Structure of the Human Cytochrome c Oxidase Subunit Vb Gene and Chromosomal Mapping of the Coding Gene and of Seven Pseudogenes

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Subunit Vb of mammalian cytochrome c oxidase (COX; EC 1.9.3.1) is encoded by a nuclear gene and assembled with the other 12 COX subunits encoded in both mitochondrial and nuclear DNA. We have cloned the gene for human COX subunit Vb (COX5B) and determined the exon-intron structure by both hybridization analysis and DNA sequencing. The gene contains five exons and four introns; the four coding exons span a region of approximately 2.4 kb. The 5’ end of the COX5B gene is GC-rich and contains many HpaII sites. Genomic Southern blot analysis of human DNA probed with the human COX Vb cDNA identified eight restriction fragments containing COX Vb-related sequences that were mapped to different chromosomes with panels of human × Chinese hamster somatic cell hybrids. Because only one of these fragments hybridized with a 210-bp probe from intron 4, we conclude that there is a single expressed gene for COX subunit Vb in the human genome. We have mapped this gene to chromosome 2, region cen–q13.

INTRODUCTION

Cytochrome c oxidase (COX; EC 1.9.3.1), the terminal enzyme of the electron transport chain, transfers electrons from reduced cytochrome c to oxygen, in the process generating an electrochemical gradient across the mitochondrial inner membrane (reviewed in Hahne, 1985; Capaldi, 1988). The mammalian enzyme is composed of 13 polypeptide subunits—3 encoded in mtDNA and 10 in nuclear DNA (reviewed in Kadenzbach et al., 1987; Capaldi, 1988; Chomyn and Attardi, 1987). The mammalian COX subunits can be divided into two groups: those with muscle-specific isoforms (reviewed in Capaldi et al., 1988; Lomax and Grossman, 1989) and those that are identical in all tissues (Yanamura et al., 1988). The nuclear genes for these latter ubiquitous subunits are expressed constitutively and probably represent examples of “housekeeping” genes (Bird, 1986).

It has been possible to map human COX genes by means of Southern blot analysis of DNA from panels of somatic cell hybrids. For example, the single-copy gene for COX subunit VIII (COX8) was mapped to chromosome 11, region q12–q13 (Rizzuto et al., 1989), and the gene for COX subunit IV (COX4) to chromosome 16 region q22–qter (Darras et al., 1987; Lomax et al., 1990). The isolation of a human COX Vb cDNA (Zeviani et al., 1988) provided a probe for cloning and mapping the COX5B gene, whose chromosomal location was obscured by the presence of several COX5B-related genes or pseudogenes. We report here the isolation and DNA sequence of the expressed gene for human COX subunit Vb (COX5B) and the chromosomal location of the expressed gene and seven pseudogenes, as determined by analysis of panels of somatic cell hybrids with cDNA, genomic, and intron probes.

MATERIALS AND METHODS

Materials

Restriction enzymes were from BRL, New England Biolabs, or Boehringer-Mannheim. The DNA labeling system for “random-primer” labeling and [α-32P]dCTP for DNA sequencing were from Amersham. [α-35S]dATP for DNA sequencing were from Amersham. [α-32P]dCTP for random primer labeling was from NEN. The Sequenase DNA sequencing kit was from USB.

