CYTOCHROME C OXIDASE:
TISSUE SPECIFICITY OF THE BOVINE ENZYME
AND MOLECULAR BIOLOGY OF HUMAN SUBUNIT VIb

by

Roque Daniel Carrero-Valenzuela

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APPROVED:

Nancy G. Kennaway, D. Phil., Mentor

Professor of Medical Genetics

Michael Forte, Ph.D., Mentor

Associate Professor of Medical Genetics

Scientist, Vollum Institute

John Resko, Ph. D., Chairman, Graduate Council

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D. ABBREVIATIONS

ATP: adenosine triphosphate.

C-terminal: carboxy-terminal.

cDNA: complementary deoxyribonucleic acid.

dATP: deoxyadenosine triphosphate.

dCTP: deoxycytosine triphosphate.

DNA: deoxyribonucleic acid.

EDTA: ethylenediaminotetraacetic acid.

Nt: nucleotides.

N-terminal: amino-terminal.

PAGE: polyacrylamide gel electrophoresis.

Py: pyrimidine.

RNA: ribonulecic acid.

SDS: sodium docecylsulfate.

SSC: sodium chloride, sodium citrate solution.

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F. ABSTRACT

The aim of this thesis work has been to further characterize the tissue specificity of bovine cytochrome c oxidase, and to investigate the molecular basis of the expression of subunit VIb of human cytochrome c oxidase.

It was known that some of the subunits of bovine cytochrome c oxidase have heart- and liver-specific isoforms; the characterization of the enzyme from other tissues was incomplete. We have isolated the enzyme from beef brain, kidney and skeletal muscle, separated their subunits by electrophoresis in polyacrylamide gels containing sodium dodecylsulfate and urea, and investigated the primary structure of some of them by N-terminal peptide sequencing. The results demonstrate that the isoforms of subunits VIa, VIIa and VIII detectable in skeletal muscle differ from the isoforms expressed in liver and are similar to the ones present in heart; and that the isoforms of those subunits that are expressed in brain and kidney differ from the heart-specific isoforms, and are similar to those in liver. These results support the existence of at least two tissue-specific isozymes of bovine cytochrome c oxidase.

As part of a multicenter effort to clarify the molecular basis of the expression of human cytochrome c oxidase, the study of subunit VIb was undertaken. A cDNA was recovered from a human heart library, and used to investigate the presence of homologous transcripts in total RNA from six human tissues by Northern blot analysis, the gene copy number by Southern blot analysis, chromosomal location by

screening of two somatic cell hybrid panels, and recovery of homologous sequences from a human genomic library. Sequences were partially characterized by restriction digestion and DNA sequencing. Single-sized messages appeared by Northern blotting, and multiple homologous sequences by Southern analysis; most of the fragments were assigned to different chromosomes, and fragments recovered from the library were identified as pseudogenes. These results do not provide evidence for tissue-specific expression of human cytochrome c oxidase subunit VIb, but prove that a human multigene family exists.

I. INTRODUCTION

In 1895, Rohmann and Spitzer discovered an enzyme able to catalyze the oxidative synthesis of indophenol blue in animal tissues. The enzyme later received the name of indophenol oxidase. Batelli and Stern, between 1911 and 1914, found a definite parallel between tissue respiratory and indophenol oxidase activities, and proposed that the enzyme was linked to the main tissue respiration [1]. In the late 1920s, Warburg characterized the "Atmungsferment", believed to activate oxygen for biological oxidations, as a ferroporphyrin compound [2]. At the same time, Keilin was studying indophenol oxidase as the enzyme responsible for the oxidation of cytochrome. By 1938, he and Hartree had enough evidence to propose that indophenol oxidase and the "Atmungsferment" were the same enzyme and, given that the only catalytic property of this enzyme was the oxidation of cytochrome, to suggest for it the name cytochrome oxidase [3].

Cytochrome c oxidase (ferrocytochrome c: oxygen oxidoreductase, E.C. 1.9.3.1; cytochrome oxidase) is an extremely important enzyme. It is responsible for the transformation into water of about 90% of the molecular oxygen consumed by aerobic organisms, in the final step of respiration [4]. It is also an extremely complex enzyme: it contains two to thirteen polypeptide subunits, up to five redox centers [5], copper, zinc and magnesium. The function of several of these enzyme components is unknown.

Cytochrome c oxidase is, finally, an extremely interesting model to study. It is a constituent of all aerobic organisms and is the

end point of respiration. In eukaryotes, it requires the coordinated expression of elements belonging to the mitochondrial and the nuclear genomes. Its occurence as tissue-specific isozymes makes mammalian cytochrome c oxidase a suitable system for studying the mechanisms which control differential gene expression. Finally, patients have been identified with cytochrome c oxidase deficiency in whom the age of onset of the disease and the clinical phenotype vary widely; the biochemical and molecular bases of this are not understood.

The objective of this thesis is to clarify the patterns of tissue specificity of the bovine enzyme, and the molecular basis of the expression of one of the subunits of the human enzyme. The thesis is organized as a collection of papers, each of which constitutes one of the main chapters. They are preceded by this Introduction, which is intended to give some background about the topics that are most relevant to my work. And they are followed by a discussion, in which I try to address in more detail the possible implications of the data presented.

I.1. Cytochrome c oxidase: structure and function

Cytochrome c oxidase is the enzyme responsible for the last step of aerobic respiration. It catalyzes the transfer of four electrons from its substrate, ferrocytochrome c, to molecular oxygen, which is reduced to water [6]. The following equation accounts for these phenomena [4]:

$$4\ \text{Cyt}\ c^{2+} + 4\ H^{+} + O_2 \longrightarrow 4\ \text{Cyt}\ c^{3+} + 2\ H_2O$$

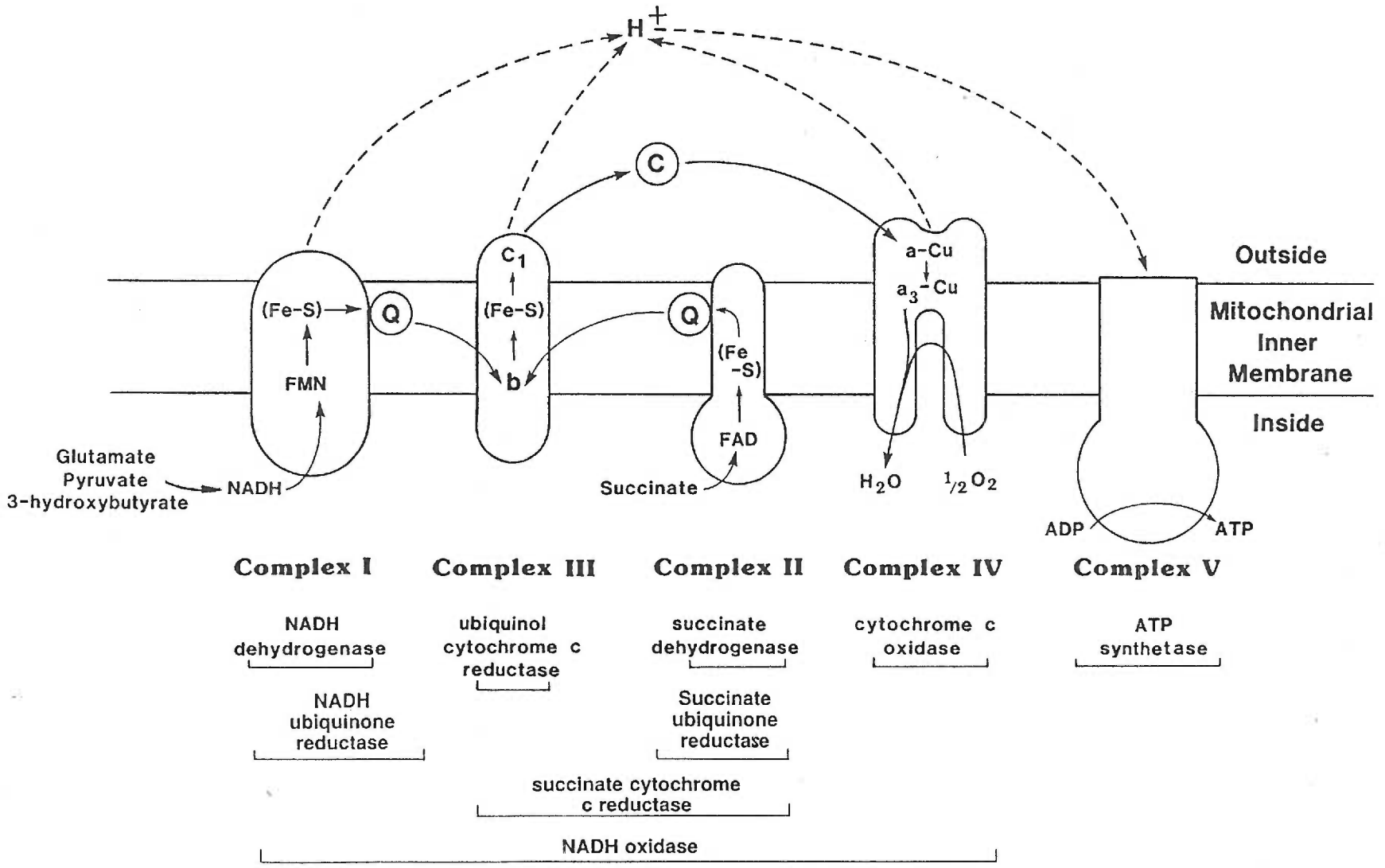
The eukaryotic enzyme corresponds to Complex IV of the electron transport chain (Fig. I.1), and is located in the mitochondrial inner membrane; the electrons enter the enzyme from the cytosolic side, while the protons consumed in the reaction are taken from the matrix; moreover, the enzyme also acts as an electrogenic proton pump that transports up to four additional protons from the matrix to the intermembrane space for every molecule of oxygen reduced [4]. The electrochemical proton gradient thus generated provides in turn the energy for the synthesis of ATP and for ion transport [7].

Cytochrome c oxidase can be isolated as stable monomers or dimers. Preparations of both forms show biphasic kinetics corresponding to the oxidative activity [8], when assayed at low ionic strength; this non-hyperbolic kinetics is currently explained by proposing the existence of two conformations of the enzyme which can accept electrons, although only one of those conformations would be able to pump protons [4]. It has been suggested that anions induce conformational changes in the enzyme [9]: all the naturally occuring anions tested stimulated the enzyme at low concentrations and inhibited it at high concentrations, although to a different extent; simultaneous changes in the visible spectrum of the oxidized enzyme are attributed to such conformational changes.

Lipid-free preparations of cytochrome c oxidase do not show oxidative activity [10]. The activity is restored by adding phospholipids, cardiolipin, or even a single synthetic lipid, dimyristoyl phosphatidylcholine [11]. In the case of cardiolipin, it has been proposed that it attracts cytochrome c and protons to the

Figure I.1:

Organization of the mitochondrial electron-transport chain. ATP-synthase is included as Complex V. Orientation of each complex on the mitochondrial inner membrane with respect to matrix and intermembrane space, and functional interrelations are schematized. The lower part of the figure offers a synopsis of how the different segments of the chain are tested, in terms of enzymatic activity.


H+
C
C1
(Fe-S)
b
(Fe-S)
FMN
Q
Q
(Fe
-S)
FAD
a-Cu
a3-Cu
Outside
Mitochondrial
Inner
Membrane
Inside
Glutamate
Pyruvate
3-hydroxybutyrate
NADH
Succinate
H2O
1/2 O2
ADP
ATP
Complex I
Complex III
Complex II
Complex IV
Complex V
NADH
dehydrogenase
ubiquinol
cytochrome c
reductase
succinate
dehydrogenase
cytochrome c
oxidase
ATP
synthetase
NADH
ubiquinone
reductase
Succinate
ubiquinone
reductase
succinate cytochrome
c reductase
NADH oxidase

vicinity of the enzyme by electrostatic forces, and also facilitates conformational changes of the enzyme [11].

Electron microscopy of two-dimensional crystals of dimeric and monomeric forms of bovine heart cytochrome c oxidase, together with image analysis, has suggested a three-dimensional model of the complex in which the monomer appears as a Y-shaped structure [7]. A single large domain, C, extends 55 Å from the cytoplasmic surface of the mitochondrial inner membrane into the intermembrane space; its largest part is at the membrane surface, and comprises two subdomains (C_1 and C_2) of equal size; the third subdomain (C_3) rises into the intermembrane space. The two other domains, M_1 and M_2, become separated close to the outer surface of the mitochondrial inner membrane, span this membrane completely, are surrounded by phospholipid and extend into the matrix 20 Å at most. In the dimeric form, monomers contact each other only through the C domains.

Structurally, mammalian cytochrome c oxidase contains thirteen polypeptide subunits, two heme irons and two or three copper ions that act as redox centers, in addition to a zinc atom and magnesium [4,5].

Among the polypeptide subunits, the three that also exist in prokaryotes are encoded by the mitochondrial genome, bear the highest molecular mass, contain the redox-active centers, and are attributed the proton pumping activity as well as responsibility for the dimerization of the enzyme [8]. Thus, subunit I binds hemes a and a_3, Cu_B and perhaps also Cu_A, and performs the catalytic and protonmotive activities; subunit II contains either Cu_A or Cu_X, and

is essentially involved in the binding of cytochrome c [12]. Subunit III might stabilize the enzyme dimers, thereby facilitating the protonmotive activity [8,12].

