according to the

company's protocol. 0.5 μg of the pCRScript plasmid containing the transgene insert were used as the template DNA for the reaction. 5 μL of translation products electrophoresed through Laemmli gels consisting of 5 % stacking and 15 % resolving gels. Gels were processed as described previously.

I. Immunoprecipitation

20% (10 μL) and 40% (20 μL) of *in vitro* translation reactions were immunoprecipitated following the procedure published by Herman Bentlage and Anne Chomyn (Bentlage and Chomyn, 1996). Translation products were diluted 2X with buffer consisting of 10mM Tris-HCl, pH 8.0, 2% SDS, 1mM phenylmethylsulfonyl fluoride (PMSF). The lysate was precleared with 120 μL of prewashed insoluble Protein A from *S. aureus* (Cowan strain), or SAC (Sigma), as described by Bentlage and Chomyn. Immune complexes were formed by the addition of either 5 or10 μL of immune serum; 5 and 10 μL of preimmune serum were used in control reactions. Incubations with serum were overnight at 4 °C. Immune complexes were collected by the addition of 120μl of prewashed SAC as described by Bentlage and Chomyn. 30 μL of each sample were analyzed by SDS-PAGE as described previously.

III. Results

A. Construction of OCTuCOII Transgene

PCR was used to amplify the OCT leader and rat COII sequences from plasmids pOCT and pDR540, respectively. The two PCR products were spliced together in a subsequent PCR reaction. In each case, 10% of the PCR product was electrophoresed through a 1% agarose gel, Figure 3. The OCT leader sequence is 96 bp, and amplification with primers OCT fwd and OCT rev should produce a product of 130 bp; this product can be seen in lane 2 of Figure 3A. The uCOII sequence is 684 bp, and the expected size of the PCR product generated by amplification with COII fwd and COII rev primers is 700 bp, as seen in lane 2 of Figure 3B. The 800 bp transgene product is seen in Figure 3C, lane 2.

B. Subcloning of OCTuCOII Transgene

The OCTuCOII transgene was cloned into pCRScript™AmpSK(+) and pLXSN vectors for expression *in vitro* and in cell lines. Agarose gels in Figure 4 confirm that the 800 bp transgene insert is present in both plasmids, and can be removed by digestion with *Kpn*I and *Mlu*I (restriction sites engineered into primers OCT fwd and COII rev, respectively). The correct orientation of the transgene in the pLXSN vector was confirmed by restriction analysis (not shown).

To confirm the sequence of the OCTuCOII transgene, the PCRScript™ vector containing the insert was sequenced. Samples were prepared and sent for sequencing at the MOBIX Centre at McMaster University, according to sample submission guidelines. Sequencing was performed using the universal and reverse primers. Sequencing results

confirmed the OCT and uCOII sequences were correct, and the two genes were successfully spliced together in frame.

C. Retroviral Infection of C2C12 and NIH3T3 Cell Lines

Infectious virus stock was obtained by transfecting GP+E-86 packaging cells with the pLXSN retroviral vector containing the transgene, and was used for infection of C2C12 and NIH3T3 cell lines. After selection in G418, 24 clones of each cell type were picked. Of the 24 clones, 11 NIH3T3 clones and 15 C1C12 clones survived, and were maintained for analysis of mRNA and protein expression.

D. Expression of mRNA From Transgene

To assess mRNA expression from the transgene, it was necessary to distinguish between rat COII (ie. transgene) mRNA and endogenous mouse COII mRNA. This was difficult using Northern blots since the two genes are 84% homologous. Instead, an RT-PCR strategy was used. RT-PCR products (252 bp) were digested with the restriction enzyme *Hinf*I. *Hinf*I cuts the amplified region of rat COII twice, producing fragments of 18 bp and 234 bp. Digestion of the amplified region of mouse COII produces the same 18 bp fragment, but the 234 bp fragment is further digested to produce 167 bp and 67 bp fragments. When *Hinf*I digestion products are electrophoresed on an acrylamide gel, it should be possible to separate the fragments and therefore distinguish between the two genes. This method was evaluated by using different percentages of mouse and rat COII DNA as templates in the PCR reactions described in the strategy. PCR products were digested with *Hinf*I, and the acrylamide gel is shown in Figure 5. The 234 bp, 167 bp, 67 bp and 18 bp fragments can be identified on the gel.

The RT-PCR technique was used to screen the 11 NIH 3T3 and 15 C2C12 clones infected with the transgene (Figure 6). For each sample, a control reaction was performed,

in which RNA was added directly to PCR reactions. No signal was detected on acrylamide gels from the control reactions, indicating there was no DNA contamination of the RNA samples. In all clones analyzed, mRNA was expressed from the transgene, as indicated by the presence of the 234 bp fragment.

The intensity of the bands was quantitated, and mRNA expression levels from the transgene relative to expression of endogenous mouse COII nRNA were determined for each clone (Table 1). For NIH3T3 clones, rat COII mRNA levels (expressed from transgene) were between 1.4 and 6.7 X higher than endogenous mouse COII mRNA levels. Rat COII mRNA levels in C2C12 cells were lower, ranging between 0.5 and 3.7 X endogenous mouse COII mRNA levels.

E. Expression of COII Protein From Transgene

To detect expression of COII protein, an antibody that recognizes rat COII protein was required. A synthetic peptide corresponding to the C-terminal 11 amino acids of rat COII was used to raise polyclonal antibodies in rabbits. This region of the rat COII protein is identical to the mouse COII protein. Both preimmune and immune sera were used to probe Western blots containing mouse liver mitochondrial protein to test for the presence of rat COII antibodies (Figure 7). The rat COII peptide has a molecular mass of 25.9 kD. On the Western blot probed with immune serum (Figure 7B), a band is visible just below the 31 kDa molecular mass marker, and this band is not detectable on the blot probed with preimmune serum (Figure 7A). Thus, the immune serum contained antibodies that could detect mouse COII protein. To test the specificity of the polyclonal antibody, immune serum was incubated with either a nonspecific peptide, or the 11 amino acid COII peptide, prior to probing of Western blots (Figure 8). Incubation with the COII peptide prevented binding of the antibody to the membrane (Figure 8B). However, incubation with the nonspecific peptide did not prevent binding of the antibody to the blot (Figure 8A).

Since the antibody recognizes both the rat and mouse COII proteins, and the transgene was expressed in mouse cell lines, it was not possible to distinguish between expression of COII protein from the transgene and endogenous mouse COII expression directly on Western blots. To obviate this problem, EtBr, which specifically inhibits replication and transcription of mtDNA, was used. Previous experiments on the stability of COII transcripts in NIH3T3 and C2C12 cells showed that after growth in EtBr for 24 hours, no COII transcripts are detectable (data not shown). This means that no new COII protein can be made. Thus, over time, COII protein levels would be expected to decrease. In this way, expression of endogenous COII protein could be "turned off" and expression from the transgene should be detectable on Western blots. To confirm that endogenous COII protein levels could indeed be abolished, C2C12 and NIH3T3 cells were grown in the presence of EtBr for one week. Cells were harvested every 24 hours and levels of COII protein were evaluated on Western blots (Figure 9). The Western blots confirm that after 5 days in EtBr for NIH3T3 cells (Figure 9A) and after 1 day in EtBr for C2C12 cells (Figure 9B), no COII protein can be detected by our antibody.

For each cell type, the two clones showing the highest levels of mRNA expression were grown in EtBr for 7 days. COII protein levels were assessed by Western blot (Figure 10). No protein was detected in either of the NIH 3T3 clones, or in C2C12 clone 7. A faint band at approximately 33 kD can be seen in the lane representing C2C12 clone 10.

F. *In vitro* Transcription and Translation of Transgene

Since no substantial protein expression from the transgene was observed in infected cells, the construct was transcribed and translated *in vitro*. Translation products were analyzed directly by SDS PAGE, Figure 11A. There are a large number of bands in lane S, representing translation products from sense mRNA. A specific band representing the recombinant protein was expected at approximately 30 kDa; no band is seen. The

background is much lower in lane AS, representing products of translation of antisense mRNA. In case the COII protein was translated, but could not be detected due to high background levels, translation products were immunoprecipitated with immune serum (Figure 11B). No protein was specifically immunoprecipitated by the antibody and not by the preimmune serum (compare Ab lanes to P lanes).