Subcloning and Restriction Mapping of the COX5B Gene

Plasmid pCOX5bR3.4 containing the human COX5B gene was constructed by cloning a 3.4-kb EcoRI fragment from the 5′ end of λCOX5B-1 into the
FIG. 1. Restriction maps of the COX5B gene. (A) Restriction map of the human COX5B locus, based on two overlapping genomic clones: λCOX5B-1 and λCOX5B-2. The hatched areas indicate the location and orientation of the COX5B gene, as defined by hybridization. Arrows define the genomic regions contained within each genomic clone. Restriction enzymes: B, BamHI; E, EcoRI; G, BglII; H, HindIII; S, SstI. (B) Restriction map of a 2.6-kb PSTI-EcoRI-fragment of a 3.4-kb EcoRI subclone of λCOX5B-1 (pCOX5bR3.4) encompassing the coding regions and 3′-untranslated region of the COX5B gene. Filled boxes represent coding (translated) exons, open boxes the 3′-untranslated region, and straight lines the introns. The open arrow (designated ALUR) denotes the position and orientation of the AluI repeat in intron 2. Restriction enzymes: E, EcoRI; P, PstI; L, SalI; M, SmaI; N, NotI; O, NcoI; R, RsaI; RV, EcoRV; S, SstI; V, PvuII. Arrows below the restriction map denote the sequencing strategy for the human COX5B gene.

DNA Sequencing

Dideoxy DNA sequencing (Sanger et al., 1977) was performed with Sequenase (USB) on duplex plasmid DNA (Chen and Seeberg, 1985) isolated by the alkaline lysis method (Birnboim and Doly, 1979) from mini-preps or from 250-ml cultures. Primers complementary to the T3 and T7 promoter sequences in BlueScriptKS was used to sequence pCOX5bR3.4 and its deletion subclones. RsaI subclones in pUC13 were sequenced using the M13 universal and reverse sequencing primers. Synthetic oligonucleotide primers (20-mers) were used to obtain the sequence of complementary strands in certain regions; they were synthesized in the University of Michigan DNA Synthesis Facility on an Applied Biosystems DNA synthesizer. DNA sequences were stored and analyzed using the ASSEMGEL program of PC/GENE (IntelliGenetics, Mountain View, CA).

Somatic Cell Hybrids

A panel of 16 hybrid clones derived from seven independent fusion experiments between Chinese hamster and human cell lines (for summary see Yang-Feng et al., 1986) was used for primary chromosomal assignment in human. Four rodent × human hybrids from series X, XVIII, and XIX containing different regions of human chromosome 2 were used for regional mapping (Alonso et al., 1988; Barton et al., 1989).

Probes

Two probes were used for primary mapping: Probe 1—a human pCOX5b.222 cDNA clone of 480 bp (Zeviani et al., 1988); and Probe 2—a 1.2-kb SstI–EcoRV fragment from genomic clone pCOX5bRS2.0 (the SstI deletion clone of pCOX5bR3.4, which retained the 2.0-kb region from SstI to EcoRI). Probe 2 contains exons 3 and 4, portions of introns 2 and 4, and all of intron 3 of human COX5B. For chromosomal assignment of the COX5B coding sequence, a 210-bp RsaI fragment (1402–1612, Fig. 2) containing a portion of intron 4 of the COX5B gene (Probe 3) was used. The probes were isolated in 0.8% low-melting-point agarose and labeled with [α-32P]dCTP by the random priming labeling method (Feinberg and Vogelstein, 1983).

Genomic Southern Blot Analysis

Human genomic DNA isolated from blood leukocytes was digested with the indicated restriction enzymes. DNA fragments (10 μg) from each digest were
FIG. 2. DNA sequence of the human COX5B gene. The nucleotide sequence of 2593 bp of genomic DNA containing exons 2 through 5 and introns 2 through 4 is presented, starting in intron 1 at the PstI site, which is designated nt 1, and continuing to the EcoRI site beyond the gene. Coding regions are underlined and the deduced amino acid sequence is presented above the DNA sequence. Consensus B-donor and 3'-acceptor splice sites, including the two potential 3'-acceptor sequences preceding the ATG initiation codon, are overlined. The Ah repeat sequence extending from base 521 to 820 is underlined.