The ten remaining subunits of the mammalian enzyme are not so well characterized in terms of their individual roles; they are encoded by the nuclear genome, synthesized by cytoplasmic ribosomes as precursors, some of them bearing N-terminal extensions, and post-translationally imported into the mitochondrion. Genetic studies in yeast have indicated that subunits IV, Va and b, VI and VIIa, are essential for catalysis, folding or assembly of the mature enzyme; these subunits are believed to correspond to bovine subunits Vb, IV, Va and VIc, respectively [13]. On the other hand, treatment of the bovine heart enzyme with alkaline detergents, which resulted in the depletion of subunits III, Vb and VIa, and of variable amounts of subunits VIIa, VIIb, VIIc or VIII, did not affect the metal content, spectral properties or oxidase activity of the bovine heart enzyme; this suggests that those subunits do not contain Cu, Fe, Zn or Mg, and that they are not essential for the electron transfer activity of the enzyme [14]. Likewise, subunit VIa and VIb have been removed without significant loss of electron transfer or redox-linked proton pumping activities [7]. Additional roles have been proposed for the cytoplasmically synthesized subunits, including modulation of catalytic activity, energy-driven Ca^{++} transport, protein uptake by the mitochondria, and intervention in the biogenesis of this organelle [7].

I.2. Nomenclature of the subunits of the mammalian enzyme

The nomenclature of the subunits of mammalian cytochrome c oxidase is confusing because of the multiplicity of systems in use. The most common nomenclatures are those of Kadenbach [15], Buse [17] and Capaldi [7]. In 1987, Buse proposed a new nomenclature to simplify the issue [17]. These are compared in Table I.1. The nomenclature of Kadenbach is used in this thesis.

Kadenbach's nomenclature is based on the relative migration of the different subunits of the bovine heart enzyme on high-resolution SDS-PAGE [15]; subunits are not only numbered in order of decreasing apparent molecular weight, but also grouped according to the close proximity between some of them.

I.3. Tissue Specificity of Mammalian Cytochrome c Oxidase

The finding of tissue-specific isoforms of some of the nuclear encoded subunits has proven that mammalian cytochrome c oxidase occurs as different isozymes. Moreover, recent data suggest that the pattern of tissue-specific expression of these isoforms differs in different mammals. A summary of the information available for several mammals is given below.

(i) Rat. Rat subunit VIa showed differential migration when the heart, kidney and liver enzymes were analyzed by SDS-PAGE in urea-containing gels [18]. Such differences were confirmed for heart and liver, and extended to subunit VIII, by chemical labelling of sulfhydryl groups with N-ethyl-[2,3-^{14}C] maleimide [19]. Quantitative immunological tests were interpreted as providing evidence of tissue specificity for the nine subunits tested; subunit

TABLE I.1: Nomenclature and comparison of cytochrome c oxidase subunits from bovine heart and liver.

SDS-PAGE H	SDS-PAGE L	Kadenbach 1983	Buse 1981	Buse 1987	Capaldi 1988	Mr
		I	I	I	Mt_I	56993
		II	II	II	Mt_{II}	26049
		III	III	III	Mt_{III}	29918
		IV	IV	IV	C_{IV}	17153
		Va	V	V	C_V	12436
		Vb	VIa	VI	C_{VI}	10668
		VIa	VIb	VIII	ASA(H) SSG(L)	9419
		VIb	VII	VII	AED	10063
		VIc	VIc	IX	STA	8480
		VIIa	VIIIc	X	C_{VII} (L) (H)	6243
		VIIb	-	XI	IHQ	6000
		VIIc	VIIIa	XII	C_{VIII}	5541 5441
		VIII	VIIIb	XIII	C_{IX} (H) (L)	4962

The photograph shows the electrophoretic separation of the 13 enzyme subunits of the bovine cytochrome c oxidase isozymes, using Kadenbach's gel system and nomenclature [15]. H, heart; L, liver. Mr, apparent molecular weight [17]. (H) and (L) indicate the isoforms from heart and liver, respectively.

Vb did not react with the antibody used [20,16]. Two different cDNAs coding for rat subunit VIa have been recovered: one of them reacts with a transcript present in heart and muscle only, while the others react with transcripts in heart and muscle as well as in liver, kidney and brain [21].

(ii) Pig. Subunits VIIa and VIII of the porcine enzyme showed differences in electrophoretic migration between heart and liver [18]. Preliminary N-terminal peptide sequencing data supported these results, and suggested that subunits VIa, VIIb and VIIc were also expressed in a tissue-specific manner [22]. An antiserum against cytochrome c oxidase from rat liver showed differential reactivity to pig heart and muscle subunits VIa and VIII, when compared to their counterparts in brain, kidney and liver [20]. Furthermore, the three subunits were protected to a different extent by cytochrome c depending upon the tissue of origin, when their accessible carboxylic acid groups were labelled with [^{14}C]glycine-ethyl ester following modification with 1-ethyl-3-[3-(dimethylamino) propyl] carbodiimide [23]. Finally, differential labelling of sulfhydryl groups confirmed that subunits VIa and VIII had heart- and liver-specific isoforms [19].

(iii) Beef. The bovine enzyme has been particularly informative. Subunits VIa, VIIa and VIII from heart and muscle migrated differently than the same subunits from liver and kidney, by denaturing gel electrophoresis [24,25]; this has been confirmed for heart and liver by N-terminal peptide sequencing [8,16]. Differential labelling of sulfhydryl groups was also found for

subunits VIa and VIIa from heart and liver [19]. Northern analysis with rat cDNA probes has shown that transcripts for subunits VIa and VIII in beef, have the same tissue distribution described above for rat subunit VIa [26 and R.A. Capaldi, personal communication].

(iv) Human. Less is known about the human enzyme. Polyclonal antibodies against subunits of the beef enzyme have been used for Western blot analysis, and the results suggest that subunits VIa and VIIa are also tissue-specific in humans, with the major isoforms in heart and muscle differing from those in liver, kidney and brain. The data also suggest that the two isoforms of subunit VIa might be expressed in heart [27]. Regarding subunit VIII, no evidence of its tissue-specific occurrence has been found: on the contrary, the sequence of the heart subunit corresponds exactly to the sequence deduced from a liver cDNA [28].

I.4. Molecular biology of the mammalian nuclear encoded subunits

During the last decade, an intense effort has been devoted to establishing the molecular basis of the expression of mammalian cytochrome c oxidase subunits. A summary of current knowledge is given below.

(i) Subunit IV. A bovine liver cDNA for subunit IV was first characterized, that included part of the sequence corresponding to the mature peptide, and additional sequence in the 5' end that corresponded to an amino-terminal polypeptide extension; the deduced extension had 22 aminoacids, was rich in basic and hydroxylated residues and devoid of acidic residues, and was partially homologous

to the corresponding sequence of several fungal precursor proteins [29]. Two bovine genes were later described, an interrupted gene and a processed pseudogene [30]. A full-length human liver cDNA, whose sequence and deduced product were 87% and 83% homologous to the bovine cDNA, was then isolated; an additional clone containing an apparently transcribed pseudogene was also recovered. Northern analysis of human tissues revealed single-sized transcripts, about 700-nt long, in HeLa cells, muscle and liver [31]. Identical cDNAs from adult rat brain and liver, and fetal liver were also isolated; these showed 80% homology with the human cDNA at the deduced peptide level [32,33].

(ii) Subunit Va. An essentially full-length cDNA, coding for a product 95% homologous to bovine cytochrome c oxidase subunit Va, was isolated from a human endothelial cell library; the deduced product included a 41-aminoacid N-terminal presequence rich in basic and hydroxylated aminoacids, and containing one acidic residue as well. Transcripts of 750 nt were identified by Northern analysis in human brain, liver and muscle, and preliminary results regarding the identification of more than one homologous gene locus were reported [34]. The deduced product from a rat heart essentially full-length cDNA was 100% homologous to the bovine mature peptide, and 96% homologous to the human deduced product [35]. A mouse pre-B cell cDNA was also isolated, that was 96% homologous to the rat heart cDNA [36].

(iii) Subunit Vb. The screening of a human fetal muscle cDNA library resulted in the isolation of an essentially full-length cDNA

encoding the putative precursor of human cytochrome c oxidase subunit Vb; this included a 31 aminoacid N-terminal presequence, rich in basic residues. The assignment was based on 85% homology of the deduced mature peptide with its bovine counterpart. A bovine brain cDNA for subunit Vb was characterized by the same authors. Its deduced product was identical to the bovine heart subunit and the nucleotide sequence was 85% homologous to the human cDNA. Northern analysis of human liver, muscle and brain RNA showed 550-nt transcripts. Southern analysis of human genomic DNA revealed seven Pst I fragments, comprising more than 30 Kb [37].

(iv) Subunit VIa. cDNAs corresponding to subunit VIa of the rat enzyme from heart and liver were only 50% homologous to each other at the deduced peptide level. The peptide deduced from the heart cDNA showed 75% homology with the corresponding bovine subunit. The rat heart cDNA included an upstream in-frame stop codon suggesting the absence of a leader sequence in the precursor. Northern analysis showed that the heart cDNA hybridizes to a 520-nt transcript in rat heart and muscle, while the liver cDNA revealed a slightly larger, single-sized transcript in rat brain, heart, kidney, liver and muscle [21]. Two human cDNAs, one from a liver library and the other from an endothelial cell library, were identical to each other; their deduced products were 87% homologous to the rat liver cDNA and had an upstream in-frame stop codon suggesting absence of a presequence [38].

(v) Subunit VIb. Two cDNAs for mammalian cytochrome c oxidase subunit VIb have been described. Neither the bovine heart nor the

human muscle cDNA is full-length, and the existence of a cleavable presequence is uncertain [39,40].

(vi) Subunit VIc. Identical cDNAs corresponding to rat liver subunit VIc were characterized by different authors [41,42]. One of them includes part of the 5' untranslated region, and shows an upstream in-frame stop codon. The overall homology between the rat deduced mature peptide and the bovine heart subunit VIc is 79.4%. Single-sized transcripts about 400-450 nt in length were found by Northern analysis of rat heart, liver, kidney and muscle. At least eight restriction fragments of high molecular weight were evident in Southern blots of rat genomic DNA. An interrupted gene and two processed pseudogenes [43,44] have been characterized in rat. A human fibroblast cDNA that lacks the upstream in-frame stop codon mentioned above has also been isolated [45].

(vii) Subunit VIIa. A bovine liver cDNA whose deduced product corresponds to the bovine liver subunit VIIa has been sequenced [46]. It has a 23-aminoacid presequence, rich in basic and hydroxylated residues. Similar features have been found in a human endothelial cell cDNA, 83% homologous to the bovine liver cDNA at the deduced mature peptide level [47].

(viii) Subunit VIIb. A 27 or 32-aminoacid N-terminal leader sequence has been predicted for bovine heart cytochrome c oxidase subunit VIIb, upon sequencing of a cDNA whose deduced mature peptide corresponds to the N-terminal sequence of the bovine heart peptide [48].

(ix) Subunit VIIc. A bovine heart cDNA corresponding to bovine heart subunit VIIc has also been isolated that indicates the existence of a 16-aminoacid presequence, rich in basic and hydroxylated residues [49].

(x) Subunit VIII. A partial rat liver cDNA, whose deduced product lacked the N-terminus and showed 60% homology to the bovine heart peptide, revealed 400-450-nt transcripts on Northern analysis of rat RNA from heart, liver, kidney and muscle, and at least six restriction fragments of high molecular weight in Southern blots of rat genomic DNA [50]. A human liver cDNA corresponding to the human heart subunit VIII has been characterized [51]. A bovine heart cDNA encoding a putative leader 24-aminoacids long and rich in basic residues, and a bovine liver cDNA encoding a 25-aminoacid leader have been characterized. They correspond to the mature peptides which are 52% homologous to each other. A 480-nt transcript is revealed by the bovine heart cDNA in bovine heart and muscle RNA by Northern analysis, while the liver cDNA hybridizes to a transcript 690-nt long in bovine brain, heart, kidney, liver and muscle [52].

I.5. Research outline and statement of the problems

The research leading to this thesis was developed in two stages.

The objective of the first stage was to further characterize the tissue-specific patterns of bovine cytochrome c oxidase. This work began when differences between bovine liver and heart had just been confirmed; the question which we wished to address was whether or not

there were differences between the subunits of the mammalian enzyme from heart and skeletal muscle.

We were interested in this particular issue because patients had been described in whom cytochrome c oxidase deficiency appeared to be restricted to skeletal muscle, suggesting that the expression of the enzyme in skeletal muscle differed from that in other tissues. One attractive hypothesis was that a defect of a tissue-specific subunit caused the peculiar distribution of the abnormalities in humans.

The difficulty of working directly with human material prompted us to use the bovine system. The reasons for this were that our collaborators had experience with the system, that methods had been developed for the isolation of the heart enzyme that could be applied to skeletal muscle, and that large amounts of starting material were readily available. The necessary assumption was that the bovine and human systems were similar with respect to the patterns of tissue-specific expression of cytochrome c oxidase; at that time, there was no convincing evidence to suggest otherwise.