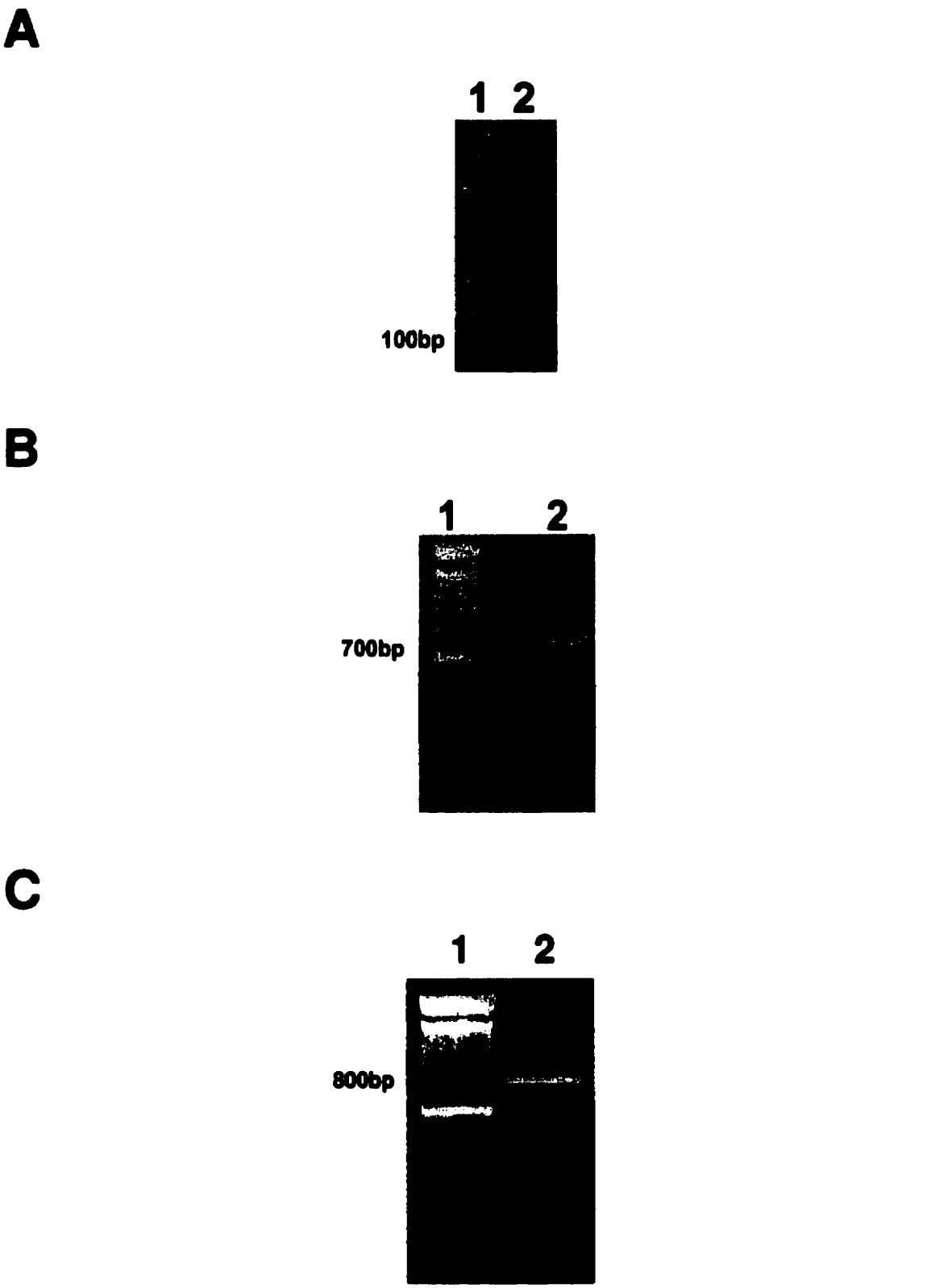

Figure 3: Agarose gel analysis of PCR products. A: Amplification of OCT presequence from pOCT. B: Amplification of rat COII coding sequence from pDR540. C: SOEing of OCT presequence to COII. Lane 1: 100 bp ladder; lane 2: 10% of respective PCR product.

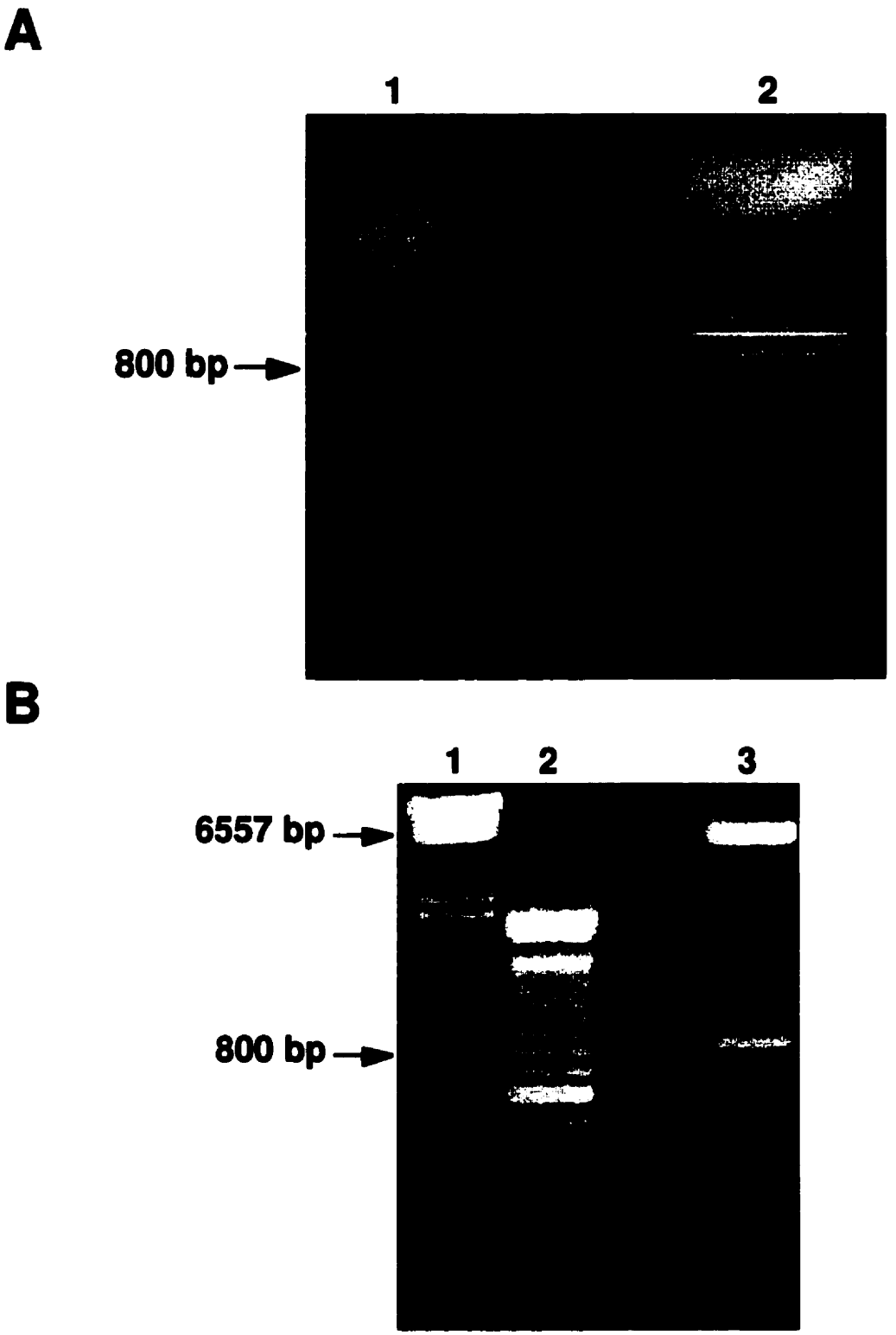

Figure 4: *Kpn*I and *Mlu*I restriction analysis of pCRScript (A) and pLXSN (B) vectors containing COII transgene insert. A. Lane 1: 100 bp ladder; lane 2: digestion products. B. Lane 1: λ/HindIII ladder; lane 2: 100bp ladder; lane 3: digestion products.

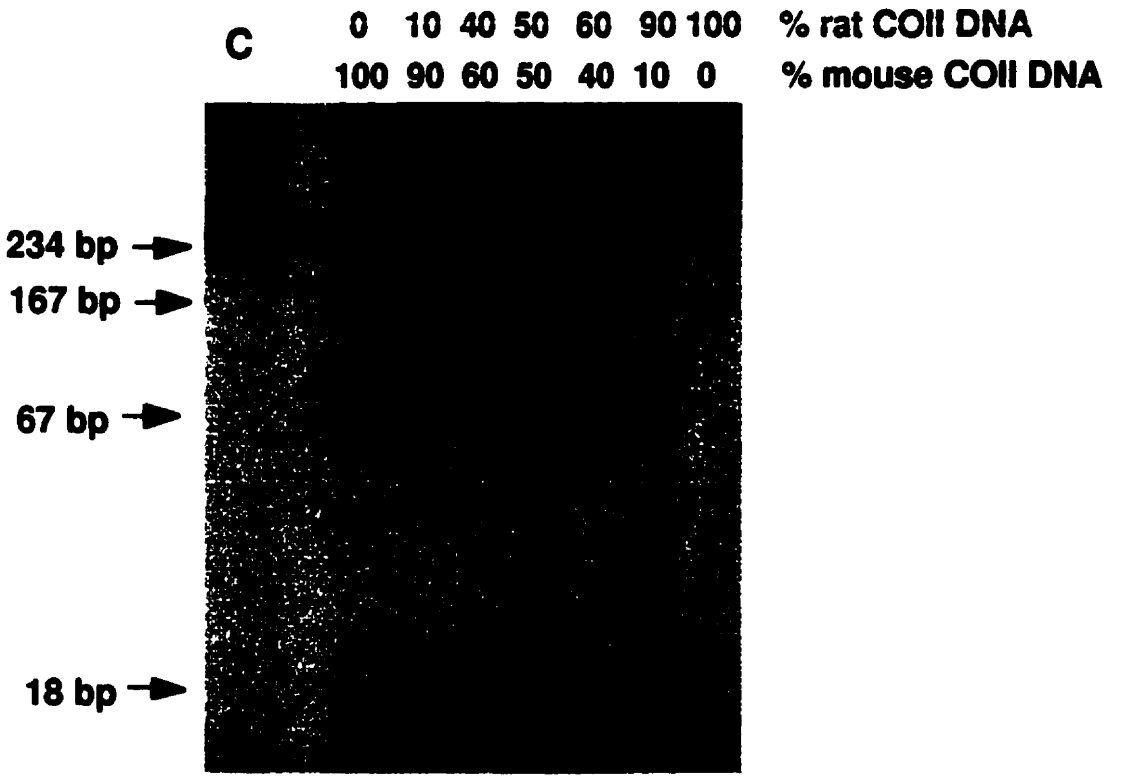

Figure 5: Assessment of RT-PCR strategy. Different percentages of rat and mouse COII DNA were used as templates in PCR reactions. Radiolabeled PCR products were digested with *Hinf*I and run on an acrylamide gel. C: amplified mouse COII DNA (undigested).