separated by electrophoresis on 0.8% agarose gels, denatured, and transferred to Nytran nylon membranes (Schleicher & Schuell) by blotting. Southern blot hybridization analysis was performed at 42°C for 18–24 h in 50% formamide, 5× SSPE, 1% SDS, 50 μg/ml denatured salmon testis DNA with 32P-labeled cDNA probe. After hybridization, membranes were washed at 60°C with decreasing concentrations of SSC containing 0.1% SDS. Restriction enzymes HindIII and PstI were used for primary mapping, and HindIII was used for regional mapping. DNA from each of the hybrid and parental control cell lines was digested with enzyme, and then fragments were separated on 0.8% agarose gels and transferred to Hybond-N membranes by the method of Southern (1975). The conditions for prehybridization, hybridization, and washing of filters were described previously (Hsieh et al., 1990). All filters were exposed to Kodak X-Omat AR films at −70°C for varying lengths of time.

RESULTS

Isolation of Genomic Clones for Human COX Vb

Southern blot analysis of human genomic DNA digested with PstI revealed many fragments that hybridized with the pCOX5b cDNA (Zeviani et al., 1988). We therefore anticipated that a genomic library would contain clones encompassing different genomic regions, including several pseudogenes. To isolate the human COX5B gene, we screened a human genomic library (Lawn et al., 1978) containing Alu–HaeIII partial digest fragments of human DNA
cloned into the EcoRI site of bacteriophage λCharon4A (obtained from A. S. Lee with permission of Dr. T. Maniatis, Harvard University). Approximately $10^6$ plaques were plated and screened by plaque hybridization techniques according to standard procedures (Benton and Davis, 1977) with Probe 1, the full-length human COX Vb cDNA insert from pCOX5b (Zeviani et al., 1988), as hybridization probe. Phage DNA was isolated from small-scale lysates (Maniatis et al., 1982) of the six plaques that gave duplicate hybridization signals and characterized by restriction mapping and Southern blot analysis with the pCOX5b cDNA (Probe 1), as hybridization probe. Phage DNA was isolated from small-scale lysates (Maniatis et al., 1982) of the six plaques that gave duplicate hybridization signals and characterized by restriction mapping and Southern blot analysis with the pCOX5b cDNA (Probe 1). EcoRI digestion revealed only two types of clones, designated λCOX5B-1 and λCOX5B-2 (Fig. 1A). Southern blot analysis of phage DNA revealed that each clone contained an approximately 6-kb PstI fragment that hybridized with the cDNA; therefore every clone isolated in this screen contained only one genomic region. Southern blot analysis with hybridization probes encoding either the 5' end (a 115-bp EcoRI–Neol fragment) or the 3' end (a 100-bp PvuII–EcoRI fragment) of the cDNA indicated that both λCOX5B-1 and λCOX5B-2 contained the entire COX5B coding region. Further restriction mapping confirmed that the two clones represented overlapping clones of the same genomic region (Fig. 1A). We therefore subcloned a 3.4-kb EcoRI fragment from the 5' end of λCOX5B-1 for more detailed analysis.

The exon–intron structure of the COX5B gene and the sequencing strategy used are presented in Fig. 1B. This 3.4-kb EcoRI fragment contained the four coding exons, designated exons 2, 3, 4, and 5. The sequence of these four exons and that of the three introns are presented in Fig. 2. Exon 2 encodes the 31 amino acids of the presequence (amino acids -31 to -1) plus amino acids 1 to 3 of mature COX Vb; exon 3 encodes amino acids 3 to 28; exon 4 encodes amino acids 29 to 62; and exon 5 encodes amino acids 62 to 97 plus the 83-bp 3'-untranslated region. The DNA sequence of the coding exons and the 3'-untranslated region in this genomic region is essentially identical to the sequence of the cDNA, confirming that this gene encodes the pCOX5b cDNA. The only discrepancy between the genomic and the cDNA sequences occurred in exon 5. The sequence we determined for codons 77–78 of the human COX5B gene is GGC–GAC, which predicts the sequence Gly–Glu, whereas the published cDNA sequence is GGG–CAG or Gly–Gln. Since the bovine (Zeviani et al., 1988), rat (Goto et al., 1989), and mouse (Basu and Avadhani, 1990) cDNAs have GAC (Glu) at position 78, we assume that our genomic sequence (GGC–GAC) is correct and that the sequence (GGG–CAG) in the cDNA probably represents a sequencing error.