The scope of the search was initially limited to characterizing the enzyme isolated from bovine skeletal muscle by SDS-PAGE and N-terminal peptide sequencing the nuclear encoded subunits. This was accomplished, although sequencing results for six of them are still preliminary, and one cannot be sequenced because its N-terminus is blocked. No difference was found between the enzyme from bovine muscle and heart. At this point, we decided to widen the search to include bovine kidney and brain, organs that are also affected in some patients, although no isolated deficiency has been described;

however, I had to limit the evaluation to the three subunits whose tissue-specific occurrence in heart and liver had already been demonstrated. Our findings are described in the first of the two manuscripts that are included in the main section of the thesis.

When the first stage was completed, we learned by means of a personal communication that subunit VIII from human heart had been sequenced, and found to correspond exactly to a human liver cDNA already isolated [28]. Since subunit VIII is one of the tissue-specific subunits in beef, these data provided clear evidence that the tissue-specific patterns of expression of the nuclear encoded subunits of the bovine and human enzymes might not be identical.

By then, a multicenter effort to elucidate the molecular basis of the expression of human cytochrome c oxidase was in progress; the intention was to promote sharing of tools and to prevent duplication of efforts. We decided to study human subunit VIb, since a bovine heart cDNA coding for it was available. Thus, our objectives for the second stage were to begin the characterization of human subunit VIb with respect to the organization of the corresponding genomic sequences, and to examine their expression at the mRNA level in different tissues.

My first goal was to isolate a human cDNA for subunit VIb, because I was reluctant to use the bovine probe for investigating human genomic sequences, since the degree of homology between the bovine and human transcripts was unknown. By December 1988, I had isolated and sequenced a human heart cDNA whose deduced mature

product showed sufficient homology with the bovine peptide to identify it as a human cDNA for subunit VIb; this was confirmed by the publication of a practically identical sequence from a human muscle cDNA in January 1989.

My next goal was to estimate the number of human genes coding for cytochrome c oxidase subunit VIb. Since multiple fragments homologous to the human cDNA appeared in Southern blots of human genomic DNA at high and low stringency with all of the restriction endonucleases used, I suspected the existence of a multigene family. To prove it, I screened two human-rodent somatic cell hybrid panels with the human heart cDNA, and identified several chromosomal loci.

Afterwards, I wished to further characterize these genes by sequence analysis; thus, I screened a human genomic library, recovered three different clones, and subcloned the homologous fragments for sequencing. The data identified these genomic elements as pseudogenes; sequence analysis also permitted some speculation regarding the structure of a homologous active gene.

Finally, I decided to investigate whether members of this gene family were differentially expressed at the mRNA level among several human tissues. Northern analysis was performed on RNA from human cerebellum, heart, kidney, liver, skeletal muscle and fibroblasts, and showed that single-sized transcripts are present in all of them.

Results from this stage are described in the second manuscript.

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II. BOVINE CYTOCHROME C OXIDASE: FURTHER EVIDENCE OF TISSUE-SPECIFIC ISOZYMES

Roque D. Carrero-Valenzuela, Mark L. Wagner, and Nancy G. Kennaway.

Department of Medical Genetics, Oregon Health Sciences University, Portland, Oregon, U.S.A..

SUMMARY

We have examined the tissue specificity of cytochrome c oxidase (ferrocytochrome c:oxygen oxidoreductase, EC 1.9.3.1), isolated from five bovine organs. Two patterns of expression were seen by SDS-PAGE and confirmed by N-terminal sequence analysis; they differed with respect to the migration and sequences of subunits VIa, VIIa and VIII. Additionally, preliminary N-terminal sequencing of six of the remaining nuclear encoded subunits of the skeletal muscle enzyme revealed no differences compared to the heart or liver enzyme. The results clearly demonstrate that the major isoforms of subunits VIa, VIIa and VIII from bovine kidney and brain differ from those in heart, whereas the major isoforms from skeletal muscle differ from those in liver. The results also suggest that identical isoforms of these subunits are expressed in heart and skeletal muscle, while the alternative isoforms are expressed in liver, kidney and brain; however, the possibility that apparently identical isoforms may differ in the C-terminal region of the polypeptide has not been ruled out. We conclude that at least two major isozymes of cytochrome c oxidase are present in the tissues studied, one common to heart and skeletal muscle, and the other common to liver, kidney and brain.

INTRODUCTION

Cytochrome c oxidase (ferrocytochrome c:oxygen oxidoreductase, EC 1.9.3.1) is the terminal member of the respiratory chain in aerobic organisms from bacteria to mammals [1]. The mammalian enzyme is embedded in the mitochondrial inner membrane, and transfers electrons from reduced cytochrome c to molecular oxygen, with generation of an electrochemical proton gradient used for ATP synthesis and ion transport. Structurally, it includes up to five redox centers [2] and thirteen polypeptide subunits [3]. Subunits I, II and III, which are homologous to those present in the bacterial enzyme, are coded by the mitochondrial genome, synthesized within the mitochondrion, and believed to be responsible for the main functions of the complex. The remaining subunits are found only in eukaryotes, coded by the nuclear genome, made in the cytoplasm, and post-translationally inserted into the mitochondrion; their function is unclear [4].

During the last decade, evidence has been obtained that mammalian cytochrome c oxidase occurs as tissue-specific isozymes. This was first indicated by the differential migration of several of the nuclear-coded polypeptides, when the enzyme from different tissues and several mammalian species was examined under highly resolving conditions of polyacrylamide gel electrophoresis [5,6]. Additional evidence of tissue specificity was provided by chemical labelling [7], and by N-terminal peptide sequencing which suggested up to five tissue-specific subunits in pig cytochrome c oxidase [8]. More recent immunological studies led Kuhn-Nentwig and Kadenbach to

propose that all nuclear-coded subunits of the mammalian enzyme may occur as tissue-specific isoforms [9].

Cytochrome c oxidase from beef heart and liver were recently compared by Western blot analysis using tissue-specific polyclonal antibodies, and by N-terminal peptide sequencing of the nuclear encoded polypeptides [10]. The results showed that subunits VIa, VIIa and VIII (nomenclature of Kadenbach et al. [11]) indeed occur as different isoforms in these bovine tissues.

We have now extended the scope of this study to include beef brain, kidney and skeletal muscle cytochrome c oxidase. In this paper, we report tissue-specific differences in the electrophoretic migration and N-terminal peptide sequence of mature forms of subunits VIa, VIIa and VIII from those bovine organs, compared to heart and liver.

MATERIAL AND METHODS

Cytochrome c oxidase isolation

Adult beef brain cortex, kidney cortex, and skeletal muscle were obtained at the slaughter house and transported to the laboratory on ice within 2 hours of death. The tissues were homogenized in a Waring blender at 4°C in 0.25 M sucrose, 0.05 M Tris-HCl, 0.01 M EDTA (pH 7.4). Mitochondria were isolated by differential centrifugation, resuspended in 0.25 M sucrose, 0.01 M Tris-HCl (pH 7.4), and adjusted to a protein concentration of 20 mg/ml.

Cytochrome c oxidase from different preparations was purified according to published methods [10,12], with minor modifications. Electron transport particles were obtained by sonication in the presence of glass beads, recovered by differential centrifugation, and resuspended in 0.25 M sucrose, 0.01 M Tris-HCl (pH 7.4), before treatment with deoxycholate, 0.3 mg/mg protein [12]. The green pellets were resuspended in 0.1 M Tris-HCl (pH 7.4), at a protein concentration of 20 mg/ml, and 20% potassium cholate added to give 1 mg/mg protein [13]. Following ammonium sulfate fractionation, the pellets were resuspended in 0.09 M NaCl, 0.02 M Tris-HCl (pH 7.4), and stored at -70°C.

SDS-PAGE

Cytochrome c oxidase subunits were separated in urea-containing gels according to Kadenbach et al. [11]. Samples were dissociated in 0.125 M Tris-HCl (pH 6.5), 2.5% SDS, 4 M urea, 1.67% 2-mercaptoethanol, 0.01% bromphenol blue, for 30 minutes at room temperature, run at 100 V until stacked, and then at 200 V for 18-19 hours.

N-terminal peptide sequencing

Proteins resolved by SDS-PAGE were transferred electrophoretically onto polyvinilidene difluoride membrane (Immobilon, Millipore) as described by Matsudaira [14], using a Hoefer TE22 Transblot apparatus, at 400 mA for 2 hours. After staining, the blots were air-dried and stored at -20°C. Polypeptides of interest were cut from the membranes and sequenced in a gas phase

protein sequencer (Applied Biosystems Model 470A) equipped with an on-line phenylthiohydantoin analyser (Model 120A).

Other assays

Protein content, cytochrome aa_3 and cytochrome c oxidase activity determinations were performed according to published procedures [15,16,17].

RESULTS

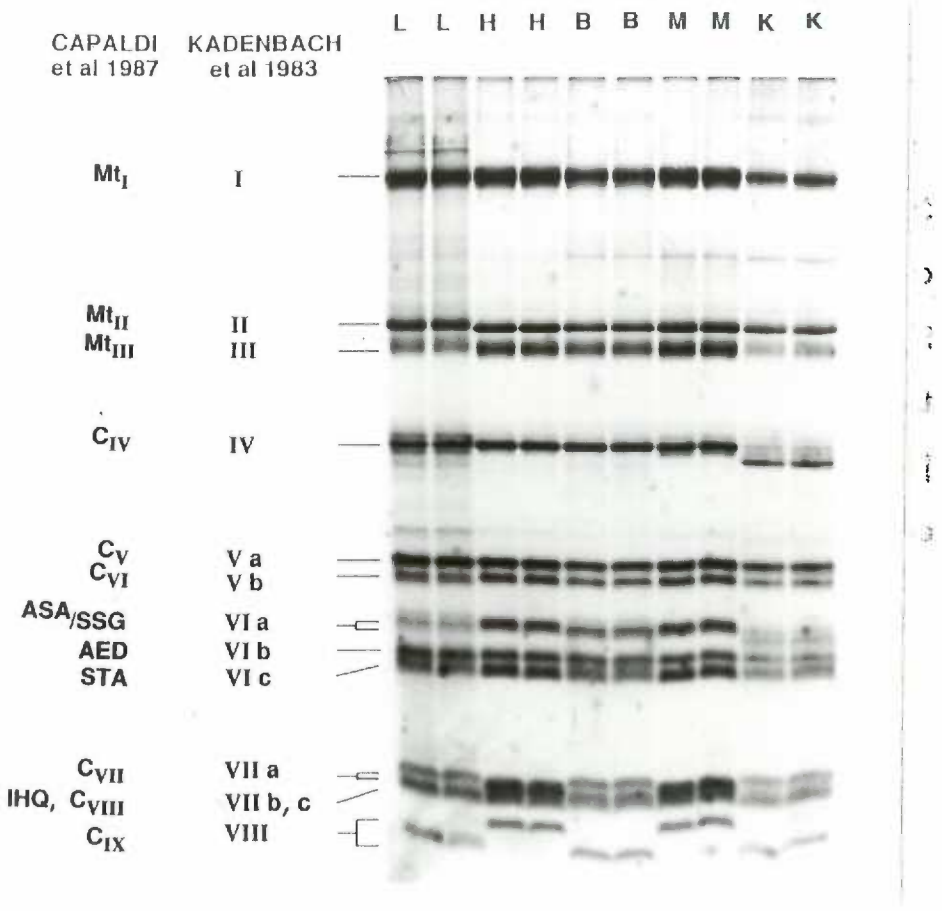
Cytochrome c oxidase was purified from bovine brain cortex, kidney cortex and skeletal muscle, by the general procedure of Fowler et al. [12]. This method depends on the separation of complex I, the bc_1 complex and ATP synthase from membrane-bound cytochrome c oxidase by differential solubilization in the presence of detergent.

The subunit composition of bovine cytochrome c oxidase from several tissues was analyzed by SDS-PAGE, and is shown in Fig. 1. Three subunits can be seen to differ in their migrations between tissues; in heart and skeletal muscle, subunits VIa and VIII migrate relatively slower, and subunit VIIa faster, than their counterparts in brain, kidney and liver.

Subunit IV and also VIa shows a faint band at the expected position plus a few extra bands of higher mobility in our kidney preparations. This has been described by Sinjorgo et al. [18] and attributed to partial proteolysis, since subunit IV has been shown to be particularly sensitive to such degradation in rat liver [19]. No other electrophoretic difference is found among preparations from

Figure II.1:

Subunit structure of bovine liver (L), heart (H), brain (B), skeletal muscle (M) and kidney (K) cytochrome c oxidase. Subunits were separated on a 19.5% SDS-polyacrylamide gel containing 6 M urea and stained with Coommassie Brilliant Blue. The nomenclature of Kadenbach et al. [11] is shown on the right column and used in the text; Capaldi's nomenclature [10] is shown on the left for comparison. Heart and liver enzyme samples were kindly provided by R.A. Capaldi.


CAPALDI et al 1987
KADENBACH et al 1983
L L H H B B M M K K
Mt$_I$
I
Mt$_{II}$
Mt$_{III}$
II
III
C$_{IV}$
IV
C$_V$
C$_{VI}$
V a
V b
ASA/SSG
AED
STA
VI a
VI b
VI c
C$_{VII}$
IHQ, C$_{VIII}$
C$_{IX}$
VII a
VII b, c
VIII

different tissues; in particular, we find no evidence of an additional band in heart, migrating between subunits VIa and VIb, as described by Sinjorgo et al. [18].