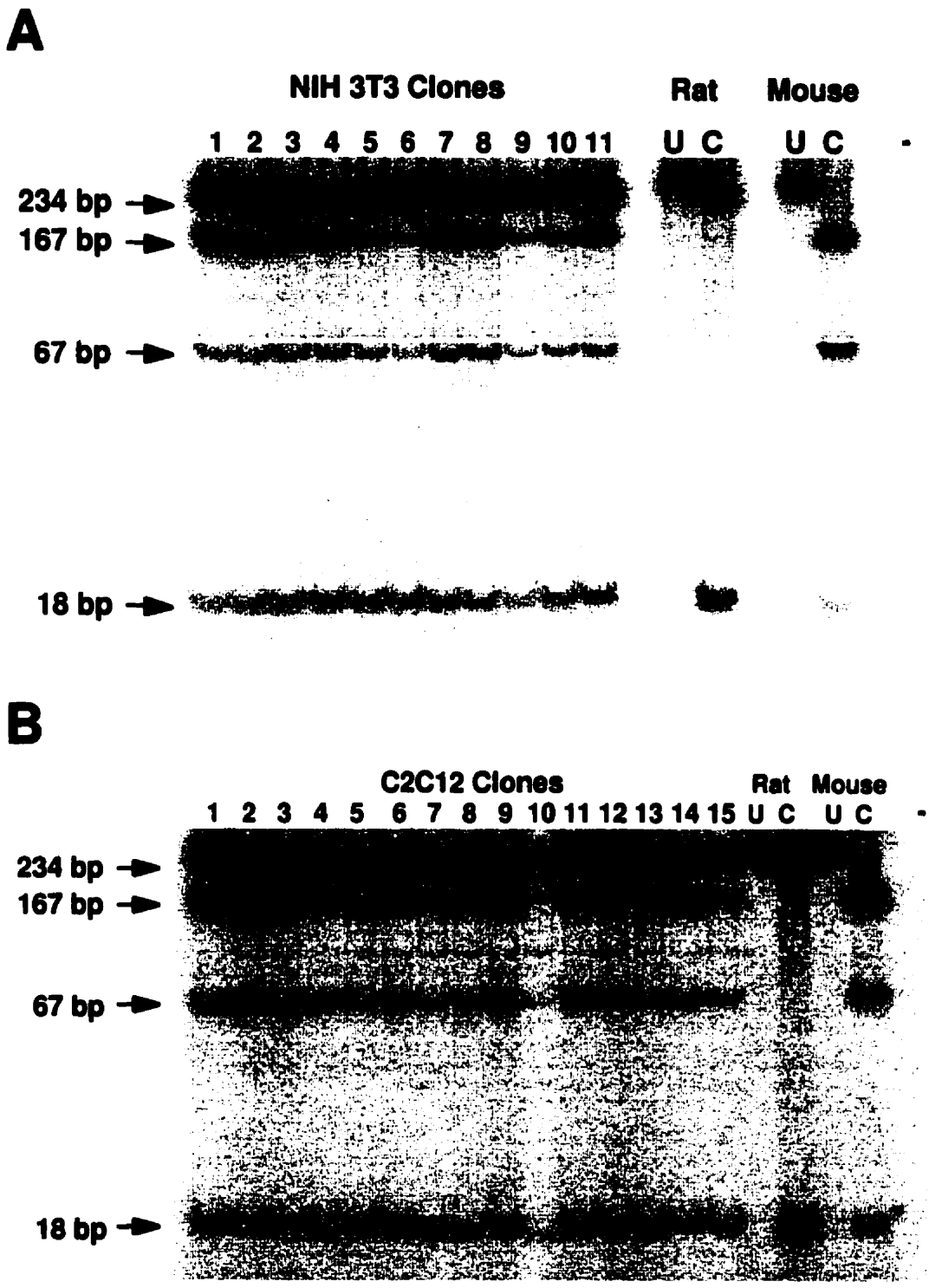

Figure 6: RT-PCR analysis of COII mRNA expression in infected mouse cells. A: NIH3T3 clones. B: C2C12 clones. Radiolabeled PCR products were digested with *Hinf*I and run on acrylamide gels. Rat and mouse DNA were used as control templates in PCR reactions and products were either run directly on gels (U), or digested with *Hinf*I (C) prior to electrophoresis. -: no template added to PCR reaction.

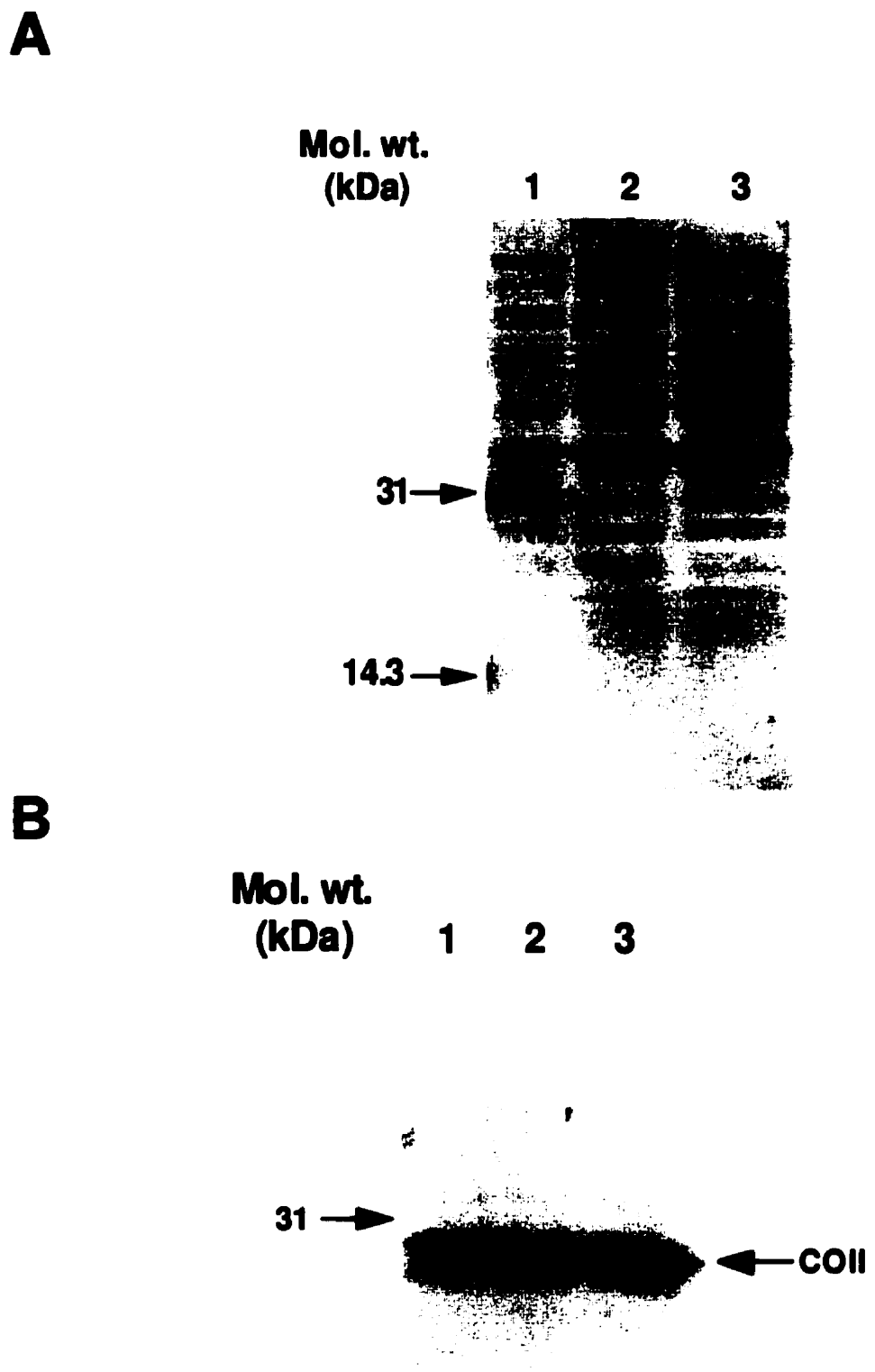

Figure 7: Presence of COII antibody in rabbit serum. 25μg (lane 1), 50μg (lane 2) and 75μg (lane 3) of mouse liver mitochondrial protein were analyzed on Western blots. Blots were probed with preimmune serum (A), or immune serum (B).

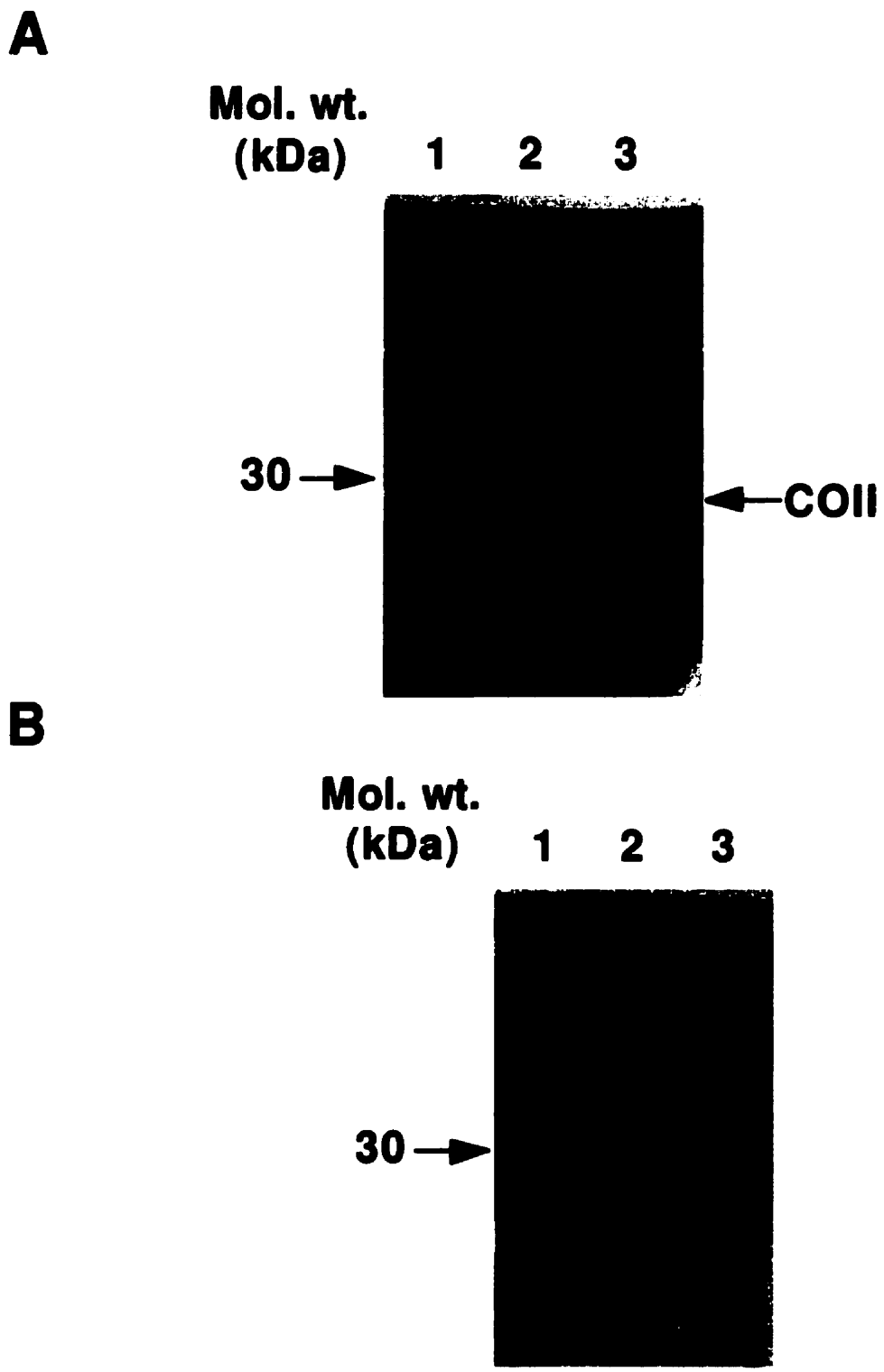

Figure 8: Characterization of rat COII antibody. 25μg (lane 1), 50μg (lane 2) and 75μg (lane 3) mouse liver mitochondrial protein were analyzed on Western blots. Immune serum was incubated with either a nonspecific peptide (A), or the specific COII peptide used in generation of the antibody (B), prior to probing of blots.

