

*CHAPTER III*

**CHARACTERIZATION AND IDENTIFICATION OF  
PROMOTER ELEMENTS IN THE MOUSE COX17  
GENE**

## III-1. INTRODUCTION

Mitochondrial DNA contains only 13 respiratory chain polypeptide, 2 ribosomal RNA and 22 transfer RNAs, however, a large number of proteins are involved in mitochondrial function (Clayton *et al.*, 1991). Most of respiratory proteins are encoded by nuclear genome. Several transcriptional regulatory elements in these respiratory genes have been identified to date (Carter and Avadhani, 1991; Virbasius and Scarpulla, 1991; Carter *et al.*, 1992; Seelan and Grossman, 1992; Basu *et al.*, 1993; Virbasius *et al.*, 1993; Bachman, 1995). And it has become apparent that a majority of the respiratory genes requires intact Sp1, NRF-1 and NRF-2 sites in their proximal promoter for optimal activity (Bachman, 1995; Seelan *et al.*, 1996; Sucharov *et al.*, 1995; Lenka *et al.*, 1998; Seelan and Grossman, 1997).

Recently, the expression analysis of mouse Cox17p mRNA was reported (Kako *et al.*, 2000). The intense signals of Cox17p mRNAs were detected in heart, kidney and brain where the metabolic activity is relatively high. Therefore, it is suggested that the regulation of the *COX17* gene expression may be related with the amount of the cellular energy consumption. In previous chapter, I isolated a mouse genomic DNA fragment encoding the full length of mouse *COX17* and determined its structural organization and chromosomal localization. I also cloned a fragment of 5'-upstream region (about 7kb from potential transcription start site) of mouse *COX17* gene. In this chapter, I investigated the transcriptional regulation of the mouse *COX17* gene.

## III-2. MATERIALS AND METHODS

### III-2-1. Materials

SuperScript<sup>TM</sup> Reverse Transcriptase, Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from GIBCO

BRL (NY, USA). PicaGene Basic Vector 2, The PicaGene Control Vector 2, PicaGene Basic Vector 2, PicaGene Cell Culture Lysis Reagent LC $\beta$ , and PicaGene Luminescence Kit were purchased from Toyo Ink (Tokyo, Japan). FuGENE 6 transfection reagent was purchased from Roche Diagnostics (Tokyo, Japan). The pRSV $\beta$  and the pCMV $\beta$  plasmids were kindly provided by Dr. Fukamizu (University of Tsukuba, Japan). NIH3T3 mouse fibroblast cells were purchased from RIKEN Cell Bank (Tsukuba, Japan). AtT-20 mouse pituitary derived cells were kindly provided from Dr. Nakayama (University of Tsukuba, Japan). All other chemicals were of reagent grade.

### **III-2-2. Cloning of the 5'-flanking region of the mouse *COX17* gene**

The mouse *COX17* genomic clone isolated as described in CHAPTER II was digested with *Eco* RI and *Not* I and the 7.5 kb digested fragment was subcloned into *Eco* RI/*Not* I digested pBluescript II SK vector.

### **III-2-3. RNA preparation**

Total RNA was extracted from cells and tissues as described in CHAPTER II.

### **III-2-4. Primer extension analysis**

A synthetic oligonucleotide corresponding to nucleotide -11 to -31 (5'-TGTGCGCTCATAGTTGCTTTC-3') was 5' end labeled with fluorescein isothiocyanate (FITC). The FITC-labeled probe (35 pmol) was mixed with 20  $\mu$ g of total RNA from AtT-20 cells or NIH3T3 cells or mouse heart and denatured at 80°C for 15 min, annealed with 20  $\mu$ g of total RNA from AtT-20 cells or NIH3T3 cells or mouse heart by heating at 45°C for 12 h in buffer containing 50% (v/v) formamide, 40 mM Pipes (pH 6.8), 400 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.0), and then precipitated with

ethanol. The dried pellet was dissolved in reaction buffer (20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 10 mM dithiothreitol (DTT), 0.4 mM dNTPs (0.4 mM each dATP, dCTP, dGTP, dTTP)). Extension reaction was carried out at 42°C for 1 h by reverse transcriptase (SuperScript™ Reverse Transcriptase). The extended products were denatured and subjected to electrophoresis on a 6% poly-acrylamide/8 M urea sequence gel. A PCR-based double-stranded DNA sequencing reaction was run in parallel with the primer extension product.