N-terminal sequencing of isolated subunits confirms the tissue-specific patterns shown by electrophoresis. Fig. 2 shows that bovine cytochrome c oxidase subunits VIa, VIIa and VIII from heart and skeletal muscle differ from their brain, kidney and liver counterparts at several positions.

Preliminary N-terminal sequencing of 12 to 25 residues of six of the remaining nuclear encoded subunits of bovine skeletal muscle cytochrome c oxidase was also carried out; the sequences are identical to those corresponding to heart and liver [10] (Data not shown).

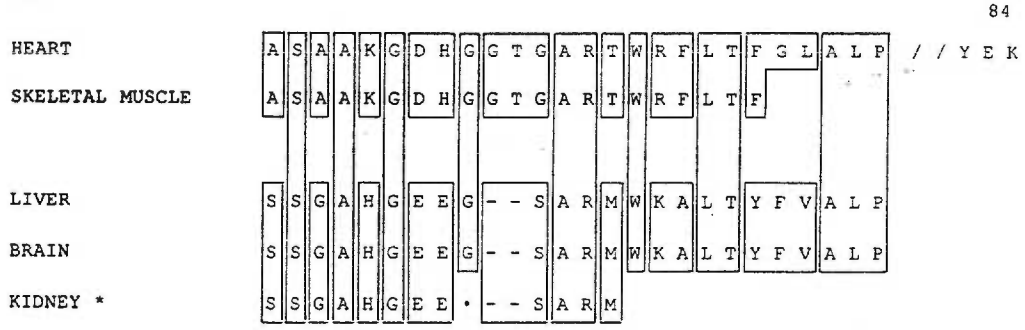
DISCUSSION

Our interest in the tissue-specific expression of mammalian cytochrome c oxidase stems from a number of factors: 1) the critical role of this enzyme in mitochondrial respiration and energy transduction; 2) the complexity of its composition in comparison to that of prokaryotes and lower eukaryotes; 3) the lack of understanding of the function of the nuclear encoded subunits in the mammalian enzyme or the significance of isoforms of these subunits in different tissues; and 4) the association of cytochrome c oxidase deficiency with human diseases which affect certain tissues while sparing others.

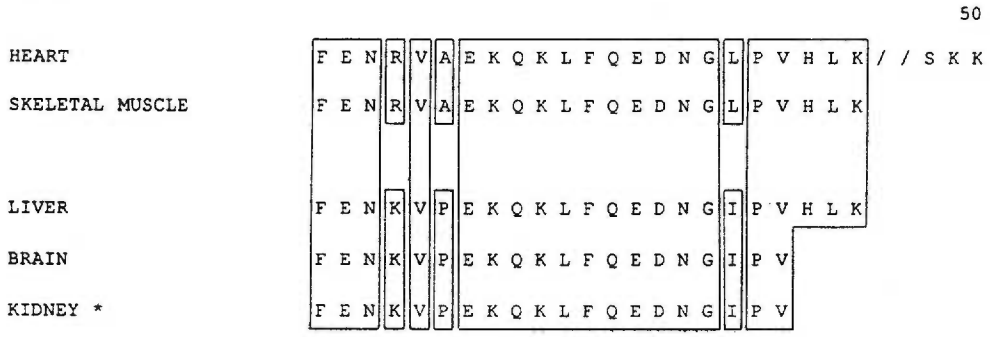
Figure II.2:

N-terminal peptide sequences of cytochrome c oxidase subunits VIa, VIIa and VIII from several bovine tissues. Sequences were obtained from at least two runs, except when indicated by *. Numerical superscripts indicate the total number of aminoacids in the mature peptides from bovine heart. The dot represents an unidentified residue and dashes are used to maximize homology. Sequences of heart and liver subunits are from published data [10]. Position 14 (underlined) of the liver enzyme subunit VIII has been shown to be polymorphic, occuring as either aspartic or glutamic acid [20].

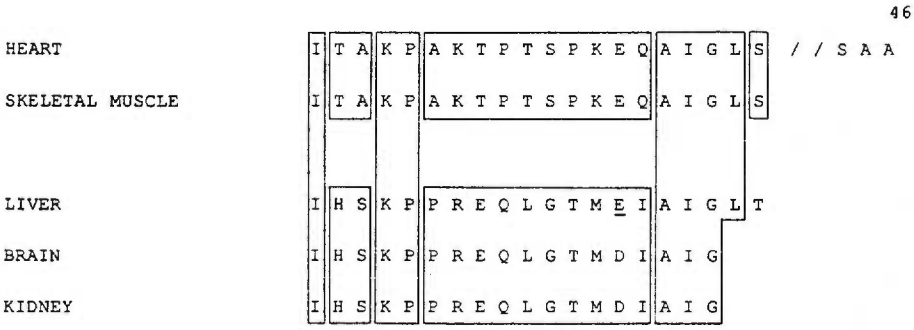
VIa



VIIa



VIII



The existence of isoforms of at least three subunits of mammalian cytochrome c oxidase has been recognized for a number of years. Antibody binding studies, using polyclonal antibodies raised against bovine heart subunits VIa, VIIa and VIII showed little or no cross-reactivity with the corresponding subunits from bovine liver; N-terminal peptide sequencing identified changes at several positions, confirming the tissue-specific expression of these subunits [10].

Our results demonstrate similar migrations of subunits VIa, VIIa and VIII in bovine heart and skeletal muscle on the one hand, and in liver, kidney and brain on the other. They extend the findings of Sinjorgo et al. [18], by showing that brain cytochrome c oxidase manifests a "liver-type" pattern. Although we find no evidence of an additional band in heart, migrating between subunits VIa and VIb as described by these authors, subunit VIa from brain does sometimes migrate as a closely spaced doublet; however, N-terminal sequencing of the two bands together gives only a single sequence, suggesting that these represent differently migrating forms of the same polypeptide, differing for example in the extent of sulfhydryl reduction (R.A. Capaldi, personal communication). This illustrates the importance of peptide sequencing to confirm that differences in migration of a particular subunit in different tissues do indeed correspond to distinct isoforms.

N-terminal sequence analysis demonstrates clearly that brain and kidney isoforms of subunits VIa, VIIa and VIII differ from their counterparts in heart and that the skeletal muscle isoforms differ

from those in liver. The results also suggest that the heart and muscle isoforms are identical and that the same is true for those of liver, kidney and brain. Although these data do not rule out the existence of additional subunit isoforms, differing in residues other than those occuring at the N-termini, or the presence of small amounts of the alternative isoform of any of these subunits in a given tissue, the absence of significant cross-reactivity of polyclonal antibodies against the heart forms of bovine subunits VIa, VIIa and VIII with the enzyme from liver suggests the absence of significant amounts of those isoforms in this tissue [10 and unpublished data]. Thus the evidence to date leads us to conclude that at least two patterns of tissue-specific expression, and therefore at least two major isozymes of bovine cytochrome c oxidase exist, one present in heart and skeletal muscle, and the other in liver, kidney and brain.

In recent years, evidence has accumulated that the patterns of tissue-specific expression of cytochrome c oxidase subunits vary between different mammals [5-10; 21-24]. Both "heart" and "liver" isoforms of subunits VIa, VIIa and VIII appear to exist in pig as well as beef. In contrast, although corresponding isoforms of subunits VIa and VIII have also been identified in rat, chemical labelling of subunit VIIa from rat heart and liver failed to show differences that characterize the beef isoforms [22]. SDS-PAGE and N-terminal sequencing also failed to show such differences (R.A. Capaldi, personal communication). In humans, our own immunological studies suggest that subunits VIa and VIIa are expressed in a tissue-specific manner [23]. However, no evidence of tissue-specific

expression in humans has been found for subunit VIII; indeed, peptide sequencing and cDNA analysis suggest instead that a single form of this subunit is expressed in human heart and liver [25]. Thus at least three different patterns of tissue-specificity of cytochrome c oxidase appear to occur among mammals.

The existence of more than one pattern of tissue-specific expression of cytochrome c oxidase among different mammals raises the possibility that different isoforms of a certain subunit may be structurally and functionally equivalent, with no effect at the mature enzyme level. The lack of evidence for any functional difference at the subunit or enzyme level [18] supports this proposition. Definition of the role of the tissue-specific subunits will be required to settle this issue.

The availability of cDNAs to several subunits of cytochrome c oxidase has permitted examination of the expression of these isoforms in different tissues. A single transcript corresponding to the liver isoform of subunit VIII has been identified in bovine liver, kidney and brain, but two transcripts, corresponding to the heart and liver isoforms of this subunit, were found in heart and skeletal muscle [20]. However, our data indicate that only one of these two isoforms of subunit VIII is incorporated into the cytochrome c oxidase complex in bovine heart and skeletal muscle, suggesting that regulation of the expression of these subunits in liver, kidney and brain occurs at the level of transcription or transcript processing, whereas in heart and skeletal muscle it occurs post-transcriptionally. A similar pattern of expression was obtained in beef [R.A. Capaldi, personal

communication] and rat [26] for subunit VIa. This illustrates the value of data such as we have presented in this paper, which complement molecular studies by identifying the isoforms actually incorporated into the cytochrome c oxidase complex in different tissues.

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III. HUMAN CYTOCHROME C OXIDASE SUBUNIT VIb: CHARACTERIZATION AND MAPPING OF A MULTIGENE FAMILY

Roque D. Carrero-Valenzuela[1], Franklin Quan[3], Robert Lightowlers[4], Nancy G. Kennaway[1], Michael Litt[1,2] and Michael Forte[2,3]

[1]Department of Medical Genetics, [2]Department of Biochemistry, [3]Vollum Institute for Biomedical Research, Oregon Health Sciences University, Portland, OR, and [4]Institute of Molecular Biology, University of Oregon, Eugene, OR.

SUMMARY

We have isolated a human heart cDNA for cytochrome c oxidase subunit VIb that includes 50 base pairs upstream from the region corresponding to the mature peptide. By Northern analysis, homologous transcripts of identical size are identified in six human tissues, providing no evidence of tissue-specific expression for this subunit. Southern blot analysis of human genomic DNA shows multiple fragments homologous to the cDNA, and analysis of DNA from several human-rodent somatic cell hybrid lines demonstrate their cosegregation with five or six different human chromosomes. Clones representing two human genomic elements have been isolated; sequence analysis suggests that both elements are pseudogenes.

INTRODUCTION

Cytochrome c oxidase (Ferrocytochrome c:oxygen oxidoreductase, EC 1.9.3.1) is the terminal component of the mitochondrial respiratory chain in aerobic organisms. It catalyses the transfer of electrons from reduced cytochrome c to molecular oxygen, and contributes to the electrochemical proton gradient used for mitochondrial ATP synthesis and ion transport [1].

Cytochrome c oxidase is a complex enzyme embedded in the mitochondrial inner membrane, that includes 13 different polypeptide subunits in mammals. The three largest subunits are encoded by mtDNA and are believed to be responsible for the electron transfer and proton pumping activities, acting as apoproteins for the hemes and coppers that constitute the redox centers of the functional complex. The remaining 10 subunits are encoded by nuclear genes, synthesized by cytoplasmic ribosomes and translocated into the mitochondrion [1]. Many of the cytoplasmic precursors contain an N-terminal leader sequence which is cleaved during their uptake into mitochondria.

Several nomenclatures for mammalian cytochrome c oxidase subunits are currently in use. The nomenclature of Kadenbach [2], used in this paper, is based upon their relative electrophoretic migration in highly resolving, urea-containing SDS-polyacrylamide gels.

The function of the nuclear encoded subunits is presently unclear. It has been proposed that they intervene in the regulation

of the rate of electron transport and stoichiometry of proton pumping, according to the concentration of multiple intracellular effectors [3]. Additional roles unrelated to the catalytic activity, such as energy-dependent calcium uptake and protein import by the mitochondrion have also been suggested [1]. Indeed, support for this is provided by the recent demonstration that subunit I of the cytochrome reductase complex of Neurospora crassa is identical to the processing enhancing protein which stimulates the activity of the matrix processing peptidase of that organism [4].

Genetic studies have shown that most of the six nuclear coded subunits of the yeast enzyme are essential for the catalytic activity, folding or assembly of the functional complex [5]. Four additional subunits occur in the mammalian enzyme [5,6]; furthermore, several of the nuclear encoded subunits are expressed in mammals as tissue-specific isoforms, differing in electrophoretic migration and N-terminal peptide sequence [7,8,9]. Roles in differential modulation of the catalytic activity, in response to different metabolic environments, have been suggested for the tissue-specific subunits [10].

The human enzyme is particularly interesting because patients with cytochrome c oxidase deficiency have been identified, in whom the defect has been found in one or in a few tissues, while the others seem to be spared. Involvement of tissue-specific subunits has been proposed [11]; however, the apparent tissue distribution of the defect in those patients did not correspond to any known pattern of tissue-specific occurrence of the mammalian enzyme. Furthermore,

when molecular and peptide sequencing studies of subunit VIII of the human enzyme, failed to reveal the tissue-specific expression observed in other mammals [12], the need to evaluate the expression of all of the nuclear-encoded subunits of the enzyme in humans became apparent.