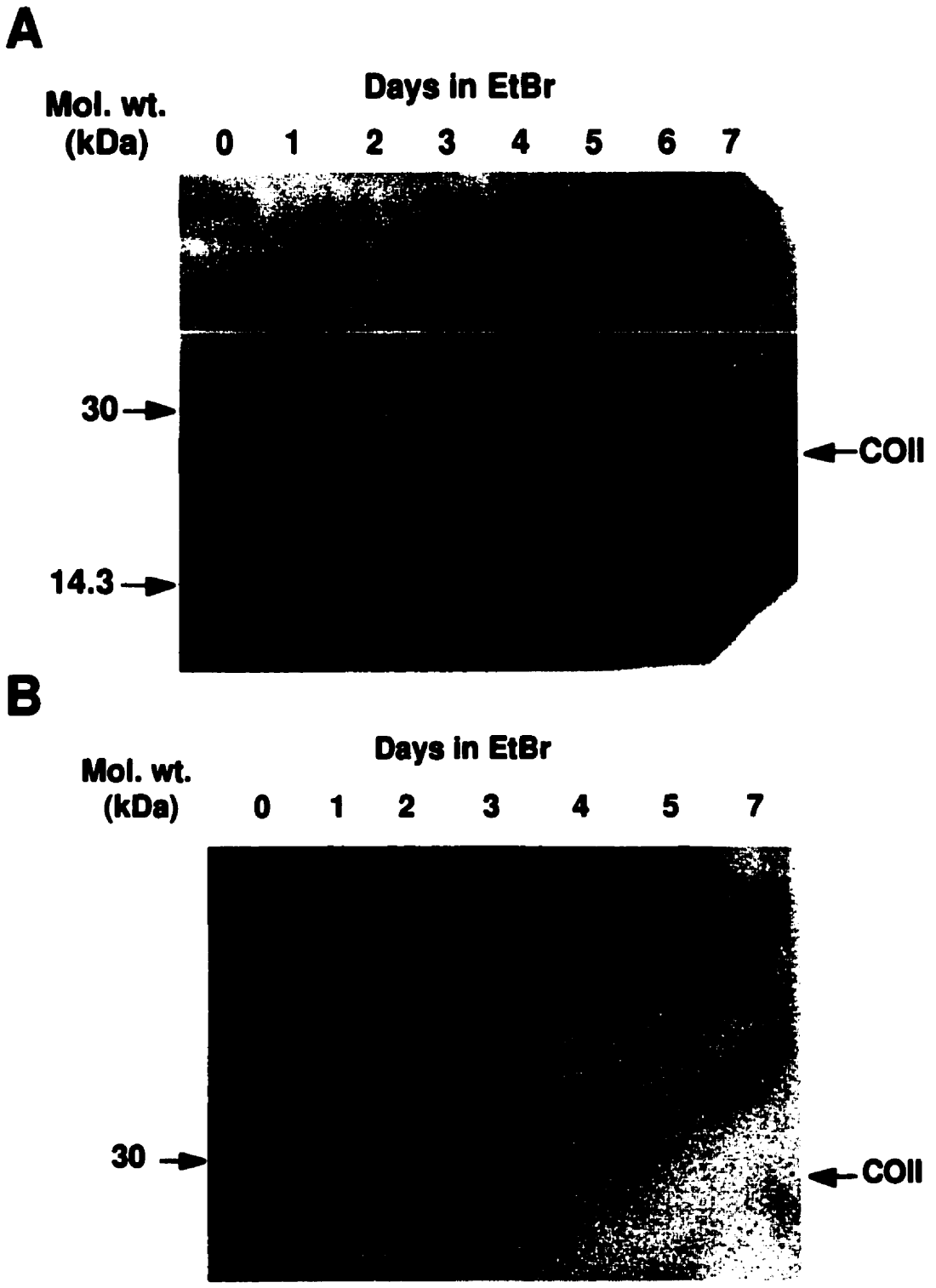

Figure 9: Western analysis of endogenous COII protein levels in NIH 3T3 (A) and C2C12 (B) cell lines. Cells were grown in the presence of ethidium bromide and harvested every 24h for one week. 50 μg total cellular protein was loaded in all lanes. Western blots were probed with immune serum.

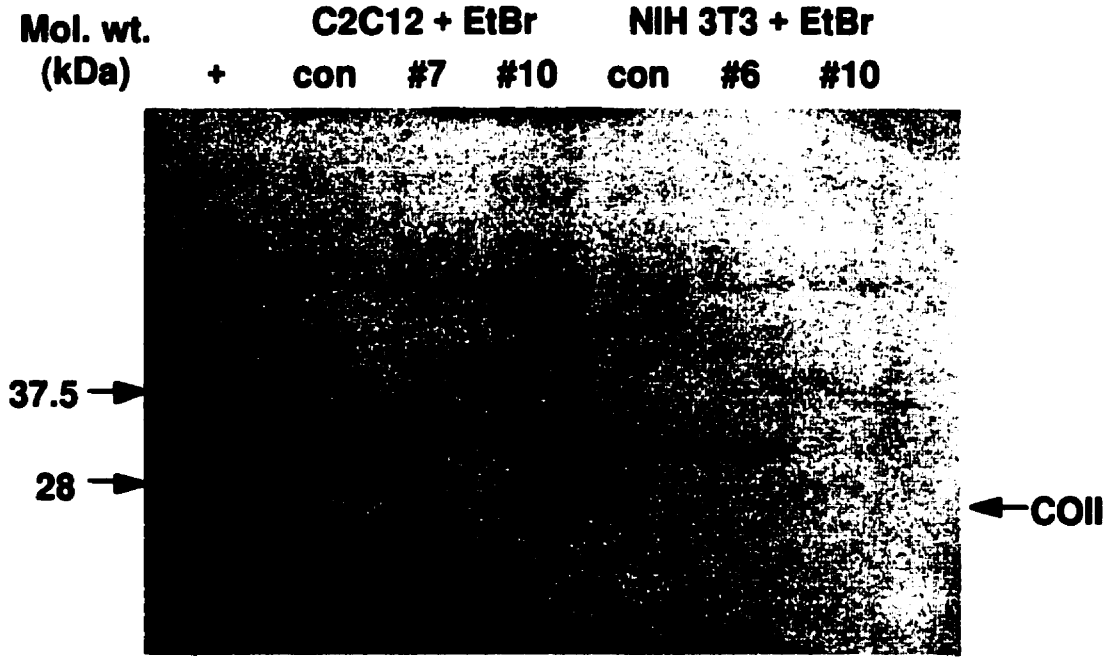

Figure 10: Expression of COII protein from transgene in infected cells. Infected C2C12 clones (7 and 10) and NIH3T3 clones (6 and 10), as well as control cells (con) were grown in ethidium bromide for one week. 50 μg total cellular protein was analyzed on Western blot. Blot was probed with immune serum. +: C2C12 cells not exposed to ethidium bromide.