### III-2-5. Cell culture

NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 mg/ml glucose, 10 % fetal bovine serum (FBS), 100 µg/ml streptomycin and 100 U/ml peniciline. Cells were incubated in a humidified atmosphere of 5 % CO<sub>2</sub> and 95 % air at 37°C.

AtT-20 cells were cultured in DMEM with 4.5 mg/ml glucose, 10 % FBS, 100 µg/ml streptomycin and 100 U/ml peniciline. Cells were incubated in a humidified atmosphere of 15 % CO<sub>2</sub> and 85 % air at 37°C.

### III-2-6. Plasmid construction

The 7.5 kb *Eco* RI/*Not* I fragment of phage clone containing the ~7 kb 5'- promoter region and 90 bp coding region (described in III-2-2) was used for all constructions. PCR with this fragment and two primers, CP-7 (5'-GGAAAGCCACTCAATGCTGT- 3') and CP-10 (5'-AAGCTTCCGCAGT-CAATCGAAG- 3'), was used to obtain the short 5'-flanking fragment (-78 to -921) and to introduce a *Hind* III site to facilitate cloning. This PCR fragment was direct subcloned into a pGEM-T Easy vector. The plasmid was then cleaved at the endogenous *Sac*I site and *Hind* III site. The resulting fragment was directly ligated into the *Sac* I/*Hind* III-digested PicaGene Basic Vector 2 to

generate pPG/0.8K. To obtain the longer promoter fragments (3.4 kb and 6.8 kb), the 7.5 kb *Not* I fragment was subcloned into a *Not* I-digested pBluescript II SK vector. This plasmid (named pBS/7K) was cut at the *Hind* III (derived from the endogenous sequence) and *Sac* I site and then the *Hind* III-*Sac* I fragment (2.6 kb) was ligated into a *Hind* III/*Sac* I-digested pBluescript II SK vector. After that, the 2.6 kb fragment was cut out from this plasmid with *Kpn* I/*Sac* I and ligated into the *Kpn* I/*Sac* I-digested pPG/0.8K to obtain pPG/3.4K. The previous pBS/7K plasmid was cut at the *Sal* I site (derived from the  $\lambda$ FIX II polylinker) and *Sac* I site and then the *Sal* I-*Sac* I fragment (6.0 kb) was ligated into a *Sal* I/*Sac* I-digested pBluescript II SK vector. The 6.0 kb fragment was released from this plasmid with *Kpn* I/*Sac* I and ligated into the *Kpn* I/*Sac* I-digested pPG/0.8K to generate pPG/6.8K. The pPG/0.8K plasmid was cut *Kpn* I site and *Sac* I site and used as a starting template for deletion mutants. Deletions were made as described CHAPTER II. Site-directed mutagenesis was performed with pPG/0.8K plasmid, using the overlap extension method (Ho *et al.*, 1989) with some modifications. I designed synthetic oligonucleotides: 5'-TTCTTTTGCGCACGCGCTTCTTAG-3' (-158 to -135) for mutation of the NRF-1 binding site (-150 to -148; CGC→TAA, -143 to -141; CGC→AAT) and 5'-TCCGGAGAGGCGGGACTTCC-3' (-123 to -104) for mutation of the Sp1 binding site (from -115 to -113; GGC→TTA). The mutated fragments were subcloned into PicaGene Basic vector 2. The nucleotide sequences of the all constructs were verified by the dideoxynucleotide chain termination method as described in CHAPTER II.