Mammalian cytochrome c oxidase subunit VIb is one of the four subunits which lacks a counterpart in yeast [5,6]. The mature subunit from bovine heart is an 85-residue, 10,063 Da polypeptide and the only one of the nuclear coded subunits whose N-terminus is blocked [13]. It has the characteristics of an extrinsic protein, being hydrophilic and readily extracted from the enzyme complex in a water soluble form; moreover, it is accesible to proteolytic cleavage from the cytoplasmic side of the mitochondrial inner membrane, suggesting that the entire mass of VIb is on the cytoplasmic side of this membrane [14]. Proteolytic removal of subunits VIa and VIb is associated with loss of calcium transport in reconstituted vesicles [1].

We have been studying human cytochrome c oxidase subunit VIb, as part of a multicenter effort aimed at clarifying the molecular basis of the tissue specificity of the human enzyme. Recently, a cDNA encoding the mature sequence of this subunit was isolated from a human muscle cDNA library [15]. However, no information was available on the 5' sequences upstream from the methionine codon which preceeds the coding sequence of the mature polypeptide, so that the presence of a cleavable presequence could not be ruled out. Moreover, the expression of this subunit in different tissues was not

studied, and no information was available on the copy number or chromosome localization of the corresponding genomic sequences.

In this paper, we report the characterization of a human heart cDNA coding for cytochrome c oxidase subunit VIb; a Northern blot study of human RNA from six tissues showing single-sized homologous transcripts; a Southern blot analysis of genomic DNA and screening of a human-rodent somatic cell hybrid panel with the identification of several human genomic loci homologous to the cDNA; and the characterization of two of the corresponding genomic elements.

MATERIAL AND METHODS

Enzymes and reagents. Restriction enzymes were from Bethesda Research Laboratories and Boehringer-Mannheim; the Sequenase sequencing kit was from United States Biochemical Corporation; the nick-translation kit was from Bethesda Research Laboratories. [alpha-^{32}P]dCTP (3,000 Ci/mmol) and [alpha-^{35}S]dATP (1094 Ci/mmol) were from Dupont-New England Nuclear Corporation.

cDNA library screening. 300,000 clones of a preamplified λ-gt11 human heart cDNA library (kind gift of M. Benjamin Perryman, Baylor College of Medicine, Houston, TX) were screened with the bovine heart cDNA for cytochrome c oxidase subunit VIb [16]. The purified insert (250 ng in 20 ul) was nick-translated to a specific activity of 0.16 x 10^6 cpm/ng with [alpha-^{32}P]dCTP (3,000 Ci/mmol), using the BRL kit and protocol. Hybridization was performed in 50% formamide, 50 mM sodium phosphate (pH 7.0), 5X SSC, 5X Denhardt's solution, 0.1% SDS, 0.1 mg/ml of sonicated salmon sperm DNA and 1% non-fat dry milk, with

0.8×10^6 cpm/ml of the probe, at 37°C for 60 hours. Nitrocellulose filters (Schleicher and Schuell) were washed twice in 2X SSC, 0.2% SDS, for 10 minutes at room temperature, and finally washed four times in 0.2X SSC, 0.2% SDS, for 30 minutes at 50°C, before autoradiography.

cDNA subcloning and sequencing. Phage DNA was obtained from liquid lysates as described [17]. cDNA inserts were recovered by Eco RI digestion, and subcloned into pBSKS(M13-) (Stratagene). Double-stranded sequencing was carried out following enzymatic linearization of the vector, by the dideoxynucleotide chain terminator method [18], using [alpha-^{35}S]dATP (1094 Ci/mmol) and the Sequenase kit. Sequence analysis and homology searches used programs included in the IG or GCG packages, in a VAX/VMS version 4.5 system (Digital Equipment Corp.).

RNA isolation. Bovine tissues were frozen in liquid nitrogen within 15 minutes of death, then stored at -70°C. Human tissues were obtained at autopsy with informed consent; they were frozen in liquid nitrogen within six hours of death, and kept at -70°C. Human skin fibroblasts were obtained from confluent cultures. HPLC-grade water was used instead of DEPC-treated water for the RNA work. Frozen tissues were ground under liquid nitrogen and homogeneized in denaturing solution prior to RNA isolation. Total RNA from frozen 0.5 g samples of human cerebellum, heart, kidney cortex, liver and skeletal muscle, and from cultured human fibroblasts was isolated as described [19]. Poly(A)$^+$ RNA was isolated from frozen bovine brain cortex and skeletal muscle with the Fastrack kit (Invitrogen).

Northern blot analysis. RNA electrophoresis was done as described [17], except that the 20-microgram samples were resuspended into 50 mM HEPES (pH 7.8), 1 mM EDTA-Na_2, 17% formaldehyde, 49% formamide for denaturing, the gel being 1.5 % agarose, 42 mM HEPES (pH 7.8), 0.84 mM EDTA-Na_2, 6% formaldehyde, and the electrophoresis buffer being 50 mM HEPES (pH 7.8), 1 mM EDTA-Na_2, 6% formaldehyde. Capillary transfer onto Nytran membranes (Schleicher and Schuell), prehybridization, hybridization, washing and removal of probes were performed as suggested by the manufacturer. The probes were the human cDNA for cytochrome c oxidase subunit VIb, and a rat cDNA for cyclophylin (provided by John P. Adelmann, Vollum Institute for Advanced Biomedical Research, Portland, OR); both probes were labelled by nick-translation as described above to specific activities of 0.3 x 10^6 cpm/ng, and purified by ammonium acetate precipitation [20].

Southern blot analysis. Protocols for the preparation of total human DNA blots, prehybridization with total human DNA, and hybridization have been described [21]. The probe, human cDNA for cytochrome c oxidase subunit VIb, was labelled to a specific activity of 0.24 x 10^6 cpm/ng by random priming [22] with [alpha-^{32}P]dCTP (3,000 Ci/mmol). Filters were washed twice in 2X SSC, 0.2% SDS, for 10 minutes at room temperature, and finally washed in 0.2X SSC, 0.2% SDS, for 30 minutes, at 55°(low stringency) or 65°(high stringency) C, before autoradiography.

Somatic cell hybrid panel screening. Southern blots containing DNA from two different human-rodent somatic cell hybrid panels were used.

The cell lines have been characterized and described [23,24,25]. Prehybridization, probe labelling and hybridization were done as for Southern blots of total human DNA (see above). Filters were washed twice in 2X SSC, 0.2% SDS, for 10 minutes at room temperature, and finally washed in 0.2X SSC, 0.2% SDS, for 30 minutes, at 55°C.

Genomic DNA library screening. Duplicate filters (NEN hybridization transfer membrane for colony plaque screen) including 300,000 clones of a preamplified λ-EMBL 3 human leukocyte genomic DNA library (provided by David Grandi, Vollum Institute for Advanced Biomedical Research, Portland, Oregon), were screened with the human heart cDNA for cytochrome c oxidase subunit VIb. 500 ng of the purified cDNA insert were nick-translated to a specific activity of 0.23×10^6 cpm/ng with [alpha-^{32}P]dCTP (3,000 Ci/mmol), using the BRL kit and protocol. Filters were prehybridized with 1 M NaCl, 1% SDS, 50% formamide, 0.1 mg/ml denatured salmon sperm DNA, for 4 hours at 37°C. Probe was added up to 4.7×10^5 cpm/ml, and the hybridization performed at 37°C for 64 hours. Filters were washed twice in 2X SSC, 0.2% SDS, for 10 minutes at room temperature, and once in 0.2X SSC, 0.2% SDS, for 1 hour at 65°C, before autoradiography.

DNA subcloning and sequencing. Phage DNA was obtained from liquid lysates as described [17], digested with different restriction enzymes, and analyzed by Southern blotting. Reactive Hinc II fragments were subcloned into pBSKS(M13-) (Stratagene). Digestion, analysis and subcloning were repeated until fragments of suitable size for sequencing were obtained. Sequencing was carried out as described above.

RESULTS AND DISCUSSION

We have isolated two positive clones from a human heart cDNA library screened with a bovine cDNA probe (BHcDNA) for cytochrome c oxidase subunit VIb (Fig. III.1). Both clones bear identical 458-nt cDNAs (HHcDNA) that contain a typical polyadenylation signal (AATAAA) beginning at residue 420, and include a poly (A) tract of 22 residues 12 nt downstream from this signal. Several open reading frames are present, the longest of which comprises residues 48 to 308. No in-frame stop codon is present 5' from the putative initiation codon of this open reading frame.

The deduced translation product is 86 aminoacids long and has four cysteines, at positions 30, 40, 54 and 65 of the deduced peptide, as does the bovine heart subunit. It is 84.7% homologous to this subunit, with differences in 13 residues resulting in a net gain of three negative charges. Given this homology, we believe that we have recovered a human heart cDNA for cytochome c oxidase subunit VIb.

To our knowledge, this cDNA is the longest available for a mammalian cytochrome c oxidase subunit VIb, and includes 50 nt of sequence preceding the region coding for the mature peptide. A 359-nt species had been obtained from a bovine heart/liver cDNA library [16], and a 418-nt cDNA from a human skeletal muscle library [15]. The human heart cDNA is 86.7% homologous at the nucleotide sequence level to the bovine heart cDNA; nucleotides at positions 347, 380, 381 and 387 of the human sequence, and at position 288 of the bovine sequence are not present in the other species. The human

```
HHPEP  :                                                        M  A  E  D  M      5
HHcDNA : ACATTGAGCTGCAGGTTGAATCCGGGGTGCCTTTAGGATTCAGCACCATGGCGGAAGACATG    62
BHcDNA :                                                .......T...A.........C    23
BHPEP  :                                                        +  +  +  +  I      5

HHPEP  :  E  T  K  I  K  N  Y  K  T  A  P  F  D  S  R  F  P  N  Q  N  Q     26
HHcDNA : GAGACCAAAATCAAGAACTACAAGACCGCCCCTTTTGACAGCCGCTTCCCCAACCAGAACCA    124
BHcDNA : C..G.................C....T..T.................................     85
BHPEP  :  Q  A  +  +  +  +  +  Q  +  +  +  +  +  +  +  +  +  +  +  +     26

HHPEP  :   T  R  N  C  W  Q  N  Y  L  D  F  H  R  C  Q  K  A  M  T  A      46
HHcDNA : GACTAGAAACTGCTGGCAGAACTACCTGGACTTCCACCGCTGTCAGAAGGCAATGACCGCTA    186
BHcDNA : ...C..G.....................................G.................     147
BHPEP  :   +  +  +  +  +  +  +  +  +  +  +  +  +  +  E  +  +  +  +  +      46

HHPEP  : K  G  G  D  I  S  V  C  E  W  Y  Q  R  V  Y  Q  S  L  C  P  T     67
HHcDNA : AAGGAGGCGATATCTCTGTGTGCGAATGGTACCAGCGTGTGTACCAGTCCCTCTGCCCCACA    248
BHcDNA : .......T...G....C.....T..........G..........A..............T.    209
BHPEP  : +  +  +  +  V  +  +  +  +  +  +  R  +  +  +  K  +  +  +  +  I     67

HHPEP  :  S  W  V  T  D  W  D  E  Q  R  A  E  G  T  F  P  G  K  I  *       86
HHcDNA : TCCTGGGTCACAGACTGGGATGAGCAACGGGCTGAAGGCACGTTTCCCGGGAAGATCTGAAC    310
BHcDNA : .........T..ACT.....C..T.GC.....A..............T.............     271
BHPEP  :  +  +  +  S  T  +  +  D  R  +  +  +  +  +  +  +  +  +  +  *       86

HHPEP  :
HHcDNA : TGGCTGCATCTCCCTTTCCTCTGTCCTCCATCCTTCT-CCCAGGATGGTGAAGGGGGACCTG    371
BHcDNA : .....C....C.A.C.-.T..........G..............G....T..........G.    332
BHPEP  :

HHPEP  :
HHcDNA : GTACCCAG--TGATC-CCCACCCCAGGATCCTAAATCATGACTTACCTGCTAATAAAAACTC    430
BHcDNA : .GTA.AT.GT.....T.......T...                                       359
BHPEP  :

HHPEP  :
HHcDNA : ATTGGAAAAAAAAAAAAAAAAAAAAA                                        458
BHcDNA :
BHPEP  :
```

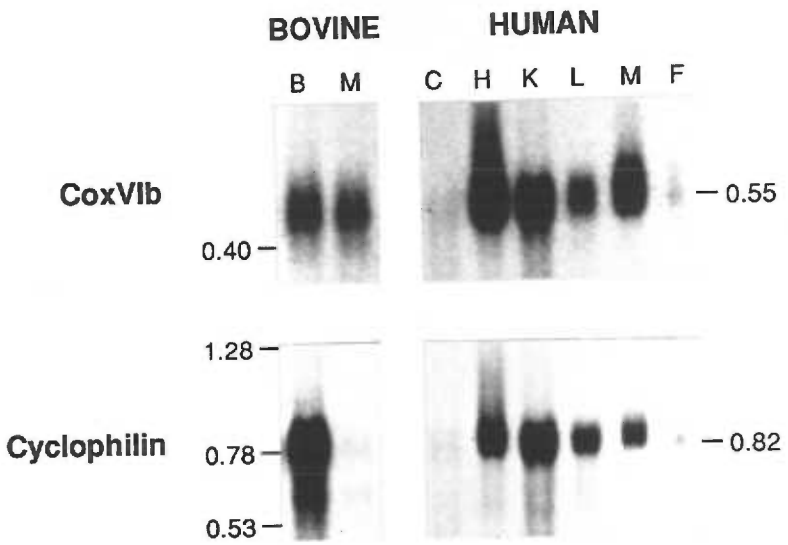
heart cDNA is 99.3% homologous to the 418-nt human skeletal muscle one; the differences correspond to positions 440, 441 and 442 of the heart cDNA where a AAA triplet is replaced by GTG in the skeletal muscle cDNA. Such a sequence difference at the 3' end suggests that the cleavage and polyadenylation of the corresponding skeletal muscle transcript occurred at least six nucleotides downstream from the putative homologous site in the human heart transcript. Since we have not found any in-frame stop codon upstream from the putative translation initiation one, these data leave open the question about whether or not mammalian cytochrome c oxidase subunit VIb has an N-terminal cleavable leader sequence.

