CHAPTER II

GENOMIC STRUCTURE OF MOUSE COX17P
II-1. INTRODUCTION

Cox17p was first cloned as a cytoplasmic copper chaperone from yeast mutant and recent works suggested the existence of mammalian homologues (CHAPTER I). In the yeast system, it is thought that copper ions carried by Cox17p from the cytoplasm are (directly or indirectly) transferred to Sco1p (Synthesis of CCO I) at the mitochondrial intermembrane space, and then transmitted to Cox2p, the copper center of CCO (Glerum et al., 1996). Despite this peptide has already been cloned or purified from human (Amaravadi et al., 1997), mouse (Nishihara et al., 1998; Kako et al., 2000), rat (Kako et al., 2000) and porcine (Chen et al., 1997; Takenouchi et al., 1999), the function of Cox17p in the mammalian system has not yet been elucidated. Recently, the isolation of mouse COX17 cDNA and the tissue distribution profiles of its mRNA were reported (Kako et al., 2000). The COX17 gene was expressed ubiquitously, and especially, tissues such as heart, kidney and brain that requires high amount of energy strongly expressed COX17 mRNA. These facts suggest a possibility that Cox17p is also involved in cellular respiration in mammalian cells. Then, is mammalian Cox17p really involved in mitochondrial copper trafficking such as the model of yeast? Generally speaking, the genetic investigation often initiates the elucidation of the physiological function of novel gene. The study on the mouse COX17 gene may provide a useful clue to the solution of the above question. In this chapter, I characterized the exon-intron organization and chromosomal localization of the mouse COX17 gene.

II-2. MATERIALS AND METHODS

II-2-1. Materials

Restriction endonucleases were purchased from Nippon Gene (Tokyo, Japan), Takara (Tokyo, Japan), Toyobo (Tokyo, Japan). Klenow fragment,
TaKaRa kilo-sequencing deletion kit and TaKaRa LA PCR™ Kit Ver.2 were purchased from Takara. [α-^32P]dCTP was purchased from Bresatec (Adelaide, Australia). λ FIXII mouse spleen genomic library and pBluescript II SK (+) vector were purchased from Stratagene (CA, USA). GeneScreen Plus was purchased from NEN (MA, USA). Mouse/Hamster Radiation Hybrid Panel was purchased from Research Genetics (AL, USA). SuperScript™ Preamplification System, RNase Mix, dCTP, terminal deoxynucleotidyl transferase, 5’ RACE Abridged Anchor Primer and Abridged Universal Amplification Primer were purchased from Gibco RRL (NY, USA). pGEM-T Easy vector was purchased from Promega (WI, USA). Genomic DNA prepared from 129SvJ mouse embryonic stem cells was kindly provided by Dr. T. Baba. All other chemicals were of reagent grade.

II-2-2. Genomic Southern hybridization analysis

Five μg of total genomic DNA prepared from 129SvJ mouse embryonic stem cells was digested with restriction endonucleases, electrophoresed on a 0.6% agarose gel, blotted onto nylon membrane (GeneScreen Plus) and hybridized using a 0.26 kb mouse Cox17p cDNA fragment as a probe. The probe was labeled [α-^32P]dCTP with the Klenow fragment by the random-priming procedure (Feinberg et al. 1983). After overnight hybridization, the membrane was washed with moderate agitation as follows; 2× standard saline citrate (SSC, 1×SSC (0.15 M NaCl- 15 mM sodium citrate (pH 7.4))) for 10 min at room temperature, 2×SSC, 1% sodium dodecyl sulfate (SDS) for 20 min at 60°C (two times), 0.2×SSC, 0.1% SDS for 5 min at 60°C, 0.2×SSC for 5 min at room temperature. And then, the blots were imaged and analyzed by using a BAS 2000 Bio-Image Analyzer (Fuji Photo Film, Tokyo, Japan).
II-2.3. Screening of mouse genomic DNA library

Approximately $9.0 \times 10^5$ recombinant plaques from a 129SvJ mouse spleen genomic DNA library, partial Sau3A I digests in $\lambda$ FIXII, were screened by the plaque hybridization method (Benton et al., 1977), using mouse Cox17p cDNA fragment as a probe, described above. The phage DNA was prepared from the positive clones, digested by various restriction enzymes, and subcloned into pBluescript II SK for further characterization. In order to sequence the long DNA fragment, 5'- and 3'-gradual deletion mutants were obtained using TaKaRa kilo-sequencing deletion kit. The nucleotide sequence was determined by the dideoxynucleotide chain termination method (Sanger et al., 1977, 1980). Analyses of nucleotide and deduced amino acid sequences were done using GENETYX software (SDC, Tokyo, Japan).

