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Structure and chromosomal location of the bovine gene for the heart muscle isoform of cytochrome *c* oxidase subunit VIII

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Abstract. We have isolated the bovine *COX8H* gene for the heart/muscle isoform of cytochrome *c* oxidase (COX) subunit VIII from a library of bovine genomic DNA cloned into lambda EMBL3. Primer extension assays on bovine heart mRNA mapped the 5′ ends of *COX8H* transcripts to a CA dinucleotide 62-bp upstream from the ATG codon. The gene thus spans 1565-bp and comprises two exons and one large intron of 1227 bp. Exon 1 encodes the 5′ untranslated region, a 24-amino acid presequence, and the first 13 amino acids of the mature COX VIII-H protein. Exon 2 encodes the remainder of the cDNA: amino acids 14 to 46 plus the 66-bp 3′ untranslated region. The exon-intron boundaries matched the consensus splice junction sequences. Two protein polymorphisms were seen: an Ala/Val polymorphism at position –6 in the presequence and the previously noted Lys/Arg polymorphism at residue 7 of the mature protein. A *Taq*I polymorphism occurs in the intron. The *COX8H* gene was mapped by bovine × rodent somatic cell hybrid mapping panels to bovine (BTA) Chromosome (Chr) 25 with 100% concordancy. BTA 25 is conserved relative to the long arm of human (HSA) Chr 11, which contains COX8, the gene for the single human COX VIII subunit that is homologous to the liver isoform.

Introduction

Cytochrome c oxidase (COX; EC 1.9.3.1), the terminal enzyme complex of the mitochondrial electron transport chain, catalyzes the transfer of electrons from reduced cytochrome *c* to molecular oxygen (reviewed in Hatefi 1985). In addition, the enzyme is involved in proton translocation across the mitochondrial inner membrane. In mammals, cytochrome oxidase contains 13 nonidentical polypeptide subunits. The three large subunits (I–III) are mitochondrial gene products that perform the catalytic functions of cytochrome *c* oxidase (Chomyn and Attardi 1987). The ten smaller subunits are nuclear gene products that have been proposed to modulate cytochrome oxidase activity in response to different physiological signals or metabolic environments (reviewed in Capaldi et al. 1987; Poyton et al. 1988; Capaldi 1990; Kadenbach et al. 1991).

COX subunits VIa, VIIa, and VIII are now known to have tissue-specific isoforms, on the basis of amino acid sequence differences between bovine heart and liver cytochrome *c* oxidase subunits (Yanamura et al. 1988). With isoform-specific cDNA probes, several laboratories have demonstrated that the gene for the heart (H) isoform is expressed only in striated muscle, for example, heart and skeletal muscle (Schlerf et al. 1988; Lightowlers et al. 1990; Ewart et al. 1991; Fabrizi et al. 1992). The gene for the liver (L) form is expressed in all tissues, albeit at low levels in contractile muscle (Lomax et al. 1990a; Seelan and Grossman 1991; Taanman et al. 1992).

Heart/muscle isoforms of COX subunit VIII are present in the cow (Lightowlers et al. 1990), rat (Scheja and Kadenbach 1992), and mouse (Van den Bogert et al. 1992; Hegeman, Brown, Lomax, unpublished data). The genes for these isoforms probably arose by gene duplication before the mammalian radiation. We have designated the isoform genes *COX8H,* for the heart/muscle isoform, and *COX8L,* for the liver isoform. Surprisingly, humans have a single COX8 gene, located on human Chr 11q12-q13, that is expressed at high levels in all tissues, including striated muscle (Rizzuto et al. 1989; Taanman et al. 1992). Human COX8 is assumed to be the paralog of the *COX8L* gene, since the human COX VIII protein is more similar to the bovine and rat L isoforms than to the H isoform.

As part of our studies on tissue-specific expression of COX nucleus-encoded subunits, we have begun to characterize the genes for heart/muscle-specific COX isoforms. In this report we present the isolation and structure of the bovine *COX8H* gene. We note that, in addition to the previously described Arg/Lys polymorphism at residue 7 (Lightowlers et al. 1990), the protein sequence deduced from the genomic sequence contains an additional change from the sequence predicted by the bovine heart cDNA. We also map the *COX8H* gene to bovine Chr 25.