### III-2-7. Transient transfection

Transfection was performed using of FuGENE 6 transfection reagent. Cells were plated at a density of  $2.0 \times 10^4$  cells/well in 24-well plates and were cultured for 24 h before transfection. Each Plasmids were prepared by using

Midi-prep columns (QIAGEN). Transfection of plasmid DNA (1.2  $\mu\text{g}$ ) into each cells were performed using 3  $\mu\text{l}$  of FuGENE 6 reagent. The PicaGene Control (SV40-driven enhancer/promoter) Vector 2 and PicaGene Basic Vector 2 were utilized as positive and negative controls, respectively. LacZ genes driven by the enhancer/promoter of the major immediate-early gene of Raus sarcomavirus and cytomegalovirus (pRSV $\beta$  and pCMV $\beta$ ) were co-transfected with luciferase constructs to correct for transfection efficiency for NIH3T3 and AtT-20, respectively.

### **III-2-8. Luciferase and $\beta$ -galactosidase assay**

24 h after transfection, cells were washed with phosphate-buffered saline (PBS) and lysed in the plate using 100  $\mu\text{l}$  of a detergent solution (PicaGene Cell Culture Lysis Reagent LC $\beta$ ). The cell extract was then centrifuged for 5 s and the supernatant was collected. The cell lysate (20  $\mu\text{l}$ ) was mixing with luciferin/ATP/CoA solution (PicaGene Luminescence Kit, 100  $\mu\text{l}$ ) in the chamber of a luminometer (AutoLumat, Berthold, MA, USA). Light production was measured for 10 s.

The  $\beta$ -galactosidase assay was set-up in 204  $\mu\text{l}$  containing 53 mM Na<sub>2</sub>HPO<sub>4</sub>, 35 mM NaH<sub>2</sub>PO<sub>4</sub>, 1.5 mM MgCl<sub>2</sub>, 66 mM, 2-mercaptoethanol and 20  $\mu\text{l}$  of cell lysate. The reaction was started by addition of 66  $\mu\text{l}$  of 4 mg/ml o-nitrophenyl- $\beta$ -D-galacto-pyranoside in 60 mM Na<sub>2</sub>HPO<sub>4</sub>, 40 mM NaH<sub>2</sub>PO<sub>4</sub>, and was incubated at 37°C until a yellow color developed. After addition of 500  $\mu\text{l}$  of 1 M Na<sub>2</sub>CO<sub>3</sub>, the absorbance of the solution was measured at 420 nm.

All experiments were performed in triplicate, and all results were obtained from at least three separate experiments.

### **III-2-9. Preparation of nuclear extracts**

Nuclear extracts were prepared as previously described (Kako *et al.*,

1998) with some modifications. To prepare nuclear extracts, the cells or tissue is homogenized in homogenizing buffer containing sucrose (250 mM sucrose, 60 mM KCl, 15 mM Tris-HCl (pH 7.9), 15 mM NaCl, 5 mM EDTA, 1 mM ethyleneglycol bis (2-aminoethylether) tetraacetic acid (EGTA), proteinase inhibitor cocktail (70 µg/ml *N*-tosyl-L-phenylalanyl chloromethyl ketone (TPCK), 10 µg/ml trypsin inhibitor, 5 µg/ml aprotinin, 1 µg/ml pepstain A, 0.5 µg/ml leupeptin, 1 mM DTT, 0.5 mM phenylmethanesulfonyl fluoride (PMSF)). The swollen cells are resuspended in hypotonic buffer (10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.9), 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, protease inhibitor cocktail) to disrupt cell membrane and then nuclei are pelleted. The cytoplasmic fraction is removed and stored, and the precipitated nuclei are resuspended in low-salt buffer + 0.5 M KCl (0.5 M HEPES (pH 7.9), 0.5 M KCl, 0.75 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 12.5% glycerol, protease inhibitor cocktail). Gentle agitation of the nuclei in this buffer permits the release of soluble proteins from the nuclei. Following extraction, the nuclear and plasma membranes are removed by centrifugation, the nuclear extract (supernatant) is dialyzed against a moderate salt buffer (12.5% glycerol, 10 mM Tris-HCl (pH 7.9), 5 mM MgCl<sub>2</sub>, 1 mM EDTA, 50 mM NaCl, 1 mM DTT, 0.5 mM PMSF), and any insoluble matter is removed by centrifugation.