All intron–exon junctions of the COX5B gene (Fig. 2) conformed to the consensus splice junction sequences (Sharp, 1981; Padgett et al., 1986), except for the 3'-splice junction preceding exon 2. The sequence preceding the ATG initiator Met codon in the pCOX5b cDNA is (T),,CCA-ATG; only the CA (nucleotides 42–43, Fig. 2) preceding the ATG initiator Met codon is present in the genomic sequence at the end of intron 1. There is no consensus 3'-splice sequence preceding the CA, although there are at least two potential 3'-acceptor splice sequences just upstream at nucleotides 19–20 and 31–32. This observation and the poly(dT) tract in the 5'-untranslated region of this cDNA and other cDNAs isolated from this human endothelial cell library (e.g., see Fabrizi et al., 1989) suggested that the cDNA contained a cloning artifact at the 5' end. Additional support for this hypothesis is that the genomic sequence preceding the ATG initiation Met codon agrees well with the consensus sequence proposed by Kozak (1987) for initiation of translation of eukaryotic genes, suggesting that this sequence should be part of exon 2. This artifact in the cDNA precluded mapping exon 1 (encoding the 5'-untranslated region) by hybridization with the pCOX5b cDNA.

**Chromosomal Mapping**

Southern blots of human genomic DNA probed with the pCOX5b cDNA (Probe 1) revealed several
FIG. 4. Mapping of COX5B sequences. Hybridization of human COX5B cDNA (Probe 1) to PstI-digested (A) and of the genomic exon/intron Probe 2 to HindIII-digested (B) Chinese hamster × human somatic cell hybrid and control DNA revealed discordant segregation of all human fragments. The chromosomal assignments for each band are marked on the left, and sizes in kb on the right. (A) The closely migrating 7.6- and 7.9-kb human fragments are separated in the hybrids: lanes 3, 4, 6, 10, and 11 are positive for the 7.6-kb band. Similarly, the 6.4-kb human fragment containing the COXSB coding gene is separable from the 6.2-kb pseudogene fragment. Hybrids positive for the 6.4-kb fragment are in lanes 3, 8, and 11. (B) The heavy 15-kb HindIII fragment appeared to cosegregate with human chromosomes 2 and 4. After hybridization of the same filter with the intron Probe 3, the 15-kb fragment was detected in lanes 3, 4, and 9 but not in lane 6. These results led us to conclude that there are two overlapping 15.0-kb HindIII fragments that map to chromosomes 2 and 4, with that on chromosome 2 containing the COXSB coding gene. Based on comparison with results obtained with the PstI-digested panel, two 7.0-kb HindIII fragments were postulated and assigned to chromosomes 7 and 12.
<table>
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<tr>
<th>Locus</th>
<th>hybrids</th>
<th>% Discordant hybrids</th>
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<tr>
<td>COX5B</td>
<td>38 0 58 20 42 46 27 54 17 38 45 31 45 33 54 50 46 42 38 54 50 69 33</td>
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<tr>
<td>COX5BP1</td>
<td>31 25 42 0 50 38 18 46 25 31 55 23 55 42 31 42 54 50 31 62 42 46 33</td>
<td></td>
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<td>COX5BP2</td>
<td>38 42 42 20 42 0 64 54 58 38 36 15 64 50 38 33 46 42 38 23 17 23 50</td>
<td></td>
</tr>
<tr>
<td>COX5BP3</td>
<td>46 25 58 20 33 54 0 62 25 15 36 38 55 58 46 38 38 67 46 46 58 62 33</td>
<td></td>
</tr>
<tr>
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<td>46 50 67 70 50 38 45 62 67 46 0 38 82 75 62 58 38 50 46 31 17 46 33</td>
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<tr>
<td>COX5BP5</td>
<td>23 26 42 20 65 15 46 54 42 38 36 0 73 50 30 33 54 42 38 38 17 38 33</td>
<td></td>
</tr>
<tr>
<td>COX5BP6</td>
<td>69 50 50 50 33 62 55 38 33 54 82 77 0 42 38 58 46 50 54 54 83 54 83</td>
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<tr>
<td>COX5BP7</td>
<td>31 58 58 40 42 23 55 31 60 46 65 38 55 60 58 31 42 38 50 43 31 30 0 50</td>
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*Note. Data for chromosomes that are structurally rearranged (P, R, or ?) or present in fewer than 10% of cells (L) were excluded from calculation of percentage discordant hybrids. */