Materials and methods

Screening genomic library. The bovine genomic library (Clontech BL1015j), consisting of 8–22 kb *Sau*3A partial digest fragments of adult bovine genomic liver DNA cloned into the *Bam*HI site of EMBL-3 SP6/T7, was titered on strain LE392 and screened for *COX8H* genomic clones by plaque hybridization (Benton and Davis 1977; Lomax et al. 1990b). The bovine COX subunit VIII-H cDNA used as hybridization probe (Lightowlers 1990) was radiolabeled by the random primer method (Feinberg and Vogelstein, 1983). One positive plaque was purified and designated λbCOX8H-1.

DNA sequence analysis. The DNA sequence of the *COX8H* genomic region was determined by the dideoxy chain termination method (Sanger et al. 1977) on alkali-denatured double-stranded plasmid DNA with the Sequenase version 2.0 kit (U.S. Biochemicals) and [α-^{35}S]dATP (Amersham Corp.). DNA sequencing reactions were electrophoresed on 6% acrylamide–7 M urea gels. The dried gels were exposed directly to Kodak X-OMAT film at room temperature. DNA sequence was confirmed by the University of Michigan DNA Sequencing Facility on an Applied Biosystems Model 373A automated DNA sequencer (Applied Biosystems, Foster City, Calif.). DNA sequences were aligned with the ASSEMGEL program of PC/GENE (Intelligenetics, Mountain View, Calif.).

Somatic cell mapping. The bovine × rodent somatic cell panel was described previously (Womack and Moll 1986). Genomic DNA from the

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hybrid cell lines, mouse LMTK$^-$ cell line, Chinese hamster E-36 cell line, and bovine leukocytes was digested with *Hin*dIII, electrophoresed, and blotted to nylon membranes (Zetabind, CUNO) (Adkison et al. 1989). Hybridizations using the COX VIII-H cDNA were performed overnight at 42°C in 5×SSC, 1× Denhardt's, 0.02 M phosphate buffer (pH 7.0), 100 μg/ml sheared salmon sperm DNA, 10% dextran sulfate, 50% formamide, 0.5% SDS, and 10^7 dpm of labeled probe. Final washes were at a stringency of 0.1 × SSC at 60°C. Filters were placed against Kodak XAR-5 film with an intensifying screen at −70°C for 4–10 days.

Results

Southern analysis of COX8H genomic region. To obtain preliminary information on *COX8H* gene structure and copy number, we performed genomic Southern blots with the bovine COX VIII-H cDNA as probe. Four enzymes generated only one genomic fragment each under stringent hybridization conditions: unique 3.2-kb *Bam*HI, 3.9-kb *Eco*RI, 6.6-kb *Hin*dIII, and 3.0-kb *Pst*I fragments. The two *Bgl*II fragments (6.6 kb and 2.3 kb) are due to the presence of a *Bgl*II site within the intron. These results suggest that COX VIII-H is encoded by a small, single-copy gene.

Isolation and restriction mapping of the bovine COX8H gene. The *COX8H* gene was isolated by screening 10^6 phage from the Clontech bovine genomic library with ^{32}P-labeled cDNA for bovine subunit VIII-H. Restriction mapping followed by Southern blot analysis of DNA from one positive clone, λbCOX8H-1, identified a single, internal 5-kb *Eco*RI fragment containing the gene. The restriction map generated from this clone agreed with the restriction fragments seen on genomic Southern blots (Fig. 1A). We determined that the *COX8H* gene contained a single intron by PCR analysis with primers based on the sequence of the cDNA, assuming that the presequence and hydrophobic regions would constitute separate exons. Therefore, PCR primers were designed to these regions. PCR reactions were carried out with pairwise combinations of each of two different upstream and two downstream primers. Each primer pair generated a 1.8-kb fragment, suggesting that the gene contained a single, 1.2-kb exon.

Sequence and organization of the COX8H gene. We subcloned the 5-kb *Eco*RI genomic fragment from λCOX8H-1 into pUC13, generated deletions from internal restriction sites, and obtained a 2.6-kb *Eco*RI-*Sma*I fragment that contained the *COX8H* gene. The DNA sequence of the 5′-flanking region, the *COX8H* gene, and the deduced COX VIII-H protein sequence are presented in Fig. 2. The gene comprises only two exons and one intron. Each intron/exon border matches the consensus splice site sequences (Padgett et al. 1986). Thus, exon 1 encodes a 62-bp 5′UTR, a 24-amino acid presequence, and amino acids 1–13. Exon 2 encodes residues 14–46, including the 20 hydrophobic amino acids (16–35) forming the transmembrane domain, plus the 3′ untranslated region.