### **III-2-10. Electrophoretic mobility shift assay (EMSA)**

EMSAs were performed as previously described (Kako *et al.*, 1998) with some modifications. Synthetic oligonucleotides were designed for probes or competitors (Table III-1). Oligonucleotides were phosphorylated at their 5'-end using T4 polynucleotide kinase and [ $\gamma$ -<sup>32</sup>P]ATP. The radio-labeled sense-oligonucleotides were mixed with 5-fold molar excess complementary oligonucleotides, annealed as follows: heat at 80°C for 1 min, gradually cooled down to 65°C for 1 h, 37°C for 1 min, and room temperature for 1 h. The

radio-labeled and annealed oligonucleotides were used as a probes. Nuclear extracts (20  $\mu$ g) were mixed with probes (15 fmol) and incubated at room temperature for 15 min in buffer containing 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 50% glycerol, 10 mM DTT, 10 mM EDTA, 0.13  $\mu$ g/ $\mu$ l sonicated salmon sperm DNA, 67 ng/ $\mu$ l bovine serum albumin) in the presence or absence of a specific double-stranded competitor DNA. The incubation mixture was loaded on a 4% nondenaturing polyacrylamide gel in 1 $\times$ Tris-boric acid EDTA (TBE) (1 $\times$ TBE: 89 mM Tris-HCl, 89 mM boric acid, 2mM EDTA) buffer, and electrophorated. The gels were dried and analyzed by using a BAS5000 Bio-Image Analyzer (Fuji Photo Film, Tokyo, Japan). For supershift assays, 1-2  $\mu$ g of anti-NRF-1 antibody was mixed in the reaction mixture and incubated for further 15 min.

### **III-3. Results**

#### **III-3-1. Cloning and characterization of the 5'-flanking region of the mouse *COX17* gene**

The 5'-flanking region of the mouse *COX17* gene was subcloned into pBluescript II SK vector. A detail restriction map of the 5'-flanking region of the mouse *COX17* gene is shown in Fig. II-2. The *Hind* III-*Not* I fragment was completely sequenced on both strands.

To define the transcription start site of the mouse *COX17* gene, primer extension analysis was performed. Total RNA from mouse heart, AtT-20 and NIH3T3 was hybridized with the FITC-labeled primer (corresponding to nucleotides -11 to -31), respectively. The transcripts were analyzed on a 6% poly-acrylamide/8 M urea sequence gel. As shown in Fig. III-1, an intense band of 69 nucleotides was extended from the end of the primer, indicating a termination of reverse transcription at nucleotide -79. This result showed good



agreement with my previous 5'-RACE data (CHAPTER II; Takahashi *et al.*, 2001). Under this band, two weaker bands of 65 and 63 nucleotides were seen, representing termination at nt -75 and at nt -73.

### III-3-2. Promoter activity of the 5'-flanking region of the *COX17* gene

To determine the features of the promoter region responsible for transcriptional regulation, AtT-20 cells and NIH3T3 cells were transiently transfected with constructs containing different length of the 5'-flanking region of the *COX17* gene (pPG/6.8K, pPG/3.4K and pPG/0.8K, Fig. III-2), and then luciferase activities in the transfected cell extracts were compared (Fig. III-3, III-4). Luciferase activity was normalized for transfection using a  $\beta$ -galactosidase expression plasmid as an internal control. The highest level of luciferase activity was detected in the cell extracts transfected pPG/0.8K encompassing the 0.8 kb fragment of the 5'-proximal region. Relative to pPG/0.8K, constructs containing additional sequences 5' of the 0.8 kb region had less activity; pPG/3.4K and pPG/6.8K which extend more than 2.6 kb and 6.0 kb beyond pPG/0.8K, gave levels of activity which were approximately 60 % and 35% that of pPG/0.8K, respectively. When this 0.8-kb fragment was subcloned in front of luciferase in the antisense orientation, there was no detectable expression of luciferase activity. It was suggested that the 0.8 kb fragment contains major *cis*-acting elements required for the *COX17* promoter. Activities of these promoter constructs exhibited a similar pattern between AtT-20 cells, which express high levels of *COX17*, and NIH3T3, which express low levels of *COX17* (Fig. III-3, III-4).