By Northern blot analysis, total human RNA from several tissues, as well as poly(A)$^+$ RNA from bovine brain and skeletal muscle run in parallel (Fig. III.2), reveal single-sized transcripts of about 550 nucleotides. Minor differences in their relative migration are probably not relevant, since they are also observable for the transcripts recognized by the control probe, a rat cDNA for cyclophilin. It is interesting that transcripts for cytochrome c oxidase subunit VIb seem to be more abundant in heart and skeletal muscle, and less abundant in liver, than in other human tissues, when compared to the control; this is consistent with the observation that cytochrome c oxidase activity is higher in heart, and lower in liver, than in skeletal muscle [11].

A rat cDNA for cyclophilin was used as a control probe to check the quality of the RNA preparations. The absence of a transcript for cyclophilin in cynomolgus monkey skeletal muscle has been described

Figure III.2:

Northern blot analysis of RNA from several human and bovine tissues. 20 micrograms of total RNA isolated from human cerebellar cortex (C), heart (H), kidney (K), liver (L), skeletal muscle (M) and fibroblasts (F) [19], were fractionated in parallel with equivalent aliquots of bovine brain (B) and skeletal muscle (M) poly(A)$^+$ RNA by electrophoresis through a 1.5 % agarose, 42 mM HEPES (pH 7.8), 0.84 mM EDTA-Na_2, 6% formaldehyde gel. Capillary transfer onto Nytran membranes (Schleicher and Schuell), prehybridization, hybridization, washing and removal of probes were performed as suggested by the manufacturer. The probes were the human cDNA for cytochrome c oxidase subunit VIb (CoxVIb) and a rat cDNA for cyclophilin (Cyclophilin), labelled by nick-translation to specific activities of 0.3 x 10^6 cpm/ng with [alpha-^{32}P]dCTP (3000 Ci/mMol). RNA molecular weight standards are indicated on the left. Ethidium bromide staining revealed that the human cerebellar RNA was degraded; this was also true for the human kidney sample, but to a minor extent. The size of the bands that hybridized to each probe are indicated on the right.


BOVINE
HUMAN
B M
C H K L M F
CoxVIb
0.40
0.55
1.28
Cyclophilin
0.78
0.53
0.82

[26], and its absence in bovine skeletal muscle was expected. Surprisingly, a homologous transcript is seen in human skeletal muscle, indicating interspecies differences in the control of the expression of this gene.

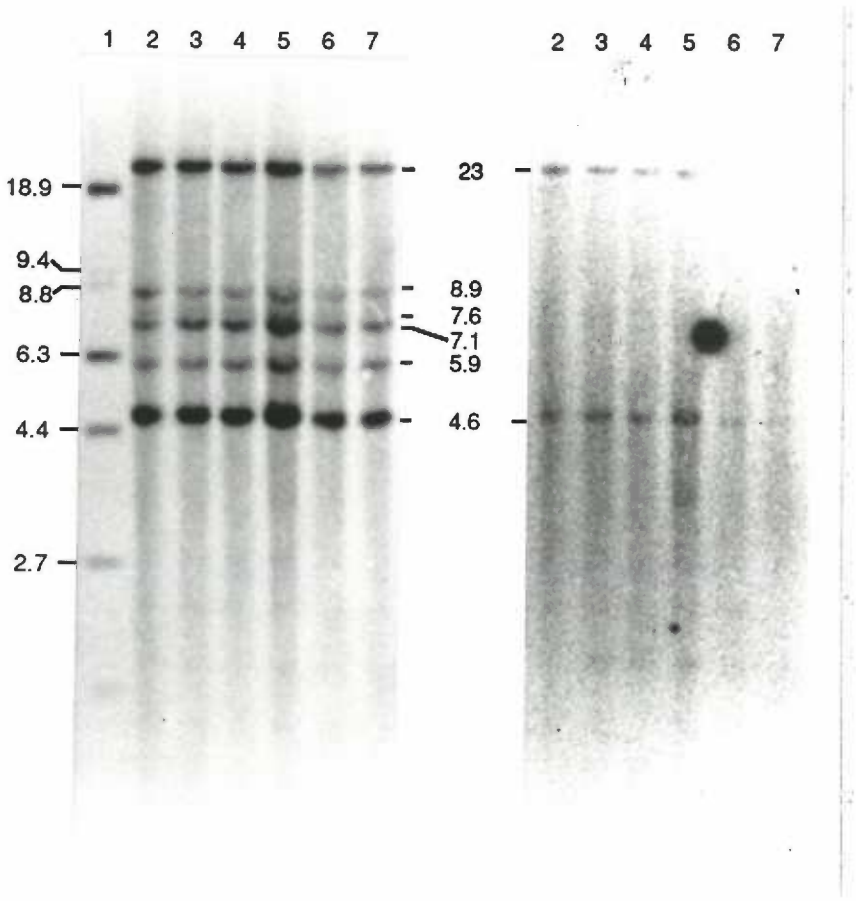
These results provide no evidence to suggest that cytochrome c oxidase subunit VIb expression is tissue-specific in humans. This is consistent with peptide sequence data on the bovine subunits, which are identical in heart and liver [8], and with Northern blot analysis of total RNA from bovine heart, kidney, liver and skeletal muscle (R.L., unpublished observations). However, transcripts differing slightly in size, or those differing in nucleotide sequence, are not distinguishable by Northern analysis. Thus, the possibility of tissue-specific isoforms has not been completely ruled out.

Southern blot analysis of total human DNA restricted with different endonucleases shows a minimum of two fragments strongly homologous to the cDNA (Fig. III.3). In particular, two Eco RI fragments about 23 and 4.6 Kb in length are detected at high stringency, suggesting that a single gene or a few related ones account for the coding of the human peptide. At lower stringency, additional fragments are visible with all enzymes (Fig 1 of the Appendix); thus, definite 8.9, 7.1 and 5.9 Kb Eco RI fragments, as well as a faint 7.6 KB Eco RI fragment, appear. While not ruling out the possibility of a single gene, this supports the existence of a multigene family.

In order to decide between these two possibilities, we analyzed a Southern blot containing Eco RI-digested DNA from several

Figure III.3:

Southern blot analysis of Eco RI-digested human genomic DNA at low stringency (left panel) and high stringency (right panel). Preparation of these human DNA blots, as well as protocols for their prehybridization with total human DNA, and for hybridization, have been described [21]. The probe, human cDNA for cytochrome c oxidase subunit VIb, was labelled to a specific activity of 0.24 x 10^6 cpm/ng by random priming [29] with [alpha-^{32}P]dCTP (3,000 Ci/mmol). This blot was washed twice in 2X SSC, 0.2% SDS, for 10 minutes at room temperature, and finally washed in 0.2X SSC, 0.2% SDS, for 30 minutes, at 55°(low stringency), or 65°(high stringency) C. DNA molecular weight standards are shown (column 1), and their sizes indicated on the left. Genomic DNAs from six non-related individuals are included (columns 2-7). The size of every hybridizing fragment is indicated between both panels.


1 2 3 4 5 6 7
2 3 4 5 6 7
18.9
9.4
8.8
6.3
4.4
2.7
23
8.9
7.6
7.1
5.9
4.6

human-mouse and one human-hamster somatic cell hybrid, by hybridization with the human heart cDNA probe. As shown in Fig. III.4, the 23-Kb human Eco RI fragment (M1) cosegregates with chromosome 19, and the 4.6-Kb fragment (M6) with chromosome 7. The chromosomal asignment of the 23-Kb Eco RI fragment is supported by the analysis of the second cell hybrid panel (data not shown). Among the more weakly hybridizing Eco RI fragments, those of 5.9 Kb (M5) and 7.6 Kb (M3) cosegregate with chromosomes 22 and 15 respectively. For the two remaining fragments, (8.9 and 7.1 Kb; M2 and M4, respectivley), neither chromosome 8 nor chromosome 17 could be excluded. These data indicate that at least five different human genes share a high degree of homology with the human heart cDNA for cytochrome c oxidase subunit VIb. Furthermore, the fact that at least four of these loci correspond to single Eco RI fragments suggests a relatively simple organization of each coding unit within the human genome.

Since Southern blot analysis of human genomic DNA probed with human heart cDNA for subunit VIb at high and low stringency indicated the presence of a number of related sequences in the human genome, a human genomic library was screened with the human heart cDNA for cytochrome c oxidase subunit VIb in order to isolate and characterize these genomic sequences. Ten positive clones were recovered which on restriction digestion and Southern blotting could be organized into three groups. Partial restriction maps of representatives from two of these groups (designated 5b and 6a) are shown in Fig. III.5; preliminary restriction analysis and sequencing data suggest that the

Figure III.4:

Somatic cell hybrid panel screening. Eco RI-digested DNAs from nine human-mouse (lanes 4-12) and one human-hamster (lane 3) hybrid cell lines [23] were screened with the human cDNA for cytochrome c oxidase subunit VIb. The Southern blot (lower right) also included hamster, mouse and human parental cell line DNAs (lanes 2, 13 and 14, respectively). Sizes of molecular weight standards are shown on the left of the blot, and the size of every investigated fragment (M1-M6), on the right. Discordancy fractions are shown on the lower left; the absence of discordancy is highlighted. Chromosomes present in each hybrid are indicated on the upper left; $\pm$ means that only 7-14% of the cells of a certain line had shown control markers for a given chromosome. Each cell line was scored for the presence or absence of every fragment (M1-M6) revealed in Southern blots of Eco RI-digested human genomic DNA by the human heart cDNA for cytochrome c oxidase subunit VIb (upper right); cell line 25/8 (lane 8) was scored positive for M1, since the faint band seen in this figure becomes clearer after overexposure (not shown); only 15% of the cells in this line had been positive for control markers on chromosome 19 [24]. Prehybridization, hybridization and washing conditions were as in Fig. III.3; no high-stringency washing was done.

LANE	CELL LINE	CHROMOSOMES																						SCORING					
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	M1	M2	M3	M4	M5	M6
1	APSS																													
2	1102-Ha	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	CF -11/4	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	CF84-39/5	-	-	+	-	-	-	+	+	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	+	-	+	-	+
5	CF84-35/8	-	-	+	+	-	+	+	+	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	+	-	+	-	+
6	CF84-34/9	-	-	-	+	-	+	+	+	-	+	+	-	-	-	-	-	+	-	+	+	-	+	-	+	+	-	+	+	+
7	CF84-27/8	-	+	-	-	+	+	-	+	-	-	+	+	-	-	-	-	+	+	-	+	+	-	-	-	+	-	+	-	-
8	CF84-25/8	-	-	-	+	+	+	-	+	-	+	+	+	-	-	-	-	+	-	+	-	+	-	-	+	+	-	+	-	-
9	CF84-20/8	-	+	+	+	±	-	+	+	-	-	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	-	+	+	+
10	CF84- 7/8	-	-	+	+	-	+	±	+	-	-	+	+	-	+	-	-	+	-	-	+	-	-	-	-	+	-	+	-	+
11	CF84- 5/8	-	+	+	-	+	+	-	+	-	-	-	-	+	+	+	-	+	-	+	-	+	+	-	+	+	+	+	+	+
12	CF84- 2/8	+	+	-	+	+	+	+	+	-	-	+	+	-	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+
13	B82-Mouse	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14	IMR91-Human	+	+	+	+	+	+	+	+	+	+	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

% DISCORDANCY:

For M_1:	30	40	70	40	29	30	56	50	50	20	44	60	40	40	20	50	50	50	**0**	50	40	20	50
For M_2:	80	50	40	30	44	20	33	**0**	100	70	33	20	70	50	70	60	**0**	60	50	40	50	50	80
For M_3:	10	20	50	50	22	50	56	70	30	40	67	50	20	20	**0**	30	70	30	20	50	40	20	30
For M_4:	80	50	40	30	44	20	33	**0**	100	70	33	20	70	50	70	60	**0**	60	50	40	50	50	80
For M_5:	30	20	50	40	33	50	33	50	50	40	44	60	20	20	20	30	50	30	20	30	40	**0**	30
For M_6:	50	60	30	20	78	40	**0**	30	70	60	44	40	60	40	60	30	30	50	60	30	80	40	70