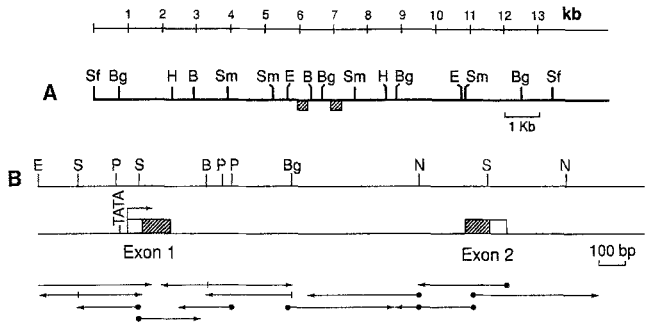

Fig. 1. Organization of the bovine *COX8H* genomic region. **A.** Restriction map of genomic clone λCOX8H-1 containing the *COX8H* gene. Restriction sites for *Bam*HI (B), *Bgl*II (Bg), *Eco*RI (E), *Hin*dIII (H), and *Sma*I (Sm) were mapped relative to known sites in the lambda arms. The distance from internal restriction sites to the junction between the lambda arms and bovine genomic DNA insert was determined by digestion with *Sfi*I. The 5-kb *Eco*RI fragment that hybridized with the cDNA probe is indicated by diagonal stripes. **B.** Organization and restriction map of the *COX8H* gene. Open boxes denote 5′ and 3′ untranslated regions; striped boxes, coding sequence. The initial transcription start site, defined by the longer of the two primer extension products, is indicated by the arrow. The location of the putative TATA-box element is indicated. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; S, *Sac*I; Sm, *Sma*I. Arrows indicate the direction and extent of DNA sequencing. The location of synthetic sequencing primers is indicated by circles.

Two nucleotide substitutions in exon 1 generated protein polymorphisms: a T–>C transition in the presequence generates a Val to Ala substitution at residue −6, and a G–>A transition predicts a Lys rather than an Arg at codon 7. The latter polymorphism was noted by Lightowlers and associates (1990). An additional G–>A transition in the third position of the Pro codon at residue 12 is silent. The only other differences between the cDNA and the genomic sequences in exon 2 were the six nucleotides immediately preceding the poly(A) tails.

Mapping COX8H to bovine linkage groups. We screened bovine × rodent somatic cell panels by Southern blot hybridization with the bovine COX VIII-H cDNA to map the *COX8H* gene to bovine syntenic groups. Test blots containing *Hin*dIII digests of bovine, mouse (LMTK$^-$) and hamster (CHO) cell line DNAs were hybridized with radiolabeled COX VIII-H cDNA under stringent hybridization and wash conditions. Only the 6.6-kb bovine fragment was detected under these conditions. We then proceeded to hybridize Southern blots of DNA from panels of bovine × rodent hybrids and performed pairwise concordancy analyses of the *COX8H* genomic fragment with markers for 29 bovine autosomal synteny groups. As shown in Table 1, *COX8H* co-segregated with U7 (BTA 25) marker LDHA with 100% concordance. The U7 syntenic group is conserved relative to two regions of HSA Chro 11: a short region on 11p and a longer region on 11q. Since the single human COX8 gene has been mapped to HSA Chr 11q, which is syntenic with BTA U7, we assume that the bovine heart gene is located in this region (Fig. 3).

Identification of a TaqI polymorphism in cattle. Digestion of genomic DNA from crossbred cattle with *Taq*I revealed a restriction fragment length polymorphism. The A allele contains a *Taq*I site within the intron of the *COX8H* gene, yielding two *Taq*I fragments: 2.0 kb and 3.0 kb. The B allele has lost this *Taq*I site, yielding a 5.0-kb fragment. Breeds represented in this test include Angus, Hereford, Holstein, Jersey, Red-polled (*Bos taurus*) and Brahman (*Bos indicus*). In these animals, the A allele is most common (Fig. 4).