The proximal sequence of the 0.8 kb fragment was analyzed with TRANSFAC (Heinemeyer *et al.*, 1998). The proximal sequence of the 5'-flanking region is devoid of canonical *cis*-acting elements such as TATA box or CAAT box but contains potential binding sites for several transcription factors

such as Sp1, NRF-1, and NRF-2. In addition, in this region contains CA repeat (23 times) and GA repeat (18 times) (Fig. III-5).

### **III-3-3. Functional analysis of the mouse *COX17* gene promoter**

To identify the minimal promoter sequence in the 5'-flanking region of the *COX17* gene, various deletion mutants truncated 5'-flanking sequences fused to the luciferase reporter gene were made and introduced into AtT-20 by transfection (Fig. III-6). Luciferase activity was normalized for transfection using a  $\beta$ -galactosidase expression plasmid as an internal control. The fragment from bases -853 to -68 relative to the translation start site conferred constitutive luciferase expression. The luciferase activity in AtT-20 was constant in progressive 5'-deletion constructs until the deletion reached 418 bases (-436 to -68), whereas there were some characteristic sequences, such as CA repeat (-796 to -747), GA repeat (-748 to -695), and AGGAAGGAA (-535 to -527). A successive deletion to base -207 including several NRF-2 sites seemed to have a little effect on the activity. A deletion to base -181 maintained 60~70 % of the luciferase activity, but deletion of 714 bases from the 5' end of 0.85 kb (-140 to -68) resulted in a 75% reduction in activity. Deletion to base -115 abolished the promoter activity to about 5% but not 0%. These results show that at least three positive regulatory elements reside between bases -115 and -68, bases -140 and -115 and bases -181 and -140, and the region between bases -181 and -115 is responsible for the promoter activity. This tendency was also observed on using cell extracts from a non-endocrine cell line, NIH3T3 cells.

### **III-3-4. Contribution of the GC box and NRF site to the promoter activity of the mouse *COX17* gene**

Further experiments were performed to characterize the basal promoter

of the *COX17* gene. Several putative binding sites for transcription factors were found in the proximal 5'-flanking region (Fig. III-5). As described above, the region between bases -181 and -141 was responsible for the *COX17* promoter activity in AtT-20 cells and NIH3T3 cells. In this region, the consensus sequence GAGGCGGGA for Sp1 binding (Ishii *et al.*, 1986; Greene *et al.*, 1987) is present at position -117 to -109. Two candidates for the NRF-2 (Nuclear respiratory factor-2; A/CGGAA; at positions -107 to -103 and -83 to -79) site and one for the NRF-1 (Nuclear respiratory factor-1, TGCGCACGCGC; at position -152 to -141) (Virbasius and Scarpulla, 1991; Virbasius *et al.*, 1993) site were localized around the GC-box. To ascertain whether these binding sites in the mouse *COX17* gene are functionally important to the basal promoter activity, they were altered by site-directed mutagenesis (Ho *et al.*, 1989) and the mutant vectors were transfected into NIH3T3 cells. As shown in Fig. III-7, mutation of the NRF-1 binding site (-152TGtaaACGaatT-141) reduced promoter activity to 53 % of the non-mutated construct. Alteration of the proximal Sp1 binding site (-117GAttaGGGA-109) also reduced the promoter activity to 28 % of the wild-type construct. By contrast, the constructs including single mutations of NRF-2A (TTaaT) or NRF-2B (CttAA) sites retained 93% and 87% of the basal promoter activity, respectively. When both NRF-2 sites are mutated, the luciferase activity dropped further (~65 %). However, when both NRF-1 and Sp1 binding sites were simultaneously mutated, promoter activity was almost abolished (less than 10 %). Since the NRF-1 and Sp1 double mutant showed a steep drop in activity, it is suggested that these two sites are more important than the two NRF-2 sites for *COX17* transactivation. This tendency was also observed using AtT-20 cells.