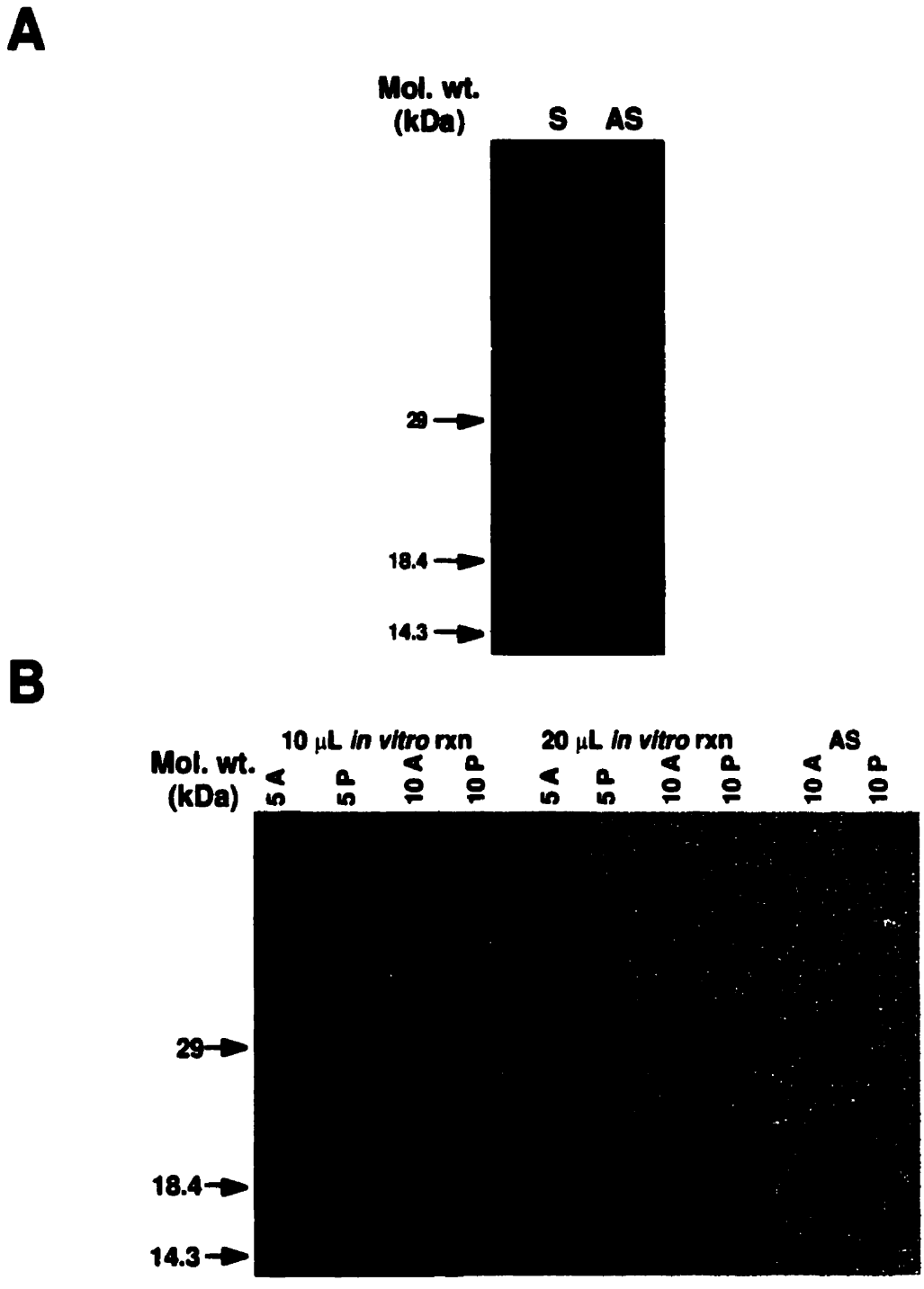

Figure 11: *In vitro* expression of rat COII transgene. SDS PAGE analysis of *in vitro* transcription/ translation reactions before (A) or after (B) immunoprecipitation with COII polyclonal antibody. A. S: translation products from sense RNA; AS: translation products from antisense RNA (control). B. 10 μL or 20 μL of *in vitro* translation products were immunoprecipitated with 5μL (5A) or 10μL (10A) of immune serum, or 5μL (5P) or 10 μL (10 P) of preimmune serum. AS: immunoprecipitation of translation products from antisense mRNA.

A

Clone	Relative expression of rat versus mouse COII mRNA
1	2.1
2	2.2
3	2.4
4	2.4
5	3.1
6	6.7
7	1.6
8	1.4
9	4.7
10	4.5
11	2.6

B

Clone	Relative expression of rat versus mouse COII mRNA
1	1.2
2	0.9
3	0.7
4	1.6
5	2.2
6	1.0
7	2.8
8	1.4
9	0.5
10	3.7
11	1.2
12	1.7
13	1.9
14	0.5
15	2.5

Table 1: Relative levels of mRNA expression from rat COII transgene and endogenous mouse COII gene. A. Infected NIH3T3 clones. B. Infected C2C12 clones. Intensity of bands on RT-PCR gels (Figure 6) was quantitated using Image Quant Software. Ratios of expression levels wre calculated by dividing the intesity of the 234 bp band (representing rat COII) by the sum of the intensities of the 167 and 67 bp bands (representing mouse COII).

IV. Discussion

A. Previous Experiments and Supporting Evidence

Previous experiments have demonstrated that mitochondrial proteins can be expressed outside the mitochondrion and subsequently targeted and imported back into the organelle both *in vitro* and *in vivo*.

In 1986, Philip Nagley and David Gearing expressed subunit 8 of yeast mitochondrial F_1F_0-ATPase (encoded in the mitochondrial genome and normally synthesized in the matrix), fused to the N-terminal targeting sequence of the *N. crassa* ATPase subunit 9 protein *in vitro* (Gearing and Nagley, 1986). The recombinant protein was successfully imported into isolated yeast mitochondria and the presequence cleaved. Similar results were obtained with subunit 9 of yeast mitochondrial ATPase (Farrell et al., 1988). Nagley and his colleagues later demonstrated the subunit 8 protein fused to *N. crassa* ATPase subunit 9 presequence could be efficiently imported, processed and incorporated into the ATPase complex of isolated mitochondria from yeast experimentally depleted of the subunit 8 protein (Law et al., 1990). Similar experiments have been done with portions of the COII protein; the N-terminal 74 amino acids of yeast COII, which consists of the N-terminal tail and the first transmembrane domain of the protein, fused to the mouse dihydrofolate reductase gene and linked to the matrix targeting sequence of ATPase subunit 9 has also been expressed *in vitro* and imported into isolated yeast mitochondria (Herrmann et al., 1995).

In vivo studies of import of mitochondrial proteins expressed outside the organelle have also employed subunit 8 of ATPase. Both *S. cerevisiae* and *Aspergillus nidulans* subunit 8 peptides, when fused to the N-terminal presequence from *N.crassa* ATPase

subunit 9, rescued a respiration defect when expressed in yeast strains deficient in subunit 8 (Straffon et al., 1994; Law et al., 1988).

Throughout eucaryote evolution, gene transfer is thought to have relocated the majority of genes for mitochondrial proteins from the organellar to the nuclear genome, and only a small number of genes for inner membrane proteins remain in mtDNA (Fox 1983). The gene for COII is consistently encoded in mtDNA in most organisms. However, the COII gene has been functionally transferred to the nucleus during flowering plant evolution. COII gene transfer is estimated to have occurred between 60 and 200 million years ago (Nugent and Palmer,1991). Many legumes have COII genes in both nuclear and mitochondrial genomes; however, in mung bean and cowpea, COII gene transfer is complete and the gene is no longer present in the mitochondrial genome. Northern blot experiments show no evidence of simultaneous expression of COII from both mitochondrial and nuclear genes (Nugent and Palmer), and with the exception of garden pea, mRNA is expressed from the nuclear gene in all cases. The COII gene transfer is proposed to occur through an RNA intermediate and involves the acquisition of a mitochondrial targeting sequence (Nugent and Palmer, 1991). In plants, mitochondrial mRNAs are edited and the transferred COII gene more closely resembles the edited mitochondrial COII mRNA than the gene encoding mRNA (Nugent and Palmer, 1991). Since mammalian mitochondrial mRNAs are not edited, we would expect that the addition of a leader sequence would be sufficient to direct the protein to mitochondria, providing it had been recoded into the universal genetic code.

B. Discussion of Present Results

The presequence used to direct COII protein to the mitochondrial matrix was that of rat OCT. This presequence was shown to direct the bacterial cytosolic enzyme asparagine synthetase to the mitochondrial matrix *in vitro* (Nguyen et al., 1986). The recoded rat COII sequence was created by oligonucleotide-directed site-specific mutagenesis; in total, 18

codons were changed (Cao et al., 1991). The two genes were spliced directly together, and the reading frame was maintained.

The results shown in Figure 3, along with the sequencing data, confirm the OCT leader and COII sequences were successfully amplified from the appropriate vectors and spliced together as intended.

Subcloning of the OCTuCOII transgene into the pLXSN retroviral vector allowed infectious retroviral stock to be collected and used for infection of mouse cell lines. Infection of the two mouse cell lines was very efficient, and 26 clones were picked and maintained in culture.

When different ratios of rat uCOII and mouse COII DNA were used as templates in PCR reactions, *Hinf*I digestion of the products allowed the two genes to be distinguished on an acrylamide gel (Figure 5). Further, the intensity of the 234 bp band compared to the combined intensities of the 167 bp and 67 bp bands gives an idea of the relative level of mRNA expression from each gene. These results confirm the RT-PCR strategy can be used to evaluate mRNA expression from the OCTuCOII transgene, compared to endogenous expression. High levels of mRNA expression from the transgene were detected in both C2C12 and NIH3T3 cells infected with the OCTuCOII transgene. Expression levels were higher in fibroblasts than in myoblasts. It is possible that the transcript is less stable in C2C12 cells, and steady-state levels of the message are lower.

The polyclonal antibody generated by injecting rabbits with the synthetic COII peptide appears to specifically detect COII (Figure 8A), and was not present in preimmune serum (Figure 7A). Results of Western blot experiments using mouse liver mitochondrial protein indicate the COII antibody cross-reacts with mouse COII; this was expected since the amino acid sequences of the two proteins are 98.7% homologous. It was therefore necessary to differentiate between protein expression from the transgene, and expression of the endogenous mouse protein.

Endogenous COII protein expression was turned off using EtBr, which specifically inhibits replication and transcription of mtDNA without affecting nuclear DNA. Experiments on ρ^0 cells, have shown that the expression of nuclear-encoded subunits of COX is unaffected by the lack of mitochondrial-encoded subunits (Nijtmans et al., 1995). Therefore, treatment with EtBr was not expected to affect expression of the transgene. The results in Figure 9, confirm that after several days of growth in the presence of EtBr, COII protein levels decrease in both cell types, and after 7 days of exposure to EtBr, no protein is present. The 7 day time course was chosen for screening of the infected clones to ensure endogenous protein levels would be undetectable, and any COII protein detected could be attributed to translation of the transgene. For each cell type, the two clones showing the highest levels of mRNA expression were analyzed. Considering the difference between mRNA expression from the transgene and endogenous mRNA COII expression, protein was expected to be translated from the transgene. In the first lane of the Western blot shown in Figure 10 (lane +), the band just below the 28 kDa marker represents endogenous COII protein in C2C12 cells. Unexpectedly, no protein was detected by the COII antibody for either of the NIH3T3 clones, or for C2C12 clone 7. A faint band at approximately 32 kDa is present in the lane representing C2C12 clone 10. This could represent the OCTuCOII recombinant protein. The increased size indicates the presequence is still present and the protein is not successfully targeted to the matrix and processed (the OCT leader peptide is 32 amino acids in length, and would be expected to increase the mass of the recombinant protein by approximately 3 kDa). However, a band at this size is also visible in the control lanes; in addition, the band is extremely faint relative to expression of endogenous COII protein, particularly when the difference in mRNA expression between the two is taken into account. Thus, although it is possible to detect high levels of mRNA expression from the transgene, it is not possible to detect significant levels of COII protein.

These data suggest that either the COII protein is unable to be translated from the OCTuCOII transgene, or that the protein is translated, but is unstable. To assess the ability

of the transgene to be translated, it was expressed in a rabbit reticulocyte lysate system in which transcription and translation are coupled. A protein of approximately 30 kDa, representing the recombinant protein, was expected to be translated. However, SDS PAGE analysis of translation products showed a large number of proteins produced by translation of sense mRNA from the construct (Figure 11A). These bands are specific to translation of the transgene since they are not present in the lane representing translation of the antisense RNA (Figure 11A). The presence of these bands suggests that initiation of protein synthesis may be leaky; that is, that translation may begin at methionine codons other than the initiation AUG codon. Inappropriate initiation of protein synthesis would result in protein products of a variety of sizes since truncated protein products would be produced if the reading frame were maintained, and protein products of larger molecular masses would be produced if the reading frame were shifted causing readthrough of the stop codon.