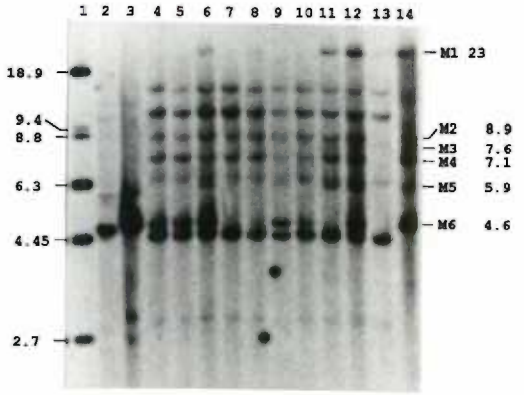
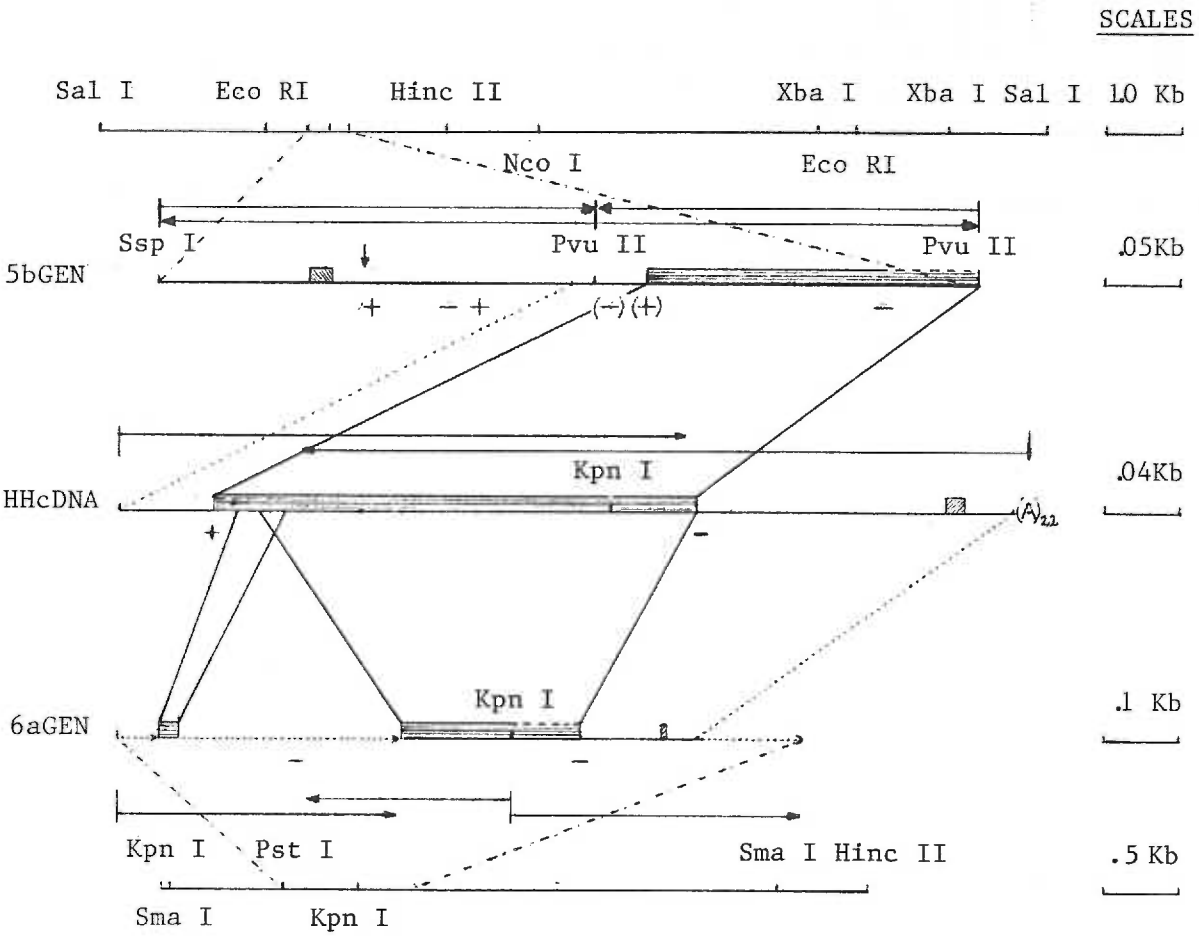


Figure III.5:

Comparison between human genomic elements respresented in clones 5b (5bGEN) and 6a (6aGEN), and the human heart cDNA for cytochrome c oxidase subunit VIb. Sequenced regions in both genomic elements are expanded from partial restriction maps of each clone. The organization of each element is shown, including regions corresponding to the mature peptide deduced from the cDNA, and to putative control elements; codons limiting open reading frames and three Alu elements present in 6b are also indicated. Scales are shown on the right.



- Restriction map projection
- Limit of homology (DNA)
- Limit of homology (DNA and peptide)
- Sequencing strategy
- Deduced mature peptide
- Translation altered by shift or stop
- Alu element
- Transcription initiation point
- TATA box
- Polyadenylation signal
- \+ ATG codon
- − Stop codon
- (+) ATG codon only in cDNA
- (−) Stop codon absent in cDNA

third group, 4a, contains inserts overlapping with 6a (data not shown).

In an attempt to identify the sequences recovered, 4a, 5b and 6a were digested with Eco RI (Fig. 2 of the Appendix); 5b and 6a contain 7.4 and 8.5 Kb fragments respectively that hybridize with the probe; these are similar in size to some of the fragments (7.1, 7.6 and 8.9 Kb respectively) seen at low stringency on Southern blots of Eco RI-digested human genomic DNA (see above). Since the homologous fragment from 4a had to be recovered as a 5.2-Kb Sal I-Eco RI digestion product, no tentative identification could be made.

Figure III.6 shows 540 bp of sequence from clone 5b which overlap the first 274 bp of the human heart cDNA for cytochrome c oxidase subunit VIb. The figure shows 273 additional bp of 5' genomic sequence. The overall homology to the cDNA at the nucleotide level is 81.9%, if gaps are counted as single events; the homology is higher in regions corresponding to the mature peptide (84.6%) than in the area of overlap extending upstream from those regions (64.9%). At the peptide level, and counting stop codons as aminoacid substitutions, the homology values are 57.8, 62.2 and 37.5%, respectively. If the sequences are optimally aligned, the putative initiation codon in the cDNA is replaced by TTG in this genomic element; two codons, including bp 392 to 397 (dashes in Fig. III.6) which code for N_{24} and Q_{25} in the peptide deduced from the cDNA, are deleted; and the codon for W_{55} (TGG) in the cDNA is replaced by a stop codon (TGA, shown as *). These observations lead us to conclude

```
5bPEP  :
5bGEN  : TTCACACTAGGTGATGGATTATTTGAAATTTTCTTCCTAATGCAGCGCTGCTGTGTAAGAAC   62
HHcDNA :
HHPEP  :

5bPEP  :
5bGEN  : ATCATCATTGTTCAGTTTGGTGGGTTTTCTCTTCACGGTGTAGATAACAGTAGAAGGGACTT  124
HHcDNA :
HHPEP  :

5bPEP  :
5bGEN  : TTGCAGGATTAGCATTTACAATGCTTTGTCAGTCAGGGTCATGGGATTAGACTCACTGAGTC  186
HHcDNA :
HHPEP  :

                                                          ▼
5bPEP  :                                  *                M  R  S    3
5bGEN  : AGTCATCAGTAGCTCCATTAACTGAGACATAAACAGATTCATTAAAGAGTGGAATGAGGTCC  248
HHcDNA :
HHPEP  :

5bPEP  :  K  V  D  V  F  A  E  G  H  T  E  L  P  A  A  S  *  A  S  L  E   24
5bGEN  : AAAGTTGATGTCTTTGCTGAGGGTCACACTGAGCTACCAGCTGCATCATGAGCATCTTTAGA  310
HHcDNA :                          ...T......G.AG.T..A...CG.G.TGC......G   37
HHPEP  :                            I  +  +  Q  V  E  +  G  V  P  +  G

                      ▼
5bPEP  :    V  S  T  L  A  K  D  M  K  T  K  I  K  N  Y  K  T  A  P  F    44
5bGEN  : AGTCAGCACCTTGGCAAAAGACATGAAGACCAAAATCAAGAACTACAAGACTGCCCCTTTTG  372
HHcDNA : .T........A....GG........G.........................C..........   99
HHPEP  :   F  +  +  M  +  E  +  M  E  +  +  +  +  +  +  +  +  +  +  +   16

                      ▲
5bPEP  : D  R  R  F  P  N  Q        T  R  N  C  W  Q  N  F  L  D  F  H   63
5bGEN  : ACAGACGCTTCCCCAACCA------GACTAGGAACTGCTGGCAGAACTTCCTGGACTTCCAC  428
HHcDNA : ....C..............GAACCA......A................A.............  161
HHPEP  : +  S  +  +  +  +  +  N  Q  +  +  +  +  +  +  +  Y  +  +  +  +   37

5bPEP  :  L  C  E  K  A  T  T  A  E  G  C  D  V  S  M  C  E  *  Y  Q  C   84
5bGEN  : CTCTGTGAGAAGGCAACAACCGCTGAAGGGTGTGATGTCTCTATGTGTGAATGATACCAGTG  490
HHcDNA : .G....C.........TG......A....AG.C...A.....G....C.....G......C.  223
HHPEP  :  R  +  Q  +  +  M  +  +  K  +  G  +  I  +  V  +  +  W  +  +  R   58

5bPEP  :    V  Y  K  F  F  C  P  A    P  G  F  Q  S  R  M  T              100
5bGEN  : TGTGTACAAGTTCTTCTGCCCTGC-TCCTGGGTTTCAGTCTAGGATGACAG               540
HHcDNA : .......C...C.C.......CA.A........CA...A..G......GCA               274
HHPEP  :    +  +  Q  S  L  +  +  T  S  W  V  T  D  W  D  E                 74
```

that this genomic sequence represents a human pseudogene for subunit VIb.

Analysis of the genomic sequences extending 5' from the above mentioned TTG codon lead to speculation that the subunit VIb protein may in fact contain a presequence which is cleaved during import into the mitochondria. A sequence in 5b, starting at nucleotide 100 (GTGTAGATAACAGTAG), fits well with the consensus TATA box sequence (G.GTATAT$\frac{A}{T}A\frac{A}{T}$.G..G); the first substitution, G for T, has been found in the mouse pseudo alpha globin gene which is not transcribed, and also shown to drastically reduce the in-vitro transcription of the conalbumin gene [27]; therefore, it is unlikely that this genomic element is utilized. Eighteen nucleotides downstream, another sequence is found (TTAGCATTTAC) that, with the exception of a single transversion (A for Py) closely matches the consensus sequence for transcriptional initiation (Py...PyAPyPyPyPyPy) [28].

Between this putative transcriptional initiation point and the region corresponding to the mature peptide deduced from the cDNA, two short in-frame open reading frames comprising nt 165-218 and 240-299 are found. Since the stop codon that closes the last of these two reading frames (TGA codon at nt 297-299) is not observed in the human heart cDNA for subunit VIb, but is replaced by a GGG triplet coding for glycine, we speculate that a presequence extending from the ATG codon at nt 240-242 to the ATG codon at nt 321-323 may exist in a homologous active gene for subunit VIb, coding for a leader peptide of 28 aminoacids including the initial methionine, and a net negative charge. Although acidic residues rarely occur in the leader

sequences of peptides targeted to the mitochondrion, they have been described in the deduced product of the cDNA for the hinge protein of the human bc_1-complex which, like subunit VIb of cytochrome c oxidase, appears to be located in the mitochondrial inner membrane, facing the intermembrane space [14,28].

Fig. III.7 shows data derived from partial sequencing of clone 6a. It contains a region of homology (6aGEN) with the human heart cDNA for cytochrome c oxidase subunit VIb, which starts nine codons downstream from the putative initiation codon in the cDNA, and extends past the point corresponding to the polyadenylation site. This region includes 399 residues; within the area of overlap, there is 81.6% homology to the human heart cDNA, but the deduced products are only 53% homologous to each other. A frameshift caused by the deletion of nt 149 accounts in part for the discrepancy; the frame is corrected by the insertion of a nucleotide before nt 223, rendering the TGA codon at nt 232-234 in-frame again.