Hybrids used only in the PstI panel were also excluded.
FIG. 5. Regional mapping of COX5B coding gene. Hybridization of 32P-labeled human intron probe (Probe 3) to a Southern blot of HindIII-digested DNA from rodent × human hybrid cell lines and controls. Lane 1, human diploid lymphoblastoid cells; lane 2, Chinese hamster cells V79/380-6; lane 3, rat hepatoma cell line; lanes 4–9, rodent × human hybrid cell lines. Lanes 5 and 8 contain the 15.0-kb human COX5B fragment. Lanes 4 and 9 (Chinese hamster × human hybrids) have no detectable COX5B signal. Lanes 6 and 7 contain only the 4.4- and 3.2-kb rat signals. DNA in lanes 4–7 was extracted from hybrids that have retained different regions of human chromosome 2; lane 4, hybrid C; lane 5, hybrid B; lane 6, hybrid A; lane 7, hybrid D (Fig. 6). DNA in lane 8 was from hybrid that has retained an intact human chromosome 2, and that in lane 9 had no chromosome 2.

ally COX5BP1 and COX5BP4, respectively (data not shown).

DISCUSSION

Structural Features of the COX5B Gene

The COX5B gene is composed of five exons and four introns. Exon 1, which has not yet been localized, encodes only the 5'-untranslated region; exon 2 the presequence; and exons 3, 4, and 5 the remainder of the cDNA. It has been suggested that exons encode functional domains of proteins; thus it is sometimes possible to predict the exon–intron structure of a gene on the basis of functional domains of the protein. One prominent feature of most proteins destined for mitochondria is a short (20–40-amino-acid) basic N-terminal extension (presequence) that directs precursors to the mitochondrial outer membrane. Thus far, all genes for mitochondrial proteins with presequences have separate exons encoding the presequence. In the COX5B gene, this functional domain also is encoded by a separate exon, exon 2, as in the bovine COX4 gene, the only other characterized COX gene encoding a subunit with a presequence (Bachman et al., 1987). Goto et al. (1989) recently reported the sequence of a rat COX subunit Vb cDNA, which they assumed to be synthesized without a presequence, based on the fact that the rat cDNA, like the human pCOX5b cDNA, had an ATG Met codon preceding the sequence of the mature protein. However, the 54-bp rat sequence preceding this Met is an open

TABLE 2

<table>
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<th>Gene name</th>
<th>Chromosome</th>
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<th>HindIII</th>
</tr>
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<td>COX5B</td>
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<td>15.0</td>
</tr>
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<td>5.2</td>
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<td>COX5BP7</td>
<td>22</td>
<td>6.2</td>
<td>16.0</td>
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reading frame with a high degree of both nucleotide
(82%) and amino acid sequence identity (84%) to the
human sequence. A longer mouse cDNA (Basu and
Avadhani, 1990) also contains an in-frame Met codon
upstream, thus predicting a presequence. We con-
clude, therefore, that the rat clone is a partial cDNA
lacking the 5'-untranslated region and the first 12 co-
dons of the presequence.