The translation products were then immunoprecipitated with the polyclonal antibody to increase the sensitivity of detection. The procedure used, published by Bentlage and Chomyn (1996), was developed specifically for immunoprecipitation of human mitochondrial translation products with peptide-specific antibodies. Further, antibodies directed against the C-terminus of human COII have been shown to immunoprecipitate the entire COX complex (Mariottini et al., 1986). The polyclonal rat COII antibody was therefore expected to immunoprecipitate COII protein. However, no protein at the molecular mass of COII was immunoprecipitated specifically by the immune serum but not by the preimmune serum. The results of the immunoprecipitations further suggest that the recombinant protein is not efficiently translated from the transgene construct. Therefore, the most likely explanation for the lack of protein detected in the infected cells is inefficient initiation of translation of the transgene mRNA. However, there are several other possible explanations.

It is possible the recombinant protein is translated, but is unstable in the cytosol. In yeast, the factors MSS2 and Sco1 are required for accumulation of COII (and COI) protein (Simon et al., 1995; Krummeck and Rodel, 1990). In yeast strains lacking these protein factors, no COII protein is detectable on Western blots; however, COII mRNA levels are unaffected in the mutant strains. This is similar to what was observed in the cells infected with the transgene, where mRNA levels were high, but no COII protein was detectable. Again, if factors like MSS2 and Sco1 were required for the stability of mammalian COII protein, they would not be present in the cytosol, and although the protein would be translated, it would be unstable and would not accumulate.

COII protein is normally synthesized in the matrix, and is inserted into the mitochondrial inner membrane. Whether membrane insertion is a co- or post-translational event remains controversial. Assuming COII is inserted into the membrane during or shortly after translation, stability of the protein may be dependent on either the presence of membranes, or on membrane insertion. Support for this hypothesis comes from experiments performed by Shelagh Ferguson-Miller and colleagues using the recoded rat COII gene (Cao et al., 1991). The group found increased COII protein levels when canine microsomal membranes were added to *in vitro* transcription/translation reactions. The data suggest that the presence of membranes may increase the stability of COII protein.

Another possibility is that the protein is undetectable by the polyclonal antibody synthesized. The rat COII protein sequence contains two glycosylation sites; one is composed of the amino acids asparagine, histidine and serine at positions 303, 304 and 305, respectively, and the second consists of asparagine, tryptophan and serine residues at positions 331, 332 and 333, respectively. For proteins translated on cytosolic ribosomes, these amino acid sequences are sites for N-glycosylation. The presence of these signals could therefore cause the protein to be glycosylated. Glycosylation of the protein could render it undetectable by the polyclonal antibody, particularly since the second glycosylation site is within the C-terminal region used to generate the antibody.

Alternatively, glycosylation may cause the protein to be degraded. However, evidence from COII expression in cowpea suggests the protein is unlikely to be glycosylated. As mentioned previously, in cowpea, the gene for COII has been functionally transferred to the nucleus. The cowpea cDNA sequence contains two glycosylation sites, but is not glycosylated. Obviously then, the presence of the mitochondrial targeting sequence is sufficient to prevent the protein from being glycosylated, and effectively targets it to the mitochondrion, where it is imported. One would therefore expect the attachment of the OCT presequence to rat COII to be sufficient to target the protein to mitochondria, and prevent glycosylation. To ensure that glycosylation of the recombinant protein was not preventing detection with the COII antibody, products of *in vitro* translation reactions were treated with glycopeptidase F, as described by Shelagh Ferguson-Miller and colleagues (Cao et al., 199), followed by immunoprecipitation. No difference immunoprecipitation products was observed (data not shown), suggesting glycosylation of the recombinant protein was not affecting it's detection.

C. Future Research

The results presented and discussed in this thesis suggest it is not possible to express rat COII protein from a nuclear transgene. Further experiments can be done to investigate whether this is due to the construct itself, or due to other factors, such as those mentioned above.

The most obvious follow-up experiment would be to determine whether the efficiency of translation from the construct could be increased. In 1987, M. Kozak compared several hundred mammalian mRNA sequences and discovered a consensus sequence for initiation of translation in higher eukaryotes (Kozak,. 1987). The consensus sequence consists of up to 10 bases upstream of the initiation codon, as well as a guanine immediately following the AUG codon (ie. (GCC)GCCAGCCAUGG). This consensus sequence could be engineered into the OCTuCOII transgene and would likely increase the

efficiency of translation. Translation of the construct could be investigated in the *in vitro* transcription/translation system initially. If the recombinant protein were translated in the cell-free system, expression could then be assessed in cell lines.

If re-engineering of the construct did not increase expression of the protein, further experiments could be done to investigate why the protein cannot be detected. Since COII expression is from a nuclear gene in cowpea, interesting data could be obtained by using cowpea COII cDNA in the *in vitro* transcription/translation system. For example, the cowpea COII leader sequence could be attached to the rat uCOII gene; the cowpea COII and recombinant rat COII genes could be expressed using the *in vitro* transcription/translation system, or even in cell lines. If the cowpea protein were expressed, but not the rat protein, this would suggest the rat COII protein cannot be expressed from a nuclear transgene, although the plant gene can. Conversely, the OCT leader sequence could be attached to cowpea COII coding sequence. Expression studies would indicate whether this presequence could direct the plant COII protein to mitochondria. For example, it is possible the COII protein requires a particular (specialized) leader sequence for targeting and import. This was the case for subunit 8 of yeast mitochondrial ATPase which could be directed to mitochondria by the targeting sequence from subunit 9 of *N. crassa* ATPase, but not by that from yeast cytochrome oxidase subunit VI (Gearing and Nagley, 1986). The authors proposed this was due to a strong tendency for the subunit 8 protein to embed itself in the mitochondrial membrane which interfered with its ability to be properly imported; this could also be the case for rat COII, as previously mentioned. Thus, the OCT presequence my not be sufficient for targeting and import, but the plant presequence may be.

To determine whether the rat COII protein is glycosylated and therefore undetectable by the polyclonal antibody, *in vitro* translation reactions could also be treated with a glycopeptidase followed by immunoprecipitation. Detectable protein after glycopeptidase treatment would indicate glycosylation of the protein product.

The dependence of COII translation or stability on mitochondrial matrix factors could be investigated by adding matrix extracts to *in vitro* transcription/translation reactions. Matrix extracts could be made from mitochondria isolated from cells treated with EtBr, so the matrix would not contain COII mRNA. The presence of COII protein after the addition of matrix extracts would indicate the requirement of matrix proteins for translation or stability of COII. Experiments could then be done to further isolate the critical component(s).

The results presented in this thesis indicate that expression of COII protein from a nuclear transgene is complex, and it may not be possible to generate a dominant negative mutation in COX. Creating transgenic mouse models for mitochondrial diseases will be challenging. The experimental procedures and molecular tools developed throughout this work will be useful for future experiments. Perhaps most importantly, the results presented in this thesis have inspired new questions to be asked, which will fuel future research on the expression of mammalian mitochondrial proteins from nuclear transgenes.

V. References

Alconada, A., Kubrich, M., Moczko, M., Honlinger, A., Pfanner, N. 1995. The mitochondrial receptor complex: the small subunit Mom8b/Isp6 supports association of receptors with the general insertion pore and transfer of preproteins. Mol. Cell Biol. 15, 6196-205.

Anderson, S., Bankier, A.T., Barrell, B.G., de Bruijn, 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. Sequence and organization of the human mitochondrial genome. Nature 290, 457-65.

Attardi, B., Attardi, G. 1967. A membrane-associated RNA of cytoplasmic origin in HeLa cells. Proc. Natl. Acad. Sci. USA 58, 1051-8.

Bauer, M.F., Sirrenberg, C., Neupert, W., Brunner, M. 1996. Role of Tim23 as voltage sensor and presequence receptor in protein import into mitochondria. Cell 87, 33-41.

Beinert, H.E., Griffiths, D.E., Wharton, D.C., Snads, R.H. 1962. Properties of the copper associated with cytochrome c oxidase as studied by paramagnetic resonance spectroscopy. J. Biol. Chem. 237, 2337-46.

Benda, C. 1898. Uber die spermatogenese der vertebrateen und hoherer evertebraten. II. Theil: Die histiogenese der spermien. Arch. Anal. Physiol (Physiol Abt), 393-8.

Bentlage, H.C.M., Chomyn, A. 1996. Immunoprecipitation of human mitochondrial translation products with peptide-specific antibodies. Meth. Enz. 264, 218-27.

Berthold, J., Bauer, M.F., Schneider, H.-C., Klaus, C., Dietmeier, K., Neupert, W., Brunner, M. 1995. The MIM Complex Mediates Preprotein Translocation across the Mitochondrial Inner Membrane and Couples It to the mt-Hsp70/ATP Driving System. Cell 81, 1085-93.

Bisson, R., Jacobs, B., Capaldi, R.A. 1980. Binding of arylazidocytochrome c derivatives to beef heart cytochrome c oxidase: cross-linking in the high- and low-affinity binding sites.

Blom, J., Dekker, P., Meijer, M. 1995. Functional and physical interactions of components of the yeast mitochondrial inner-membrane import machinery (MIM). Eur. J. Biochem. 232, 309-14.

Bollinger, L., Junne, T., Schatz, G., Lithgow, T. 1995. Acidic receptor domains on both sides of the outer membrane mediate translocation of precursor proteins into yeast mitochondria. EMBO J. 14(24), 6318-26.

Bombelka, E., Richter, F.W., Stroh, A., Kadenbach, B. 1986. Analysis of the Cu, Fe, and Zn contents in cytochrome *c* oxidases from different spcies and tissues by proton-induced X-ray emission (PIXE). Biochem. Biophys. Res. Comm. 140(3), 1007-14.

Boulet, L., Karpati, G., Shoubridge, E.A. 1992. Distribution and threshold expression of the trans-RNA(lys) mutation in skeletal muscle of patients with myoclonic epilepsy and ragged-red fibers (MERRF). Am. J. Hum. Genet. 51, 1187-1200.

Braun, H.P., Schmitz, U.K. 1995. Are the 'core' proteins of the mitochondrial bc1 complex evolutionary relics of a processing protease? Trends Biochem. Sci. 20, 171-5.

Cao, J., Revzin, A., Ferguson-Miller, S. 1991. Conversion of a mitochondrial gene for mammalian cytochrome c oxidase subunit II into its universal codon equivalent and expression in vivo and in vitro. Biochem. 30,2642-50.

Capaldi, R.A. 1990. Structure and function of cytochrome *c* oxidase. Ann. Rev. Biochem. 59, 569-96.