Discussion

We have isolated, sequenced and mapped *COX8H,* the gene for the heart/muscle isoform of bovine COX subunit VIII. The gene contains a single 1.2-kb intron and maps to BTA syntenic group U7, which corresponds to Chr 25 (Bishop et al. 1994). This study identified several nucleotide substitutions in the bovine *COX8H* gene that probably represent both previously reported and novel protein and DNA polymorphisms. Lightowlers and colleagues (1990) noted protein polymorphisms in both the heart and liver isoforms of COX VIII, namely, an Arg and Lys polymorphism at residue 7 of the mature bovine heart isoform and an Asp or Glu at residue 14 of the liver isoform. The *COX8H* gene we have analyzed contains a Lys codon at position 7, and the liver-type cDNAs contain either Arg or Lys at position 7 (Scheja and Kadenbach 1992). We also noted a previously unidentified protein polymorphism in the deduced presequence, namely, an Ala at −6 in the genomic sequence vs. a Val in the cDNA. Both the rat and mouse cDNAs contain a Val at this position, whereas the three COX VIII

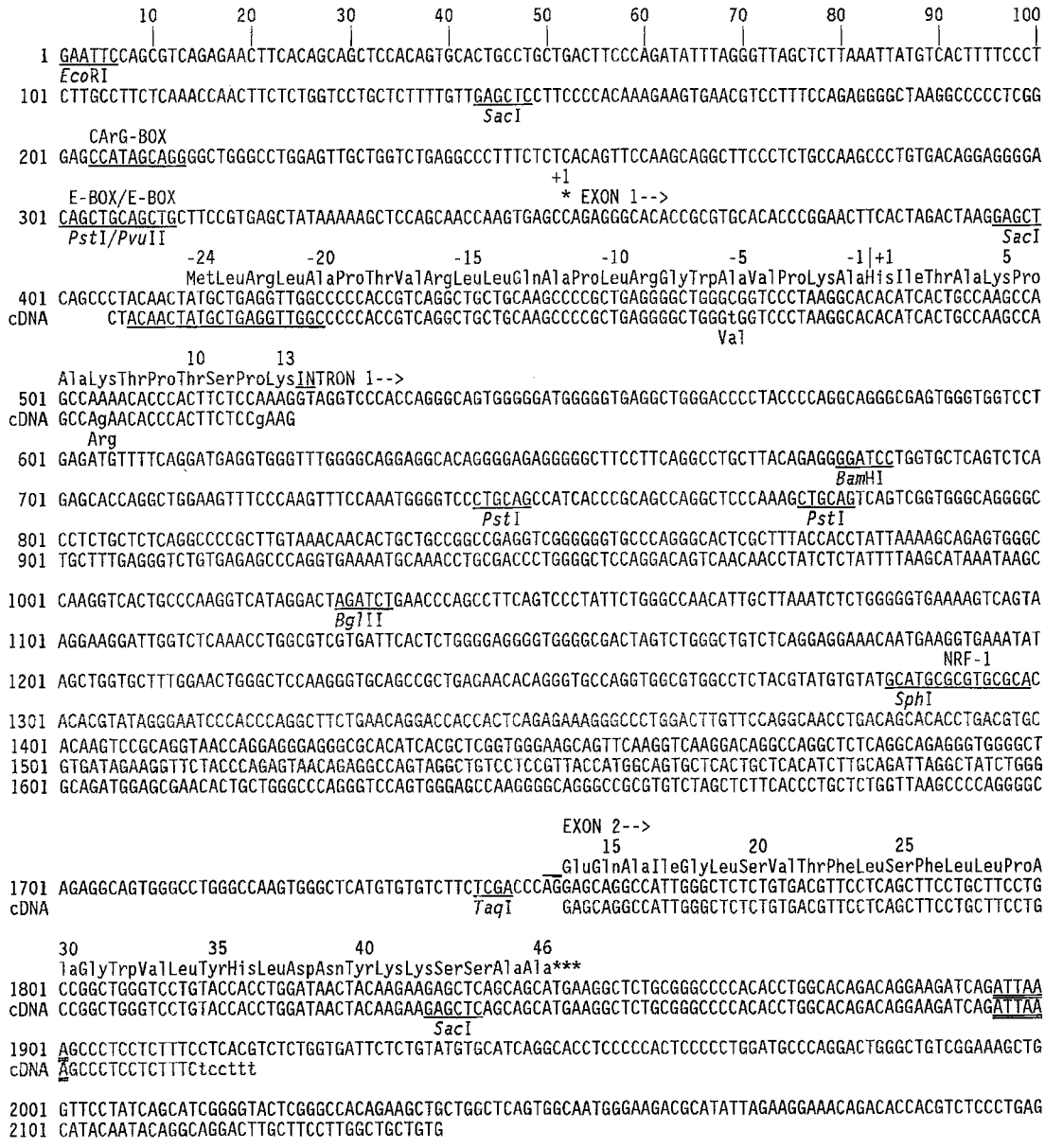

Fig. 2. Complete DNA sequence of the *COX8H* gene and its 5′- and 3′-flanking regions. The sequence of the gene is presented and numbered from the *Eco*RI site in the 5′ flanking region. The DNA sequence of the exons and the intron-exon junctions was generated by using the PCR primers for DNA sequencing; additional sequence was generated from subcloned restriction fragments and by designing sequencing primers to extend the sequence. The deduced protein sequence is presented and numbered above the DNA sequence. Sequence differences in the cDNA are indicated by lower-case letters; the resulting amino acid polymorphisms are indicated below the sequence. Intron-exon consensus splice junctions are overlined. The putative poly(A) additional signal is double-underlined. Potential TATA, myogenic, and respiratory enhancer elements are underlined and labeled above the sequence. Restriction sites indicated in Fig. 3 or the text are underlined and labeled below the sequence. The first transcription start site deduced from the longer primer-extension product (Fig. 4A) is indicated by an * above the sequence. The location of the anti-sense primer used for the primer extension experiments is underlined in the cDNA sequence.

