A Gene Specifying Subunit VIII of Human Cytochrome c Oxidase Is Localized to Chromosome 11 and Is Expressed in Both Muscle and Non-muscle Tissues*

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Subunit VIII of mammalian cytochrome c oxidase (COX; EC 1.9.3.1) exists in at least two isoforms, because different but related polypeptides have been identified in COX isolated from liver and heart of both beef and pig. We have isolated a full length cDNA specifying subunit VIII of human COX from a human liver cDNA library. Sequences hybridizing to this cDNA are present at only one site, the COX8 locus, on human chromosome 11q12-q13. The deduced human polypeptide is 58% identical with COX VIII isolated from beef liver, but only 38% identical with COX VIII isolated from beef heart. Transcriptional analysis shows that an mRNA identical with the isolated cDNA is present in abundant amounts not only in human and monkey liver tissue, but in heart and skeletal muscle as well, tissues not known previously to contain this isoform. Since the only COX VIII subunit found in human heart agrees 100% with the polypeptide deduced from this cDNA, it may well be that, in distinction to other mammals, only one form of COX VIII exists in primates.

Cytochrome c oxidase (COX)1 is the terminal enzyme of the respiratory chain, coupling the transfer of electrons from cytochrome c to molecular oxygen with the concomitant production of a proton electrochemical gradient across the inner mitochondrial membrane (1, 2). In addition to three mitochondrial encoded subunits (3), which correspond to the three subunits of the prokaryotic enzyme (4) and which perform the catalytic function, the eukaryotic enzyme contains a number of additional nuclear-encoded smaller subunits, ranging from four in Dictostelium discoideum (5) to ten in mammals (6-9). The function of the nuclear-encoded subunits is still unknown, although it has been proposed that they might be involved in the modulation of the catalytic function (6-8, 10, 11). This possibility is also in agreement with the results of Bisson and Schiavo (12), who have shown that alternative forms of COX subunits are expressed in different phases of the cell cycle of D. discoideum.

In higher organisms, COX exhibits a tissue-specific plasticity, as indicated by the different kinetic and biochemical properties of the enzyme isolated from different tissues (13, 14) and by the presence of human COX deficiency diseases in which the biochemical defect and clinical involvement are limited to one or a few tissues (15-17). At the molecular level, the tissue specificity of COX appears to reside in the nuclear-encoded subunits (6-8) and not in the mitochondrial-encoded subunits, as there is no evidence of tissue-specific differences in the mitochondrial genome.

There is strong evidence that subunits VIa, VIIa, and VIII (nomenclature of Kadenbach et al. (18)) have liver- and heart-specific isoforms in cows, pigs, and rats (6-8). In particular, COX VIII isolated from mammalian heart differs from the same subunit isolated from liver in its electrophoretic migration, thiol content, and immunological properties (19-22). The amino acid sequences of COX VIII isolated from beef heart and liver show the presence of two different but related polypeptides which are 60% identical in the sequenced region (23, 24). Amino acid sequences of the N-terminal regions of COX VIII isolated from pig heart and liver show a similar result (18). As part of an ongoing interest in the molecular basis of the COX deficiency disorders and in the tissue-specific nature of COX activity (25-27), we have isolated a full length cDNA specifying subunit VIII of human COX and have determined the chromosomal localization of the cox VIII gene.

MATERIALS AND METHODS

Enzymes and Reagents—Restriction enzymes were from Boehringer Mannheim, New England Biolabs, Bethesda Research Laboratories, and BI; the oligonucleotide "random-primer" labeling kit was from Boehringer Mannheim; the Sequenase sequencing kit was from United States Biochemicals; sequencing primer was from New England Biolabs; hybridization reagents were from Sigma; [α-32P]dNTPs (800 Ci/mmol) were from Du Pont-New England Nuclear.