II-2.4. RNA preparation

Total RNA was extracted from mouse heart as described by Chomczynski and Sacchi (1987).

II-2.5. 5'-Rapid amplification of cDNA ends (5'-RACE)

5'-RACE technique was used to obtain more sequence at the 5' end of the mCox17p cDNA (Frohman et al., 1988). With Oligo (dT)$_{10-12}$ primer as an initial primer and mouse heart total RNA (2 $\mu$g) as template, first strand cDNA was synthesized using the SuperScript$^{TM}$ Preamplification System. After first strand cDNA synthesis, the original total RNA template was removed by treatment with RNase Mix (mixture of RNase H, which is specific for RNA:DNA heteroduplex molecules, and RNase T1). The resultant cDNAs were tailed with dCTP and terminal deoxynucleotidyl transferase and amplified by PCR (initial denaturation at 94°C for 2 min, 30 cycles of PCR; denaturation at 94°C for 1 min, annealing of primers at 55°C for 1 min and primer extension
at 72°C for 1 min, and final extension at 72°C for 7 min) using TaKaRa LA PCRTM Kit Ver.2 with gene-specific primer 1 (GSP1, 5’-ATTCAAAAGTAGGACCACACCAC-3’) corresponding to 258-279 nt (Kako et al., 2000) and the 5’-RACE Abridged Anchor Primer (AAP, 5’-GGCCACGCGTGCAGCTAGTACGGAAGGGG-3’). The cDNA fragment amplified by the first PCR was diluted and re-amplified with nested gene-specific primer 2 (GSP2, 5’-CAGTGGCTTTTTC-TCTAGA-3’) corresponding to nucleotides 107-125 nt (Kako et al., 2000) and Abridged Universal Anchor Primer (AUAP, 5’-GGCCACGCGTGCAGACTA-GTAC-3’). The second PCR products were cloned into a pGEM-T Easy Vector and sequenced.

II-2-6. Radiation hybrid mapping

The chromosomal localization of the mouse COX17 gene was ascertained by a PCR-based screening of a Mouse/Hamster Radiation Hybrid Panel (McCarthy et al., 1997). For this purpose, I designed a pair of primers (SP-1, 5’-TGAAGGATAGGTGGGAATG-3’ and SP-2, 5’-CTTCCGCGAT-CATCGAAG-3’) corresponding to the 5’-upstream region of mouse COX17 (from -78 to -339) and optimized the PCR condition to amplify the mouse specific fragment. The specific PCR product (262bp) was cloned into pGEM-T Easy Vector and the sequence confirmed. After the PCR condition was fixed (initial denaturation at 94°C for 2 min, 30 cycles of PCR; denaturation at 94°C for 1 min, annealing of primers at 64°C for 1 min and primer extension at 72°C for 1, 100 radiation hybrid clones were detected in the presence or absence of this PCR product. Data were sent to The Jackson Laboratory Mapping Panels (The Jackson Laboratory Backcross DNA Panel Mapping Resources and The Jackson Laboratory Mouse Radiation Hybrid Database) for statistic analysis.
II-3. Results

II-3-1. Genomic Southern hybridization analysis

Five μg of 129SvJ mouse genomic DNA digested with Bgl II, EcoR I and Pst I was subjected to Southern blot hybridization analysis with mouse Cox17p cDNA fragment as probe (Fig. II-1). The hybridization patterns were consistent with the restriction map of the mouse COX17 (Fig. II-2). Thus, the COX17 is a single copy gene on the mouse genome.