cDNAs for the liver-type COX VIII isoform have Pro and Met residues at this position. A fourth base substitution in codon 12 near the end of exon 1 is silent: CCA (Pro) in the gene vs. CCG in the bovine heart cDNA. Additionally, we noted a *Taq*I polymorphism in the single intron. These changes represent a high degree of polymorphism at both the protein and DNA level for such a small gene.

This report completes the characterization of the three genes for heart/muscle-specific isoforms of tissue-specific COX subunits. Surprisingly, no generalizations about the regulatory elements involved in transcriptional regulation of these genes can be drawn from these analyses. Both the *COX6A1* (Smith and Lomax 1993) and *COX8H* genes share one feature of tissue-specific genes, namely TATA box elements. Although the *COX6A1* gene has both potential TATA and CCAAT elements, transcription initiation is imprecise and generates numerous transcripts that are heterogeneous at the 5′ ends. The *COX8H* gene has only a single basal promoter element, a TATA box, yet has precise transcription initiation sites. In contrast, both the bovine *COX7AH* and *COX7AL* genes (Seelan and Grossman 1992, 1993) are located in CpG islands, have no TATA or CCAAT elements, but many SP1-binding sites. Thus, the structures of these three COX heart/muscle-specific genes are quite different, implying different functional elements regulating developmental and tissue-specific expression.

The current interest in comparative genome mapping (O'Brien et al. 1993) makes the localization of bovine genes for COX a significant undertaking. For many COX genes that encode a housekeeping subunit, such as *COX5B*, mapping is complicated by the

Table 1. Concordance of *COX8H* gene with markers and bovine syntenic groups.

Syntenic group	Marker	% Concordance
U1	*GNB1*	50
U2	*ME1*	26
U3	*NKNB*	50
U4	*MPI*	30
U5	*FOS*	70
U6	*AMY1*	65
U7	*LDHA*	100
U8	*GNB2*	40
U9	*GPI*	44
U10	*SOD1*	45
U11	*VIM*	50
U12	*GPX1*	55
U13	*MET*	45
U14	*GSR*	74
U15	*CASK*	70
U16	*ABL*	45
U17	*CRYG*	45
U18	*GGTB2*	50
U19	*CAT*	80
U20	*CLO1*	35
U21	*GH*	45
U22	*AMH*	65
U23	*ALDH2*	40
U24	*TG*	45
U25	*CLTLA1*	00
U26	*OAT*	60
U27	*DU27S1B*	45
U28	*MBP*	40
U29	*RBP3*	50
X	*DMD*	45