Isolation and Characterization of cDNA Clones—Using a nick-translated (28) cDNA-encoding subunit VIII of rat COX isolated from a rat hepatoma cDNA library (29) as a probe, we screened 60,000 recombinants from a Agt11 human liver cDNA library (a kind gift of Dr. G. Ricca). Hybridization was at 42 °C, and posthybridization washing was at 50 °C, as described (25). We obtained one positive clone (XhCOX8.31) with an insert of about 500 bp. The phage DNA was isolated by the plate-by-sequence method (30); the insert was excised from the phage vector by digestion with EcoRI and was subcloned into appropriately digested M13 (31) vectors prior to DNA sequencing (32) with the Sequenase kit (United States Biochemicals). The se-
and mature polypeptide (shaded box) coding regions are shown, nucleotide sequencing strategy is shown below the and are not present in the COXVII mRNA. The EcoRI sites are linkers derived from the cDNA library (Fig. 1). Using the random-prime labeled (34) EcoRI insert of XhCOX8.21 was subcloned into the EcoRI site of the clone from (Mucaca fuscata) tissues by the guanidinium isothiocyanate/cesium chloride method (35). Northern blot and S1 nuclease protection analyses—Total RNA was prepared from human and monkey (Macaca fascicularis) tissues by the guanidinium isothiocyanate/cesium chloride method (36). Northern blot and S1 nuclease protection experiments were performed as described (34). For S1 analysis, the EcoRI insert of XhCOX8.21 was subcloned into the EcoRI site of M13mp11 and homogeneously labeled single-stranded probes (36) were used in both sense (control) and anti-sense (test) orientations, as described (25).

Chromosomal Assignment—The origin and the characterization of the somatic cell hybrids between Chinese hamster V79/380-6 cells and human diploid leukocytes, lymphoblastoid cells, or fibroblasts have been summarized elsewhere (37). The hybrids used for this study were derived from seven independent fusion experiments. Their chromosomal content was determined at the time of DNA extraction by cytogenetic analysis as well as by gene marker studies. The various hybrids used for regional mapping of COX8 on chromosome 11 have been described (38). Restriction enzyme digestion, electrophoresis, Southern transfer, and blot hybridization were carried out by standard methodology with modifications as described (38). Probe pCOX8.34 contains the EcoRI insert of XhCOX8.21 inserted into the EcoRI site of Bluescribe M13+ (Stratagene). A 250-bp BamHI-EcoRI linker subfragment from the 3′ end of the insert of pCOX8.31 (see Fig. 1), labeled by random oligomer priming (34), gave single-copy signals with little background and was used for all mapping experiments. For the in situ hybridization to human metaphase chromosomes, probe pCOX8.21 was labeled by nick translation (28) using [3H]dATP, [3H]dCTP, and [3H]dTTP to a specific activity of 2 x 10^6 cpm/μg. Hybridization, washes, and staining were carried out as described (39) with modifications (40).

RESULTS

Isolation of cDNA Clones Encoding Subunit VIII of Human COX—Using a partial length cDNA clone specifying rat COX subunit VIII (29), we screened a human adult liver cDNA library at low stringency and isolated one hybridizing clone, designated XhCOX8.31. The EcoRI insert of the clone contains (Fig. 2) a 41-bp 5′-untranslated region; a 210-bp open reading frame, beginning with an ATG codon at nucleotide position 42 situated in a favorable context for initiation of translation (41), which encodes a deduced polypeptide 69 amino acids in length, plus a TGA stop codon; and a 221-bp 3′-untranslated region, containing an AATAAA polyadenylation signal (42) 14 bp upstream from a 7-μl-long poly(A) tail. The deduced polypeptide appears to represent the precursor to subunit VIII of human COX, as the 44 C-terminal amino acids are 58% and 63% identical with the liver isoform of subunit VIII of rat COX (29) and cow COX (30), respectively. For the in situ hybridization to human metaphase chromosomes, probe pCOX8.21 was labeled by nick translation (28) using [3H]dATP, [3H]dCTP, and [3H]dTTP to a specific activity of 2 x 10^6 cpm/μg. Hybridization, washes, and staining were carried out as described (39) with modifications (40).

Fig. 1. Map and sequencing strategy of the human full length cox VIII cDNA. The presumed presquence (hatched box) and mature polypeptide (shaded box) coding regions are shown, flanked by the 5′- and 3′-untranslated regions (single lines). The nucleotide sequencing strategy is shown below the map; arrows denote the direction and extent of each sequence. Relevant restriction sites are shown. The EcoRI sites are linkers derived from the cDNA library and are not present in the cox VIII mRNA.