The human cytochrome c1 gene is the only gene in
which two exons encode the presequence (Suzuki et
al., 1989); however, because the cytochrome c1 pre-
cursor is processed in two separate steps, this two-exon
structure still correlates with the two functional do-
mains of the presequence. Thus, Gilbert’s hypothesis
for exon shuffling (Gilbert, 1985) appears to be valid
in this case.

An additional feature common to most nuclear-
coded COX subunits is a hydrophobic, putative mem-
brane-spanning region flanked by hydrophilic N-ter-
minal and C-terminal domains. COX subunits Va and
Vb, however, are the two nuclear-coded COX sub-
units that have no such hydrophobic, membrane-
spanning regions; it has also been determined experi-
mentally that these two subunits do not span the mito-
chondrial inner membrane (Zhang and Capaldi,
1989). Thus, it was not possible a priori to predict the
intron–exon structure of this gene.

None of the four introns of the COX5B gene se-
quenced thus far is larger than 1 kb. The largest, in-
tron 2, contains an Alu repeat (nts 513–820, Fig. 2)
with 92% sequence identity to the consensus genomic
Alu repeat sequence (Schmid and Shen, 1985). This
repeat is more similar to the consensus sequence for
the older Alu repeat family than to the more recently
evolved repeat family (Deininger and Slagel, 1988)
and also contributes to the high G–C content of the
intron. Intron 3 contained several interesting repeats,
a direct repeat of CTTCTG near the beginning of the
intron (nts 1111–1124, Fig. 2) and several direct re-
peats of TTGTTTT near the 3' end (nts 1256–1288,
Fig. 2) which could potentially be used to develop
PCR markers to detect RFLPs.

The COX5B Gene May Be a Housekeeping Gene

Proteins that are essential components of interme-
diary metabolism and are found in most cell types are
said to be encoded by “housekeeping” genes. Bird
(1986) noted that housekeeping genes have several
common structural features, most notably a high fre-
quency of the rare dinucleotide CpG at their 5' ends,
resulting in a large number of HpaII sites (CCGG).
The promoter regions of housekeeping genes also lack
certain consensus sequence elements such as TATA
and CAAT boxes known to be associated with tissue-
specific expression of genes (Maniatis et al., 1987),
but have multiple binding sites (G–C boxes) for the
transcription factor SP1 (Dynan and Tjian, 1985).
The COX5B gene also contains a large number of
HpaII sites near the 5' end, i.e., in exon 2 and intron 1,
and at least two consensus SP1 binding sites in the
limited region of the first intron sequenced thus far
(data not shown). These features suggest that the
COX5B gene is also a housekeeping gene. Whether
there are additional regulatory promoter or enhancer
elements for respiratory proteins, such as NRF-1
binding sites found in the promoter of the cytochrome
c gene (Evans and Scarpulla, 1989), awaits mapping
and further analysis of the promoter region.

Chromosomal Localization of the Expressed COX5B
Gene

We have identified eight different chromosomal
sites of hybridization with probes containing both
exon and intron sequences of COX5B. Since only the
gene on human chromosome 2, region cen–q13, hy-
bridized to an intron probe, we assigned the COX5B
coding gene to this site. All the human fragments hy-
bridized strongly under high stringency hybridization
and washing conditions. This suggests that these se-
quences are pseudogenes of COX5B instead of par-
tially related genes. Two other genes, INHBR (inhi-
bin-β) and MAL (T cell differentiation protein),
have been assigned to the same region in the same set
of hybrids (Barton et al., 1989; Alonso et al., 1988).
Genes for other nuclear-encoded subunits of cy-
tochrome c oxidase have been mapped to different
chromosomes: subunit IV (COX4) to chromosome 16,
region q22–qter (Darras et al., 1987; Lomax et aZ.,
1989) and subunit VIII (COX8) to chromosome 11,
region q12–q13 (Rizzuto et al., 1989).

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