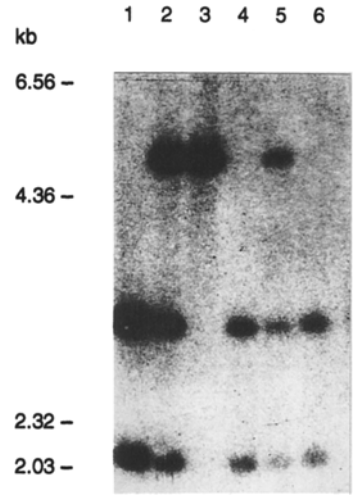

Fig. 4. Southern blot analysis of *TaqI* digests of DNA from crossbred cattle. DNA from crossbred cattle was digested with *TawI*, subjected to electrophoresis on agarose gels, transferred to nylon membranes, and hybridized with the COX VII-H cDNA as described in Materials and Methods. The A allele yields two *TawI* fragments of 2.0 kb and 3.0 kb; the B allele, a single 5.0-kb fragment. Lane 1: Brahman/Hereford = AA; lane 2: Red-polled/Angus/Brahman = AB; lane 3: Angus/Holstein = BB; lane 4: Hereford/Jersey = AA; lane 5: Hereford = AB; lane 6: Brahman/Jersey = AA.

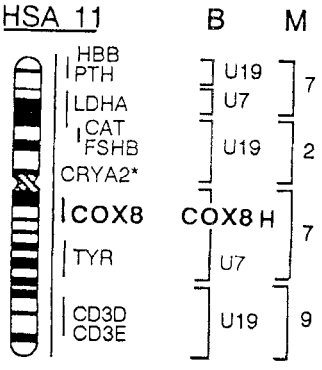

Fig. 3. Comparison of the location of COX8 genes in human, bovine, and mouse chromosomes. The idiogram of HSA 11 is shown with the location of several genes indicated. COX8 is the locus for the single human COX VIII subunit (Rizzuto et al, 1989), which is more similar to the bovine and rat liver subunits than to the heart subunits. B represents the bovine linkage groups syntenic with HSA11 and shows the location of linkage group U7; M represents the mouse chromosomal regions syntenic with HSA11.

presence of numerous pseudogenes (Lomax et al. 1991). In such cases, an intron probe is essential to distinguish the expressed gene from the processed pseudogenes. A few COX genes, such as *COX4* (Bachman et al. 1987), have a single pseudogene that can be mapped if information about the pseudogene is available (Lomax et al. 1990b). *COX4P,* the pseudogene for subunit IV, has been mapped in the cow to syntenic group U3, which is on bovine Chr 5 (Dietz et al. 1992). This study is the first to report mapping of an expressed COX gene in the cow. Mapping the COX genes for sarcomeric isoforms is straightforward in both humans and the cow, because these genes do not have processed pseudogenes. Surprisingly, *COX8H* pseudogenes are present in rodents (Scheja and Kadenbach 1992; Makris, Hegeman, Lomax, unpublished data).

The current model for the origin of tissue-specific isoforms of COX subunits is based on the observation that, with some exceptions, isoforms are present in all mammals and that the H and L isoforms retain regions of sequence conservation, particularly in the hydrophobic transmembrane domain. This model invokes a gene duplication event that occurred before the mammalian radiation, giving rise to the two isoform genes present in all mammals. Additional evidence supporting the gene duplication model comes from the conservation of gene organization. The position of the single intron in the COX8 gene is conserved in both the bovine *COX8H* gene and the single human COX8 gene (M. Lomax, unpublished data). The genes for the only COX isoform pair that has been mapped, namely, human COX7AH and COX7AL, are not linked (Arnaudo et al. 1990). The data reported here map the bovine *COX8H* gene to Chr 25, which is homologous with two regions of human Chr 11. Since the human COX8 gene homologous to bovine *COX8L* has been mapped to human Chr 11, the bovine *COX8H* and *COX8L* genes may still be physically linked in the cow. Confirmation of this hypothesis awaits the isolation and mapping of the bovine *COX8L* gene for COX subunit VIII-L. Such mapping data would provide additional support for this model.

Note added in proof. We have confirmed the bovine mapping data by mapping the mouse *COX8L* gene to mouse Chromosome 7 (Makris and Lomay, unpublished data).

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