Fig. 2. A, nucleotide (5′ to 3′ in the mRNA sense) and deduced amino acid (N- to C-terminal) of the human (H) full length and rat liver (R) partial length (29) cox VIII cDNAs. Dashes were inserted to maximize homology. Dots indicate sequences absent in the truncated rat cDNA. The EcoRI inserts flanking the cox VIII insert of XhCOX8.31 are not shown; the HindIII and BamHI sites used in the sequencing are underlined. The deduced amino acid sequence from the human cDNA is shown below its sequence; only those amino acids in the rat liver sequence that differ from the human are shown, above its cDNA sequence. Nucleotides of the human cDNA are numbered at the side; amino acids are numbered below the lines. The polyadenylation signal is overlined. B, alignment of four mammalian COX VIII subunit polypeptides. Dashes and dots as in A.
In this case the targeting signal is part of the mature protein. The targeting signal is contained in this C-terminal extension or is embodied in the rest of the mature polypeptide. A single hybridizing band was present in all tissues, with a size (about 500 nucleotides) comparable to that of the liver clone. Although equal amounts of RNA were loaded, the intensity of the hybridizing signal varied among tissues, with the strongest signal present in heart and skeletal muscle. An essentially identical pattern was obtained when the cDNA encoding a non-tissue-specific human COX subunit, COX Va (26), was used as a probe (Fig. 5, top). Thus, the difference in hybridizing signal among tissues seems to correlate with the steady state level of transcription of COX subunit genes in and adult heart tissue. Two hybridizing clones were isolated: a nearly full length clone (λhCOX8.21; 447-bp cDNA insert) from the fetal muscle library and a partial length clone from the adult heart library (λhCOX8.51; 318-bp insert). The nucleotide sequences of the two clones, isolated from libraries extracted from tissues not known to contain the liver isoform of subunit VIII, were the same as that of the liver clone.

**Gene Mapping of Subunit VIII of Human Cytochrome c Oxidase**—In genomic Southern hybridization analysis using DNA digested with BglII, we detected a single hybridizing human band approximately 21 kb in size and two Chinese hamster bands of 10.5 and 6.8 kb (Fig. 4A); in a panel of Chinese hamster × human somatic cell hybrids, only hybrids containing human chromosome 11 showed the 21-kb human BglII fragment (Fig. 4A, lanes 2 and 4). After digestion with PstI, a single human fragment was detected, while digestion with HindIII generated two fragments; in a panel of 12 Chinese hamster × human somatic cell hybrids whose DNA was digested with PstI or HindIII, the human-specific cosVIII restriction fragment was concordant with human chromosome 11 (not shown). All other chromosomes were excluded by two or more discordant hybrids (Table I).

In a regional mapping panel, the human cosVIII signal was present in hybrids that contained region 11p11.2-qter and was absent in three independent hybrids containing the short arm of chromosome 11. The result assigns the cosVIII gene to the long arm of chromosome 11 (Fig. 4B). We designate this locus COX8. In order to confirm this regional assignment and more precisely define the COX8 localization, we performed in situ hybridization to human metaphase chromosomes. Initially 66 randomly chosen cells were analyzed for localization of grains on chromosome 11. Among the total 233 grains (3.5 grains/cell), 15 grains (6%) were located at the specific site 11q12-q13. Of the 66 cells, 14 (21%) had label at this site. In addition, 38 cells were selected for the presence of a grain anywhere on chromosome 11. In these cells, 39 grains were found on chromosome 11, of which 18 (46%) were located at the specific site 11q12-q13 (Fig. 4C). These results demonstrate localization of COX8 on the proximal long arm of chromosome 11 and suggest the existence of only a single gene for COX VIII in the human genome.

The localization at 11q12-13 places COX8 in the vicinity of loci for C1 complement inhibitor, which, when defective, is responsible for hereditary angioedema; the human homolog of the INT2 integration site for the murine mammary tumor virus oncopogene, which is a member of the fibroblast growth factor family; the BCLI gene that is rearranged in translocations in chronic B lymphocytic leukemia; the human homolog of the SEA avian erythroblastosis oncogene; and a cluster of pepsinogen genes (47).

**Transcriptional Analysis of cosVIII**—We performed Northern analysis of cosVIII transcription in several monkey and human tissues, including heart and skeletal muscle, tissues which, in other mammals, are devoid of the liver isoform polypeptide. A single hybridizing band was present in all tissues, with a size (about 500 nucleotides) comparable to that of the insert of λhCOX8.31 (Fig. 5, bottom). This result indicates that this cosVIII cDNA is essentially full length. Although equal amounts of RNA were loaded, the intensity of the hybridizing signal varied among tissues, with the strongest signal present in heart and skeletal muscle.