Nucleotides 364-369 in the genomic sequence (AAGTGA) correspond exactly to the homologous region of the human skeletal muscle cDNA and are absent in the human heart cDNA for this subunit [15]. It is possible that the 3' end of the primary transcript corresponding to the cDNA includes a series of GAAA(A) repeats, only interrupted by a GUGA sequence in the second position of the series. It is tempting to speculate that any of the members of the series could be cleaved in a certain transcript to undergo polyadenylation; this would be a potential source of microheterogeneity among the mRNAs, but its relevance is unclear.

```
6aPEP  :
6aGEN  :
HHcDNA : ACATTGAGCTGCAGGTTGAATCCGGGGTGCCTTTAGGATTCAGCACCATGGCGGAAGACATG   62
HHPEP  :                                                    M  A  E  D  M    5

6aPEP  :              K  N  Y  K  T  A  L  F  D  S  S  F  P  N  Q  N  Q   17
6aGEN  :           AAGAACTACAAGACCGCTCTTTTTGACAGCAGCTTCCCCAACCAGAACCA      50
HHcDNA : GAGACCAAAATC.................C.C.........C.....................  124
HHPEP  :  E  T  K  I  +  +  +  +  +  +  P  +  +  +  R  +  +  +  +  +  +  26

6aPEP  :    P  R  N  C  L  Q  D  Y  L  D  F  H  L  C  E  K  A  V  I  A    37
6aGEN  : GCCCAGGAACTGCTTGCAGGACTACCTGGACTTCCACCTCTGTGAGAAGGCAGTGATTGCTA   112
HHcDNA : .A.T..A.......G....A.................G....C........A...CC....  186
HHPEP  :    T  +  +  +  W  +  N  +  +  +  +  +  R  +  Q  +  +  M  T  +    46

6aPEP  : K  G  D  N  V  F  V  C  E  W  Y  R  L  C  T  S  P  S  F  P  Y   58
6aGEN  : AAGGGGACAATGTCTTTGTATGTGAATGGTACCGGC-TGTGTACAAGTCCCTCATTCCCATA   173
HHcDNA : ....A.G.G..A...C...G..C..........A..G.......C........TGC....C.  248
HHPEP  : +  +  G  D  I  S  +  +  +  +  +  Q  R  V  Y  Q  S  L  C  +  T   67

6aPEP  :    P  G  S  Q  P  G  T  T  T  G  Q  K  P  H  F  P  G  K  I  *    77
6aGEN  : TCCTGGATCTCAGCCTGGGACGACCACTGGGCAGAAGCCACATTTCCCTGGGAAGATTTGAA   235
HHcDNA : ......G..A...A......T..G..AC....T....G...G......-........C....  309
HHPEP  :  S  W  V  T  D  W  D  E  Q  R  A  E  G  T  +  +   +  +  +  *    86

6aPEP  :
6aGEN  : CTGGCTGCACCCCACCTTTCCTCTGTCCTCCGTCCTTCTCCCAGGGTGGTAAAAGGGGACCT   297
HHcDNA : .........T.T.-.................A.............A....G..G........  370
HHPEP  :

6aPEP  :
6aGEN  : GGGTACATGGCGATCCCCACCCTGGAACCCTCAATCATGACTTGACTAATAATAAAA-CTTA   358
HHcDNA : ..-...CCA.T...........CA.G.T...A...........AC..GC.......A..C.   431
HHPEP  :

6aPEP  :
6aGEN  : TTGGAAAAGTGAAAGAAAGAAAGAAAAGAAAGAAAGAGGCCA                      399
HHcDNA : .......AAA...A...A...A....A                                     458
HHPEP  :
```

Our data indicate that clone 6a also represents a pseudogene. First, an Alu sequence is found immediately upstream of the region homologous to the human cDNA; second, no putative splicing signals are found in the area, suggesting that the Alu sequence does not correspond to an intron; third, an in-frame TAG stop codon that closes any potential open reading frame is located within the Alu sequence; and fourth, no in-frame ATG initiation codon occurs within either the Alu sequence or the region of homology.

The existence of multigene families has been suspected for several of the nuclear encoded subunits of mammalian cytochrome c oxidase, usually because of hybridization of suitable cDNA probes to multiple, relatively high-molecular weight bands in Southern blots of genomic DNA restricted with different restriction endonucleases [29]. In a few cases, chromosomal locations were determined and multiple loci defined; thus, human COXIV-L1 maps to 14q21-q32, and human COXIV-L2 to 16q22-q24 [30]. Genes and pseudogenes had previously been characterized by restriction digestion and nucleic acid sequencing only for subunits IV and VIc [31,32,33]. Finally, characterization of two different cDNAs has indicated the existence of at least two genes for some of the nuclear encoded subunits of the mammalian enzyme; such is the case of rat subunit VIa [34], and bovine subunit VIII [35].

Our data demonstrate the existence of a multigene family for human cytochrome c oxidase subunit VIb; we have characterized several different loci with respect to their size and chromosomal location, and partially sequenced two pseudogenes. The data suggest that an

active gene belonging to the same family may code for a precursor with an N-terminal extension; characterization of the active gene will be required to provide a more definitive answer.

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IV. DISCUSSION AND CONCLUSIONS

Cytochrome c oxidase is one of the most intensively studied membrane proteins, because of its ubiquitous and critical function in the metabolism of the cell, and because of the challenges that it offers for investigators in a wide range of disciplines. In several respects, the knowledge of this enzyme has advanced further than that of other membrane proteins, so that cytochrome c oxidase is becoming a model among them, a role similar to that of hemoglobin for soluble proteins.

Through this thesis work we have focused on two of the currently most interesting study areas concerning this complex enzyme, which are the tissue specificity and molecular basis of the expression of mammalian cytochrome c oxidase. The existence of patients with cytochrome c oxidase deficiency in whom the defect is not expressed in every tissue, prompted us first to look for a clue to explain it in the bovine model, and then to investigate the molecular basis of the expression of one of the subunits of the human enzyme.

It was known that three of the subunits of the bovine enzyme occur as tissue-specific isoforms between liver and heart; however, since compromise of a single tissue such as muscle, or multisystem disease predominantly affecting brain, had been described in humans, we wished to further characterize the bovine model to find out whether or not a peculiarity in enzyme from bovine brain, kidney and skeletal muscle offered a reasonable explanation. Later on, when

it became evident that the expression of the human subunits may differ from that in beef in terms of tissue-specific patterns, the need to improve the characterization of the human model was clear.

For studying the bovine enzyme, we used SDS-PAGE and N-terminal peptide sequencing. Our results demonstrate similar electrophoretic migrations of the three tissue-specific subunits in bovine heart and skeletal muscle on the one hand, and in liver, kidney and brain on the other. Furthermore, sequence analysis shows clearly that the brain and kidney isoforms of those subunits differ from their counterparts in heart, and that the skeletal muscle isoforms differ from those in liver.

Because of limitations inherent to the methods used, we could not rule out additional differences in the sequence beyond the N-terminus; this would require complete sequencing of every subunit. Likewise, the possibility that more than one isoform of a given subunit is expressed in a single tissue cannot be ruled out completely, although no evidence for this has been obtained by gel electrophoresis, Western blotting and peptide sequencing studies in beef.

In conclusion, our data indicate that the isoforms of three subunits that are expressed in bovine heart and skeletal muscle are different from the ones expressed in liver, brain and kidney. They also suggest that the same isoforms are expressed in heart and muscle, and their alternatives in liver, brain and kidney, thus supporting the existence of two tissue-specific isozymes of bovine cytochrome c oxidase.

Recently, evidence was obtained to suggest that human subunit VIII expresses the same isoform in heart and liver. Such a difference compared to the bovine system persuaded us to continue our work by studying the human enzyme. We limited ourselves to subunit VIb, the investigation of its expression in different tissues and the characterization of the gene or genes involved.

We isolated a human heart cDNA whose deduced mature peptide shows strong homology with the bovine subunit, and used it for Northern analysis of RNA from several human tissues, Southern analysis of human genomic DNA, screening of two human-rodent somatic cell hybrid panels, and screening of a human genomic DNA library.

Northern analysis of six different human tissues showed that single-sized transcripts are expressed. We did find some differences between tissues with respect to the abundance of transcripts homologous to the probe in relation to control transcripts; however, we could not at this point conclude that such relative differences reflect tissue-specific differences in the steady-state amount of transcript for human subunit VIb, because the control transcript used is not so well characterized as to rule out tissue-specific differences in its own expression. An alternative would be to use the ribosomal RNA bands as a reference. Furthermore, to really rule out tissue-specific differences at the mRNA level, it is likely that enzymatic amplification of the transcripts and DNA sequencing will be required.

Southern blot analysis of human genomic DNA showed the existence of several fragments homologous to the cDNA. To

investigate whether this corresponded to a single or to multiple genes, we screened two human-rodent somatic cell hybrid panels, and demonstrated that most of the fragments cosegregate with a different human chromosome, thus indicating that a human multigene family exists for cytochrome c oxidase subunit VIb.

Screening of a human genomic DNA library resulted, finally, in the isolation of clones that contain sequences corresponding to two pseudogenes. Sequence analysis of these elements helped to explain a minor difference between the two human cDNAs that have been recovered (from skeletal muscle and heart respectively), and to suggest that the precursor of human subunit VIb may include a cleavable leader sequence. To finally address this issue, the characterization of the active gene or genes of this multigene family is necessary; at that point, subchromosomal location studies by in situ hybridization will probably be done. It is likely that the isolation of the active gene will take advantadge not only of classical methods, but also of techniques based in enzymatic amplification to obtain intronic probes, or to sequence regions adjacent to the coding one by "inverted" PCR.

In conclusion, results of our approach to the molecular basis of the expression of human cytochrome c oxidase subunit VIb do not provide evidence of tissue-specific expression, although they suggest that this may be the case for the steady-state levels of transcript. They demonstrate that a multigene family related to this subunit exists in humans. Finally, they suggest that it is possible

that the human precursor for human subunit VIb does have a cleavable leader sequence.

Further studies are necessary to characterize this multigene family; this will open the door for the investigation of the control of expression of its members, which should help to clarify the "big picture" concerning the expression of human cytochrome c oxidase.

V. APPENDIX

Figure A.1:

Southern blot analysis of human genomic DNA from six unrelated individuals with the human heart cDNA for cytochrome c oxidase subunit VIb. The restriction endonucleases used are indicated above each panel. Molecular weight standards sizes are shown on the left. The blot had been washed at low stringency before autoradiography.

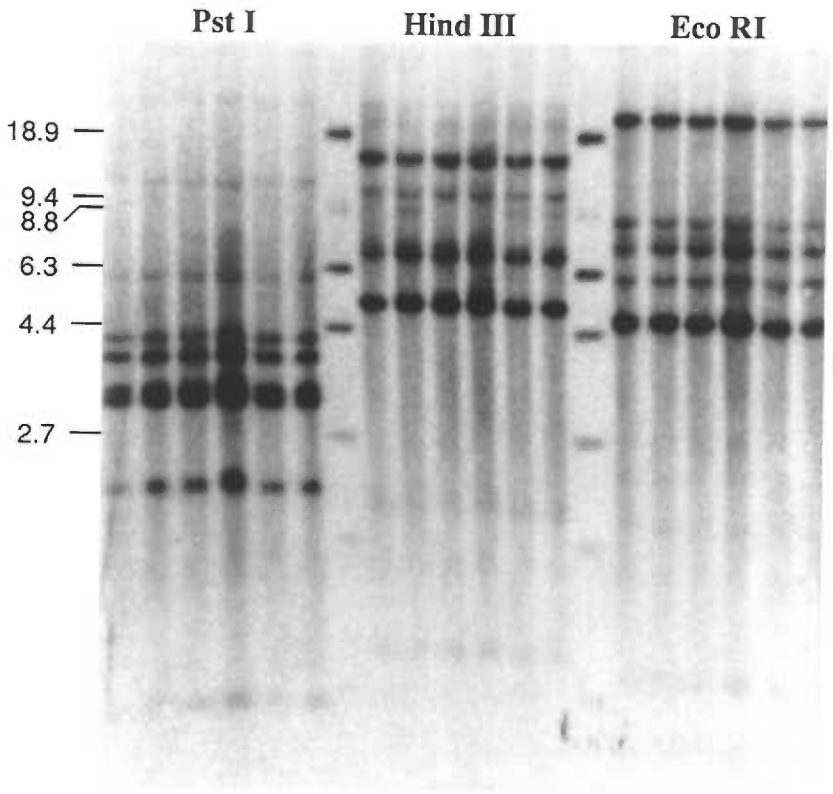
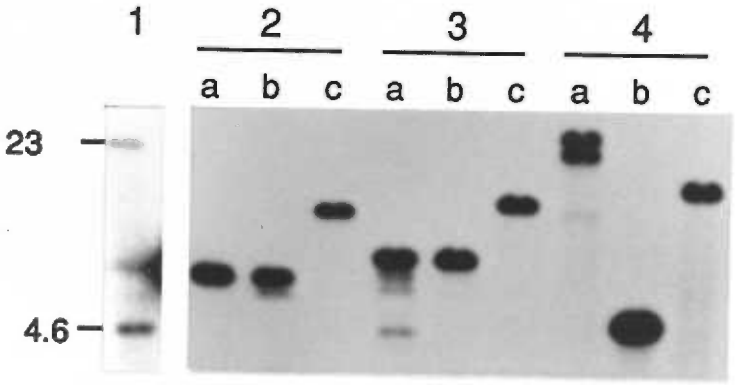

Pst I
Hind III
Eco RI
18.9
9.4
8.8
6.3
4.4
2.7

Figure A.2

Southern blot analysis of human genomic clones 5b (2), 6a (3) and 8b (4). Clone 8b is identical by restriction digestion to 4a. Eco RI (a), Eco RI/Sal I (b) and Sal I (c) digests were used. An Eco RI digest of human genomic DNA (1) was run in parallel, and that section of the gel overexposed. Hybridization conditions were similar to those used during the somatic cell hybrid panel screening; we attribute the absence of weekly hybridizing fragments in lane 1 to underloading.


1
2
3
4
a b c a b c a b c
23
4.6