II-3-2. Structure of the mouse COX17 gene

After screening of approximately 9.0 × 10^5 recombinant plaques from a mouse spleen genomic library, 3 positive clones were obtained. And these three clones were amplified and purified for further characterization. Regions of overlap were determined by restriction enzyme mapping and hybridization analysis of phage DNA. The mouse COX17 genomic region was completely sequenced on both strands (Fig. II-2). The complete gene sequence has been deposited in the GeneBank Database under the Accession No. AB047323. The first exon was determined by 5'-RACE (see II-3-3. Cloning of the 5'-noncoding region). The end of the third exon was determined by comparison with sequence data for a poly(A) tail-containing cDNA (GenBank accession number, AU051371) since the available mouse cDNAs were truncated in this region. The gene has two potential polyadenylation signals (ATTAAA) (Bhat and Wold, 1985) located 21 to 26 and 81 to 86 nt upstream of the poly(A) tail, respectively (Fig. II-5). The first exon (187nt) encodes the complete 35 amino acids; it also contains a 5' non-coding region of 80 nt. The second exon (89bp) encodes the remaining 29 amino acids of the Cox17p peptide and 4 nt of the 3' non-coding region, whereas the third encodes 129 nt of the non-coding region. All the
exon-intron boundaries conform the canonical GT/AG rule (Mount, 1982); the first and second introns are 2,033 and 3,282 bp, respectively.

The gene is located at a CpG island (Bird, 1986), based on the distribution of CpG residues. Of the 108 CpGs that span the COX17 gene, ~60% are found near the transcription start site and the first intron (Fig. II-3). CpG islands are found associated with the 5'-flanking regions of many genes transcribed by RNA polymerase II, including many genes encoding housekeeping genes (Tazi & Bird, 1990).

In this experiment, I also cloned about 7kb fragment of 5'-upstream region of this gene.

II-3.3. Cloning of the 5'-noncoding region

To isolate and characterize 5' ends of the mouse COX17 gene, 5'-RACE was performed. After reverse transcription and the following two-step PCR, a major second PCR product was seen at 170 bp. After the PCR product was subcloned, the individual clones were sequenced to confirm whether each sequence was identical or not. As a result, the mouse COX17 gene was found to contain one major transcription start site and to be located 80 bp upstream of the ATG initiation codon.

II-3.4. Chromosomal assignment

To determine the chromosomal location of mouse COX17, a radiation hybrid (RH) mapping panel, the Mouse/Hamster Radiation Hybrid Panel (McCarthy et al., 1997), was typed by PCR amplification using gene specific primers corresponding to the 5'-upstream region of mouse COX17. Statistical analysis using the Jackson Laboratory Mapping Panels showed the data had a highest anchor LOD of 21.9 to D16Mit59 (Fig. II-4, left). Around this loci is the central part of a region of well-documented homology to human 3q13-24
spanning from Gprc2a to Gap43, respectively CASR and GAP43 in the human genome (DeBry & Seldin, 1996) (Fig. II-4, right).

II-4. DISCUSSION

In this chapter, I cloned and determined the entire structure of the chromosomal DNA of the mouse COX17 gene. After the screening of a mouse genomic library and sequence analysis, the COX17 genomic structure was completely covered (Fig. II-2). As shown in Fig. II-1, the COX17 is encoded by a single-copy gene on the mouse genome. After the screening of a mouse genomic library and sequence analysis, the COX17 genomic structure was completely covered (Fig. II-2). The mouse COX17 composed of three exons and two introns and spans approximately 5.72 kb of genomic DNA (Fig. II-2). Exon 3 contains sequence of the 3' untranslated region, only. A 400 bp region containing exon 1 is extremely GC-rich and has the properties of a CpG island (Fig. II-3). Of the 108 CpGs that span this gene, 32 residues are found in the first 400 bp including exon 1; by contrast, the distribution of GpC residues appears to be fairly uniform. It is well known that the methylation of specific residues in these CpG islands can play a role in regulating gene expression and this point will be discussed later (see CHAPTER III).