**Fig. 3. Hydropathy profiles of human liver-type COX VIII, beef heart COX VIII, and the analogous yeast COX VIIa.** The hydropathy plots were calculated according to the algorithm of Chou and Fasman (53) using the Pustell software package (IBI). Positive and negative values denote hydrophobic and hydrophilic regions, respectively. The deduced presequence of human COX VIII is denoted by negative amino acid numbering.
**Human COX VIII cDNA**

**Fig. 4. COX8 chromosomal assignment.** A, BglII-digested DNA from human (H) and Chinese hamster (CH) parental cell lines, and of H × CH hybrid cell lines (lanes 1–4), hybridized with COX VIII probe. The cell hybrids in lanes 1 and 3 are negative, and those in lanes 2 and 4 are positive for the human fragment. B, regional assignment of COX8 to the long arm of chromosome 11. Rodent × human somatic cell hybrids with defined regions of human chromosome 11 (represented by vertical bars A–D) were analyzed for hybridization with COX VIII probe on Southern blots containing DNA digested with HindIII or PstI. Only hybrids with region C were positive; hybrids with regions A, B, and D were negative. The localization of COX8 based on these experiments is shown by the bracket on the right. C, localization of silver grains with respect to standard G-bands on chromosome 11 after in situ hybridization of tritium-labeled COX VIII probe to normal human metaphase spreads. Dots indicate the sites of label observed in 38 cells selected for the presence of grains on chromosome 11. Crosses indicate localization of grains in 66 unselected cells. Based on these results, we assigned COX8 to either band 11q12 or the proximal part of band 11q13.

**Table I**

| Hybridization/chromosome | 1 +/+ | 2 -/- | 3 +/− | 4 −/+ | 5 +/+ | 6 −/− | 7 +/− | 8 −/+ | 9/10/11/12/13/14/15/16/17/18/19/20/21/22X | Discordant hybrids | Informative hybrids |
|---------------------------|-------|-------|-------|-------|-------|-------|-------|-------|---------------------------------|------------------|------------------|
| 1 +/+                     | 2     | 1     | 2     | 0     | 1     | 4     | 1     | 2     | 0                              | 6                | 6                |
| 2 -/-                     | 4     | 4     | 2     | 2     | 4     | 3     | 2     | 3     | 5                              | 6                | 5                |
| 3 +/−                     | 4     | 5     | 3     | 4     | 5     | 2     | 4     | 3     | 5                              | 0                | 5                |
| 4 −/+                     | 2     | 1     | 4     | 3     | 1     | 3     | 1     | 4     | 3                              | 1                | 2                |

**Fig. 5. Northern analysis of total RNA (15 μg) isolated from monkey or human brain (B), liver (L), lung (Lu), heart (H), kidney (K), skeletal muscle (M), and uterus (U).** A single filter was probed with random primer-labeled full length cDNAs encoding COX Va (27), which migrates at about 750 nucleotides (5a, top), and COX VIII, which migrates at about 500 nucleotides (8, bottom).

**Fig. 6. S1 protection analysis of 10 μg of human brain (B), liver (L), heart (H), and muscle (M) total RNA, using the human cox VIII cDNA derived from hCOX8.21 in either the sense (S) or the antisense (A) orientation as a probe.** The sizes of the protected RNAs are consistent with protection of the entire length of the antisense cDNA (arrows). m = markers of XDNA digested with HindIII.

The human cox VIII gene appears to be highly diverged from the analogous gene in other mammals, as our cDNA did not hybridize to any mRNA species in either bovine heart or
liver, even at conditions of very low stringency (data not shown).

Since the level of sensitivity of Northern analysis does not exclude the possibility that the cDNA encoding the liver isoform cross-hybridized to an mRNA encoding a putative COX VIII heart isoform, we performed S1 nuclease protection of mRNA isolated from four different human tissues, using the cosVIII cDNA from AHCox8:21 as a probe. Only an mRNA species of about 450 nucleotides (i.e. of the same size as the entire probe) was protected in RNA isolated from human liver, brain, heart, and skeletal muscle (Fig. 6). Considering the marked difference between the two beef isoforms at the protein level, the mRNA protected in human heart and muscle (which, according to the limit of resolution of this technique, may contain, at most, single-base differences with the probe cDNA) is almost certainly not a related heterologous message encoding the putative human "heart" isoform, but rather must represent the homologous, "liver" isoform. In other words, the mRNA encoding the COX VIII polypeptide represented by our cDNA is expressed in human liver, brain, heart and muscle and, based on the Northern analysis described above, is most likely expressed in all tissues of the body.

**DISCUSSION**

The existence of tissue-specific (heart and liver) isoform polypeptides of subunit VIII of cytochrome c oxidase has been documented in three mammals, beef, pig, and rat. Thus, it was surprising to find that a gene specifying a human liver-type COX VIII polypeptide was actively transcribed in all examined tissues in primates, including humans. Moreover, this transcript is almost certainly translated and assembled into the COX holoenzyme in human heart, because the only form of subunit VIII that has been isolated from human heart has an amino acid sequence that agrees 100% with the polypeptide deduced from our cosVIII cDNA (49). Thus, all the data presently available at the chromosomal, transcriptional, and translational level indicate that only one gene specifying subunit VIII of COX is active in primates and that, in distinction to what has been observed in other mammals, human COX does not appear to express alternative isoforms of this subunit in different tissues.