Capaldi, R.A. 1996. The complexity of a respiratory complex. Nature Stuct. Biol. 3(7), 570-4.

Deshaies, R.J., Koch, B.D., Werner-Washburne, M., Craig, E.A., Schekman, R. 1988. A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. Nature, 332, 800-5.

Elvehjem, C.A. 1931. The role of iron and copper in the growth and metabolism of yeast. J. Biol. Chem. 90, 111-32.

Ernster L., Schatz,G. 1981. Mitochondria: A Historical Review. J. Cell Biol. 91(3), 227s-255s.

Ephrussi, B., Hottinguer, H., Tavlitzki, J., 1949. Action de l'acriflavine sure les levures, II etude genetique du mutant 'petite colonie'. Ann. Inst. Pasteur 76, 419-42.

Ewart, G.D., Zhang, Y.Z., Capaldi, R.A. 1991. Switching of bovine cytochrome *c* oxidase subunit Via isoforms in skeletal muscle during development. FEBS Lett. 292(1-2), 79-84.

Farrar, J.A., Lappalainen, P., Zumft, W.G., Saraste, M., Thomson, A.J. 1995. Spectroscopic and mutagenesis studies on the CuA centre from the cytochrome-c oxidase complex of Paracoccus denitrificans. Eur. J. Biochem. 232(1), 294-303.

Farrel, L.B., Gearing, D.P., Nagley, P. 1988. Studies on the import into mitochondria of yeast ATP synthase subunits 8 and 9 encoded by artificial nuclear genes. FEBS Lett. 236(2), 501-5.

Fox, T.D. 1983. Mitochondrial genes in the nucleus. Nature 301, 371-2.

Gearing, D.P., Nagley, P. 1986. Yeast mitochondrial ATPase subunit 8, normally a mitochondrial gene product, expressed *in vitro* and imported back into the organelle. EMBO Journal 5(13), 3651-55

Giles, R.E., Blanc, H., Cann, H.M., Wallace, D.C. 1980. Maternal inheritance of human mitochondrial DNA. Proc. Natl. Acad. Sci. USA 77, 6715-9.

Glick, B.S. 1995. Can Hsp70 proteins act as force-generating motors? Cell 80, 11-14.

Goping, I.S., Millar, D.G., Shore, G.C. 1995. Identification of the human mitochondrial protein import receptor, huMas20p. Complementation of Δmas20 in yeast. FEBS Lett. 373, 45-50.

Grossman, L.I., Shoubridge, E.A. 1996. Mitochondrial genetics and human disease. BioEssays 18, 983-91.

Hachiya, N., Alam, R., Sakasegawa, Y., Sakaguchi, M., Mihara, K., Omura, T. 1993. A mitochondrial import factor purified from rat liver cytosol is an ATP-dependent confomational modulator for precursor proteins. EMBO J. 12, 1579-86.

Hachiya, N., Mihara, K., Suda, K., Horst, M., Schatz, G., Lithgow, T. 1995. Reconstitution of the initial steps of mitochondrial protein import. Nature 376, 705-9.

Haltia, T., Finel, M., Harms, N., Nakari, T., Taitio, M., Wikstrom, M., Saraste, M. 1989. Deletion of the gene for subunit III leads to defective assembly of bacterial cytochrome oxidase. EMBO J. 8, 3571-9.

Haltia, T., Saraste, M., Wikstrom, M. 1991. Subunit III of cytochrome c oxidase is not involved in proton translocation: a site-directed mutagenesis study. EMBO J. 10(8), 2015-21.

Haucke, V., Horst, M., Schatz, G., Lithgow, T. 1996. The Mas20p and Mas70p subunits of the protein import receptor of yeast mitochondria interact via the tetrotricopeptide repeat motif in Mas20p: evidence for a single hetero-oligomeric receptor. EMBO J, 15(6), 1231-7.

Hayashi, J.I., Ohta, S., Kikuchi, A., Takemitsu, M., Goto, Y., Nonaka, I. 1991. Introduction of disease-related mitochondrial DNA deletions into HeLa cells lacking mitochondrial DNA results in mitochondrial dysfunction. Proc. Natl. Aca. Sci. USA 88, 10614-8.

Herrmann, J.M., Koll, H., Cook, R.A., Neupert, W., Stuart, R.A. 1995. Topogenesis of Cytochrome Oxidase Subunit II. Mechanisms of Protein Export from the Mitochondrial Matrix. J. Bio. Chem. 270(45), 27079-86.

Holt, I.J., Harding, A.E., Morgan-Hughes, J.A. 1988. Deletions of muscle mitochondrial DNA in patients with mitochondrial myopathies. Nature 331, 717-9.

Honlinger, A., Bomer, U., Alconada, A., Eckerskorn, C., Lottspeich, F., Deitmeier, K., Pfanner, N. 1996. Tom7 modulates the dynamics of the mitochondrial outer membrane translocase and plays a pathway-related role in protein import. EMBO J. 15(9), 2125-37.

Horst, M., Hilfiker-Rothenfluh, S., Oppliger, W., Schatz, G. 1995. Dynamic interaction of the protein translocation systems of the inner and outer membranes of yeast mitochondria. EMBO J. 14(10), 2293-7.

Horton, R.M. 1993. In vitro recombination and mutagenesis of DNA. SOEing together tailor-made genes, in Methods in Molecular Biology, Vol. 15: PCR Protocols: Current Methods and Applications, ed. B.A. White. Humana Press Inc., Totowa, NJ.

Horwich, A.L., Kalousek, F., Mellman, I., Rosenberg, L.E. 1985. A leader peptide is sufficient to direct mitochondrial import of a chimeric protein. EMBO J. 4(5), 1129-35.

Hurt, E.C., Pesold-Hurt, B., Schatz, G. 1984. The amino-terminal region of an imported mitochondrial precursor polypeptide can direct cytoplasmic dihdrofolate reductase into the mitochondrial matrix. EMBO J. 3(13), 3149-56.

Iwahashi, J., Yamazaki, S., Komiya, T., Nomura, N., Nishikawa, S., Endo, T., Mihara, K. 1997. Analysis of the functional domain of the rat liver mitochondrial import receptor Tom20. J. Biol. Chem. 272, 18467-72.

Iwata, S., Ostermeier, C., Ludwig, B., Michel, H. 1995. Structure at 2.8 Å resolution of cytochrome *c* oxidase from Paracoccus denitrificans. Nature 376, 660-9.

Johnson, M.A., Turnbull, D.M., Dick, D.J., Sherratt, H.S.A. 1983. A partial deficiency of cytochrome *c* oxidase in chronic progressive external ophthalmoplegia. J. Neur. Sci. 60, 31-53.

Kadenbach, B. 1986. Regulation of respiration and ATP synthesis in higher organisms: hypothesis. J. Bioener. Biomemb. 18(1): 39-54.

Keightley, A.J., Hoffbuhr, K.C., Burton, M.D., Salas, V.M., Johnston, W.S.W., Penn, A.M.W., Buist, N.R.M., Kennaway, N.G. 1996. A microdeletion in cytochrome c oxidase (COX) subunit III associated with COX deficiency and recurrent myoglobinuria. Nat. Genet. 12, 410-6.

Keilin, D., Hartree, E.F. 1939. Cytochrome and cytochrome oxidase. Proc. R. Soc. (Lond.) B127, 167-91.

Kelly, M., Lappalainen, P., Talbo, G., Haltia, T., van der Oost, J., Saraste, M. 1993. Two cysteines, two histidines, and one methionine are ligands of a binuclear purple copper center. J. Biol. Chem. 268(22), 16781-7.

Kim, K., Lecordier, A., Bowman, L.H. 1995. Both nuclear and mitochondrial cytochrome *c* oxidase mRNA levels increase dramatically during mouse postnatal development. Biochem. J. 306, 353-8.

King, M.P., Koga, Y., Davidson, M., Schon, E.A. 1992. Defects in mitochondrial protein synthesis and respiratory chain activity segregate with the transfer RNA(leu)(UUR) mutation associated with mitochondrial myopathy, encephalopathy, lactic acidosis, and strokelike episodes. Mol. Cell. Biol. 12, 480-90.

Kozak, M. 1987. At least six nucleotides preceding the AUG initiator codon enhance translation in mammalian cells. J. Mol. Biol. 196(4), 947-50.

Krebs, H.A. 1970. The history of the tricarboxylic acid cycle. Prospect. Biol. Med. 14, 154-70.

Kroneck, P.M., Antholine, W.A., Riester, J., Zumft, W.G. 1989. The nature of the cupric site in nitrous oxide reductase and of CuA in cytochrome *c* oxidase. FEBS Lett. 248(1-2), 212-3.

Kronidou, N.G., Oppliger, W., Bollinger, L., Hannavy, K., Glick, B.S., Schatz, G., Horst, M. 1994. Dynamic interaction between Isp45 and mitochondrial hsp70 in the protein import system of the yeast mitochondrial inner membrane. Proc. Natl. Acad. Sci. USA 91, 12818-22.

Krummeck, G., Rodel, G. 1990. Yeast SCO1 protein is required for a post-translational step in the accumulation of mitochondrial cytochrome c oxidase subunits I and II. Curr. Genet. 18(1), 13-5.

Künkele, K.-P., Heins, S., Dembowski, M., Nargang, F.E., Benz, R., Theiffry, M., Walz, J., Lill, R., Nussberger, S., Neupert, W. 1998. The preprotein translocation channel of the outer membrane of mitochondria. Cell 93, 1009-19.

Lappalainen, P., Watmough, N.J., Greenwood, C., Saraste, M. 1995. Electron Transfer between Cytochrome *c* and the Isolated Cu_A Domain; Identification of Substrate-Binding Residues in Cytochrome *c* Oxidase. Biochemistry 34, 5824-30.

Law, R.H., Farrell, L.B., Nero, D., Devenish, R.J., Nagley, P. 1988. Studies on the import into mitochondria of yeast ATP synthase subunits 8 and 9 encoded by artificial nuclear genes. FEBS Letters 236, 501-5.

Law, R.H., Devenish, R.J., Nagley, P. 1990. Assembly of imported subunit 8 into the ATP synthase complex of isolated yeast mitochondria. Eur. J. Biochem. 188, 421-9.

Liu, X.Q., Bell, A.W., Freeman, K.B., Shore, G.C. 1988. Topogenesis of mitochondrial inner membrane uncoupling protein. Rerouting transmembrane segments to the soluble matrix compartment. J. Cell Bio. 107, 503-9.