As described above, it has been believed that Cox17p guides Cu to the mitochondria for insertion into CCO in yeast (Glerum et al., 1996). In the yeast system, it is thought that copper carried by Cox17p from cytosol is (directly or indirectly) transferred to Sco1p (Synthesis of CCO 1) at the mitochondrial intermembrane space (Valentine and Gralla, 1997). Recent reports suggested fatal infantile cardioencephalomyopathy with CCO deficiency and mutations in hSCO2, a candidate for the human homologue of yeast Sco1p (Papadopoulou et al., 1999; Jaksch et al., 2000). Thus, it is of interest to
examine the possible genetic linkage between mammalian *COX17* and *SCO2*. The present mapping results assign the *COX17* locus to mouse chromosome 16 (Fig. II-4, left). The human chromosomal location of the *COX17* locus can be predicted based on known mouse-human linkage homologies (DeBry and Seldin, 1996). According to this prediction, *COX17* and *SCO2* lie on the human autosomes 3q13 and 22q13 (Fig. II-4, right) (Papadopoulou et al., 1999; Paret et al., 1999), respectively. This implied that there is no genetic linkage between them. However, the mRNA distribution profile of hSCO2 (Papadopoulou et al., 1999) overlapped with that of mouse Cox17p (Kako et al., 2000) indicating that the expression of these genes may be regulated by similar transcription mechanisms.

Using 5'- RACE, I found a major transcription start site located 80 bp upstream of the ATG initiation codon. Polyadenylation sites are present 81 and 21 bp downstream of two potential polyadenylation signals (AATTTA) (Bhat and Wold, 1985), yielding 3'-untranslated regions of 133 bp (Fig. II-5). The expected size of the mature mCox17p mRNA without taking account of the poly A tail is 405 nt, in good agreement with the size of the mouse Cox17p mRNA (ca. 750 bases) estimated by Northern analysis of mouse tissue RNA (Nishihara et al., 1998; Kako et al., 2000).

Recently, the expression profile of mouse Cox17p mRNA was reported: The *COX17* gene expressed in all mouse tissues, but notably intense signals were observed in heart, brain and kidney RNA samples. Furthermore, some of the neuroendocrine and endocrine cell lines showed higher expression levels of the Cox17p mRNA than fibroblasts. Besides cell/tissue specificity, embryonic and postnatal changes in the expression levels of Cox17p mRNAs were observed in the brain, whereas a constitutive and gradually increasing expression level was detected in heart during early development (Kako et al., 2000). In general, cell/tissue specific and developmentally regulated
expression of genes is controlled mainly at the transcription level (Darnell, 1982; Mitchell and Tjian 1989). Further analysis of the regulatory elements and their respective binding factors, as well as the importance of methylation at CpG dinucleotides, should provide insight into the underlying the developmental and/or tissue/cell-specific profile of COX17 gene regulation.
Fig. II-1. Southern blot analysis of the mouse *COX17* gene. The mouse genomic DNA was digested by these three enzymes and subjected to Southern blot analysis using $^{32}$P-labeled Cox17p cDNA fragment as a probe.
Fig. II-2. **Structure and exon-intron organization of the mouse COX17 gene.** A genomic clone encoding mouse COX17 has been identified from a mouse genomic library. The COX17 gene carrying three exons (closed boxes) interrupted by two introns is located in the mouse genome. The sites of restriction enzymes are shown as follows: B, Bgl II; E, EcoRI; H, HindIII; P, Pst I; X, Xba I.
Fig. II-3. *COX17* is located at a CpG island. The distribution of CpG (black bar) and GpC (white bar) dinucleotides spanning the 5.7-kb region is shown.
Fig. II-4. The position of the COX17 locus on mouse Chr. 16 (left) and a segment of human Chr. 3q with the position of the putative COX17 locus.
Fig. II-5. DNA sequence of the mouse COX17 gene. The gene contains three exons (bold) extending from the transcription start site (+1) to the polyadenylation site at 5620. The amino acid sequence is shown below the nucleotide sequence numbered from 1-63. An asterisk denotes the stop codon and the putative copper binding motif (KPCCAC) is boxed. Two potential polyadenylation signals, ATTAAA, are underlined.