At least two different mechanisms could account for such a difference among mammals. Based on comparison of the nucleotide sequence of a partial length cDNA encoding the heart-specific isoform of bovine COX VIII with that of our cDNA encoding the human liver-type COX VIII, it is likely that the mammalian heart- and liver-specific isoform genes specifying this subunit arose via a gene duplication event, as both cDNAs share regions of significant sequence identity in their respective 3'-untranslated regions (not shown). Thus, the expression of only a single COX VIII polypeptide in humans and monkeys, but of two different but related isoform polypeptides in other mammals (cows, pigs, and rats), suggests either that the putative gene duplication event that gave rise to the two cosVIII isoform genes occurred after the mammalian radiation (or more specifically, after the divergence of primates from other mammals), or that the duplication event occurred prior to the mammalian radiation, but that in primates the heart isoform gene may have been silenced in a manner similar to the silencing of the β-globin gene in Old World monkeys (50). The high degree of amino acid divergence between beef heart and liver COX VIII (58%) relative to the high degree of nucleotide and amino acid sequence identity between human and bovine COX IV, Va, and Vb (82%, 95%, and 85%, respectively (26)) favors early rather than late divergence. Moreover, gene duplication prior to the mammalian radiation is supported by the presence of liver- and heart-specific isoforms of subunit VIII in chicken (51).

In that case, there may indeed be a second heart-type isoform gene in the human genome. The fact that cosVIII sequences hybridized to a single site on chromosome 11 does not preclude this possibility, as either the locus may contain a multigene family, as is the case with the β-globins, or the two isoform genes may be so highly diverged that they do not cross-hybridize, in which case a heart isoform gene located elsewhere would not be detected in our chromosome mapping experiments using the liver-type cDNA.

Our data, however, do not exclude the possibility that, besides the liver-type COX VIII polypeptide, a second as yet unidentified COX VIII isoform is present in human heart. At the transcriptional level, significant sequence divergence between liver and heart isoform genes could prevent detection of a heart isoform mRNA in both the Northern and S1 protection experiments; at the protein level, it is possible (although unlikely) that another isoform besides the liver isoform is present in human heart, but was not detected using the specific column chromatography and high performance liquid chromatography procedures employed to isolate human heart COX VIII (49).

If a heart-type COX VIII isoform exists and is preferentially assembled into the COX holoenzyme in muscle tissues, as observed in other mammals, the presence of a liver-type mRNA in primate muscle tissues could reflect a low level of "constitutive" expression of this isoform in heart and muscle. This is presumably the case for a truly tissue-specific COX subunit, rat COX VIa. Schlief et al. (52) isolated cDNAs encoding the heart- and liver-specific isoforms of rat COX VIa in Northern analyses with these genes, the liver-specific rat cox VIa probe hybridized to mRNA derived from liver, kidney, heart, and skeletal muscle. On the other hand, the muscle-specific cox VIa probe hybridized only to mRNA isolated from heart and muscle tissue. Thus, transcription of the liver-type gene was apparently not tissue-specific, while the heart-type isoform gene showed a tissue-specific transcription pattern. A problem with this interpretation of our results is that in the case of rat cox VIa, the "inappropriate" isoform (i.e. liver mRNA in heart or muscle) was usually expressed at a lower level than was the "appropriate" isoform (i.e. liver mRNA in liver); in the case of human cox VIII, the intensity of the hybridization signal in heart relative to both the signal in other tissues and to the cox VIa signal indicated that the liver-type cox VIII mRNA is a major transcription product in heart. This result, along with the protein analysis data (49), seems to exclude the possibility that transcription of liver-type COX VIII reflects merely a constitutive low level expression of the liver-type polypeptide in human muscle tissues. However, the possibility also exists that events following transcription (e.g. at the level of translation, transport to the mitochondria, or assembly in the enzyme complex) could reduce substantially the appearance of the liver-type polypeptide in the holoenzyme in heart and muscle.

In order to further investigate the existence of tissue-specific isoform polypeptides of subunit VIII of human COX, we are using both antibodies to bovine heart COX VIII and a cDNA clone encoding this bovine subunit (kind gifts of R. Capaldi, University of Oregon) to search for the corresponding heart-type isoform polypeptide and cDNA in humans.

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