Mariottini, P., Chomyn, A., Doolittle, R.F., Attardi, G. 1986. Antibodies against the COOH-terminal undecapeptide of subunit II, but not those against the NH2-terminal decapeptide, immunoprecipitate the whole human cytochrome c oxidase complex. J. Biol. Chem. 261(7), 3355-62.

Matouschek, A., Rospert, S., Schmid, K., Glick, B.S., Schatz, G. 1995. Cyclophilin catalyzes protein folding in yeast mitochondria. Proc. Natl. Acad. Sci. USA 92, 6319-23.

Mayer, A., Nargang, F.E., Neupert, W., Lill, R. 1995. MOM22 is a receptor for mitochondrial targeting sequences and cooperates with MOM19. EMBO J. 14, 4202-11.

Mitchell, P. 1961. Coupling of phosphorylation to electron and hydrogen transfer by a chemiosmotic type of mechanism. Nature 191, 144-8.

Murakami, K., Mori, M. 1990. Purified presequence binding factor (PBF) forms an import-competent complex with a purified mitochondrial precursor protein. EMBO J. 9, 3201-8.

Nass, S., Nass, NMK. Ultramitochondrial fibers with DNA characteristics. J. Cell Biol. 19, 593-629.

Neupert, W., Hartl, F-U., Craig, E.A., Pfanner, N. 1990. How do polypeptides cross the mitochondrial membranes? Cell 63, 447-50.

Neupert W. 1997. Protein Import Into Mitochondria. Ann. Rev. Biochem. 66, 863-917.

Nguyen, M., Argan, C., Lusty, C.J., Shore, G. 1986. Import and processing of hybrid proteins by mammalian mitochondria *in vitro*. J. Bio. Chem. 261, 800-5.

Nijtmans, L.G.J., Spelbrink, J.N., Van Galen, M.J.M., Zwaan, M., Klement, P., Van den Bogert, C. 1995. Expression and fate of the nuclearly encoded subunits of cytochrome-*c* oxidase in cultured human cells depleted of mitochondrial gene products. Biochim. Biophys. Acta 1265, 117-26.

Nugent, J.M., Palmer, J.D. 1991. RNA-Mediated Transfer of the Gene coxII from the Mitochondrion to the Nucleus during Flowering Plant Evolution. Cell 66, 473-81.

Oblad, M., Selin, E., Malmstrom, B., Strid, L., Aasa, R., Malmstrom, B.G. 1989. Analytical characterization of cytochrome oxidase preparations with regard to metal and phospholipid contents, peptide composition and catalytic activity. Biochim. Biophys. Acta 975(2), 267-70.

Ono, H., Tuboi, S. 1988. The cytosolic factor required for import of precursors of mitochondrial proteins into mitochondria. J. Biol. Chem. 263, 3188-93.

Pfanner, N., Douglas, M.G., Endo, T., Hoogenraad, N.J., Jensen, R.E., Meijer, M., Neupert, W., Schatz, G., Schmitz, U.K., Shore, G.C. 1996. Uniform nomenclature for the protein transport machinery of the mitochondrial membranes. Trends Biochem Sci. 21, 51-2.

Poyton, R.O., Trueblood, C.E., Wright, R.M., Farrel, L.E. 1988. Expression and function of cytochrome *c* oxidase subunit isologues. Modulators of cellular energy production? Ann. NY Adad. Sci. 550, 289-307.

Rassow, J., Maarse, A.C., Krainer, E., Kubrich, M., Muller, H., Meijer, M., Craig, E.A., Pfanner, N. 1994. Mitochondrial protein import: biochemical and genetic evidence for interaction of matrix hsp70 and the inner membrane protein MIM44. J. Cell Biol. 127, 1547-56.

Reijnders, L. 1975. The Origin of Mitochondria. J. Mol. Evol. 5, 167-76.

Rospert, S., Junne, T., Glick, B.S., Schatz, G. 1993. Cloning and disruption of the gene encoding yeast mitochondrial chaperonin 10, the homolog of E. coli groES. FEBS Lett. 335, 358-60.

Saraste, M. 1990. Structural features of cytochrome oxidase. Quart. Rev. Biophys. 23, 331-66.

Satoh, M., and Kuroiwa, T. 1991. Organization of multiple nucleoids and DNA molecules in mitochondria of a human cell. Exp. Cell Res. 196, 137-40.

Schlossman, J., Neupert, W. 1995. Assembly of the preprotein receptor MOM72/MAS70 into the protein import complex of the outer membrane of mitochondria. J. Biol. Chem. 270, 27116-21.

Schneider, H.-C., Berthold, J., Bauer, M.F., Dietmeier, K., Guiard, B., Brunner, M., Neupert, W. 1994. Mitochondrial Hsp70/MIM44 complex facilitates protein import. Nature 371, 768-74.

Seligman, A.M., Karnovsky, M.J., Wasserkrug, H.L., Hanker, J.S. 1968. Non-droplet ultrastructural demonstration of cytochrome oxidase activity with a polymerising osmiophilic reagent, diaminobenzidine (DAB). J. Cell. Biol. 38, 1-14.

Shanske, S. et al., 1990. Widespread tissue distribution of mitochodrial DNA deletions in Kearns-Sayre Syndrome. Neurology 40, 24-8.

Shore, G.C., McBride, H.M., Millar, D.G., Steenaart, N.A.E., Nguyen, M. 1995. Import and insertion of proteins into the mitochondrial outer membrane. Eur. J. Biochem. 227, 9-18.

Shoubridge, E.A., Karpati, G., Hastings, K.E. 1990. Deletion mutants are functionally dominant over wild-type mitochondrial genomes in skeletal muscle fiber segments in mitochondrial disease. Cell 62, 43-9.

Simon, M., Séraphin, B., Faye, G. 1995. The nuclear-encoded *MSS2* gene is involved in the expression of the mitochondrial cytochrome-*c* oxidase subunit 2 (Cox2). Biochim. Biophys. Acta 1228, 95-8.

Steffens, G.C., Soulimane, T., Wolff, G., Buse, G. 1993. Stoichiometry and redox behaviour of metals in cytochrome-c oxidase. Eur. J. Biochem. 213(3), 1149-57.

Straffon, A.F.L., Nagley, P., Devenish, R.J. 1994. Rescue of Yeast Defective in Mitochondrial ATP Synthase Subunit 8 by a Heterologous Gene from Aspergillus Nidulans. Biochem. Biophys. Res. Comm. 203(3), 1567-73.

Taanman, J.-W., Herzberg, N.H., De Vries, H., Bolhuis, P.A., Van den Bogert, C. 1992. Steady-state transcript levels of cytochrome c oxidase genes during human myogenesis indicate subunit switching of subunit Via and co-expression of subunit VIIa isoforms. Biochem. Biophys. Acta 1139, 155-62.

Tatuch, Y., Christodoulou, J. Feigenbaum, A., et al. 1992. Heteroplasmic mtDNA mutation (T->G) at 8993 can cause Leigh disease when the percentage of abnormal mtDNA is high. Am. J. Hum. Genet. 50, 852-8.

Thomas, J.W., Puustinen, A., Alben, J.O., Gennis, R.B., Wikstrom, M. 1993. Substitution of asparagine for aspartate-135 in subunit I of the cytochrome bo ubiquinol oxidase of Escherichia coli eliminates proton-pumping activity. Biochemistry 32, 10923-8.

Tsukihara, R., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh, K., Nakashima, R., Yaono, R., Yoshikawa, S. 1995. Structures of Metal Site of Oxidized Bovine Heart Cytochrome *c* Oxidase at 2.8 Å. Science 269, 1069-74.

Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh, K., Nakashima, R., Yaono, R., Yoshikawa, S. 1996. The whole structure of the 13-subunit oxidized cytochrome *c* oxidase at 2.8 Å. Science 272, 1136-44.

van der Oost, J., Lappalainen, P., Musacchio, A., Warne, A., Lemieux, L., Rumbley, J., Gennis, R.B., Aasa, R., Pascher, T., Malmstrom, B.G., Saraste, M. 1992. Restoration of a lost metal-binding site: construction of two different copper sites into a subunit of the *E. coli* cytochrome *o* quinol oxidase complex. EMBO J. 11(9), 3209-17.

Van Kuilenburg, A.B., Muijsers, A.O., Demol, H., Dekker, H.L., Van Beeumen, J.J. 1988. Human heart cytochrome *c* oxidase subunit VIII. Purification and determination of the complete amino acid sequence. FEBS Lett. 240(1-2), 127-32.

Wallace, D.C., Singh, G., Lott, M.T., Hodge, J.A., Schurr, T.G., Lezza, A.M., Elsas, L.J 2d., Nikoskelainen, E.K. 1988. Mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy. Science, 242, 1427-30.

Wikstrom, M.K. 1977. Proton pump coupled to cytochrome c oxidase in mitochondria. Nature 266, 271-3.

Wilmanns, M., Lappalainen, P., Kelly, M., Sauer-Eriksson, E., Saraste, M. 1995. Crystal structure of the membrane-exposed domain from a respiratory quinol oxidase complex with an engineered dinuclear copper center. Proc. Natl. Adad. Sci. USA 92, 11949-51.

Winkler, J.R., Malmstrom, B.G., Gray, H.B. 1995. Rapid electron injection into multisite metalloproteins: intramolecular electron transfer in cytochrome oxidase. Biophys. Chem. 54(3), 199-209.

Yanamura, W., Zhang, Y.Z., Takamiya, S., Capaldi, R.A. 1988. Tissue-specific differences between heart and liver cytochrome *c* oxidase. Biochem. 27(13), 4909-14.

Yoshikawa, S., Shinzawa-Itoh, K., Nakashima, R., Yaono, R., Yamashita, E., Inoue, N., Yao, M., Fei, M.J., Peters Libeu, C., Mizushima, T., Yamaguchi, H., Tomizaki, T., Tsukihara, T. 1998. Redox-coupled crystal structural changes in bovine heart cytochrome c oxidase. Science 280, 1723-9.

Zumft, W.G., Dreusch, A., Lochelt, S., Cuypers, H., Friedrich, B., Schneider, B. 1992. Derived amino acid sequences of the nosZ gene (respiratory N2O reductase) from Alcaligenes eutrophus, Pseudomonas aeruginose and Psuedomonas Stutzeri reveal potential copper-binding residues. Implications for the CuA site of N2O reductase and cytochrome-c oxidase. Eur. J. Biochem. 208(1), 31-40.