### III-3-5. EMSAs

EMSAs were performed to detect the nuclear proteins in AtT-20 and

NIH3T3 nuclear extracts which were associated with the positive regulatory elements. Two sets of oligonucleotides encompassing the Sp1 binding site (-123 to -104; 5' TCCGGAGAGGCGG-GACTTCC 3') and NRF-1 site (-158 to -135; 5' TTCTTTTGCGCACGCGC-TTCTTAG 3') were <sup>32</sup>P-labeled and used as probes. Fig. III-8 shows the band shift when nuclear extracts of NIH3T3 were incubated with a probe containing the Sp1 binding site. Three prominent retarded bands (I, II and III) were noted. Since this region comprises a putative binding site for Sp1, a 100-fold molar excess of consensus oligonucleotide containing Sp1 sequence was preincubated with nuclear extracts prior to the addition of the probe. All bands specifically competed with unlabeled oligonucleotide containing the Sp1 consensus sequence (lane 3). In addition, the band did not compete with a 100- and 200-fold molar excess of the probe with the mutation (-117GAttaGGGA-109) (lane 4, 5), respectively. These results indicate that Sp1 and/or closely related proteins bind to the canonical Sp1 site. Fig. III-9 shows a band shift when the nuclear extracts of A<sub>9</sub>T-20 were incubated with a probe containing the NRF-1 binding site. One prominent retarded band and one non-specific band were noted (lane 2). The more retarded band disappeared when an excess of the same unlabeled probe was added (lanes 3). More importantly, the band did not compete with a 100-fold molar excess of the probe with the mutated core element (-152TGtaaACGaatT-141) (lane 4). To clarify whether this band of DNA-protein complexes contained NRF-1, we performed gel mobility shift analysis in the presence of anti-NRF-1 antibody (lane 5-8). The incubation of nuclear extracts with anti-NRF-1 antibody resulted in a supershift of the complex with a concomitant diminishment of the retarded band (lane 7, 8). These results indicate that NRF-1 is present in the retarded band.

### III-4. DISCUSSION

In this chapter, I characterized the promoter elements of the mouse *COX17* gene. A majority of the cellular respiratory genes are also TATA-less genes that have been shown to require intact Sp1, NRF-1 and NRF-2 sites in their proximal promoter for optimal activity (Bachman, 1995; Seelan *et al.*, 1996; Sucharov *et al.*, 1995; Lenka *et al.*, 1998; Seelan and Grossman, 1997). Sequence analysis of 5'-flanking region of the *COX17* gene revealed that this gene was also TATA-less gene and these *cis*-elements were also localized on this region (Fig. III-5). And it was suggested that the transcription of the *COX17* gene might be regulated by the common mechanisms of the transcription of several other respiratory genes. Then, is the transcription of the *COX17* gene really regulated by these *cis*-elements? Luciferase assays and EMSAs demonstrated that both the Sp1 and the NRF-1 binding sites are also functional on the mouse *COX17* gene, and interaction between the NRF-1 site and Sp1 site contributes to the expression of this gene. Furthermore, not only NRF-1 and Sp1, but also the two NRF-2 sites might be important for the expression of this gene. Mutation analysis of NRF-2 sites (NRF-1A and/or NRF-1B) showed that single mutation of the each NRF-2 site did not affected the promoter activity of this gene (Fig. III-7). However, double mutations of both NRF-2 sites induced about 40% reduction of the activity (Fig. III-7). In case of *COX 5b* gene whose transcripts contain several heterogenous 5' ends, the cluster of NRF-2 sites is thought to act as transcription initiator (Carter and Avadhani, 1994; Sucharov *et al.*, 1995). Therefore, NRF-2 may be involved in transcription start site positioning in the *COX17* promoter region.

Interestingly, there is no amphiphilic  $\alpha$  helical domain with basic amino acid residues or transmembrane domain in the N-terminus of Cox17p peptide (Kako *et al.*, 2000). Because these domains are characteristic for COX

subunit proteins targeted to the mitochondrial inner-membrane (Roise and Schatz, 1988), Cox17p has been regarded as a cytosolic protein (Glerum *et al.*, 1996). Independent of their localization, expression of all COX related proteins might be controlled by Sp1, NRF-1 and NRF-2 transcription factors.

Since Scarpulla *et al.* have shown that CREB (cAMP-responsive element binding protein) is also involved in expression of cytochrome c (Gopalakrishnan *et al.*, 1994; Herzig *et al.*, 2000), the transcription of *COX17* may be regulated by CREB on cAMP- or serum-stimulation. During the evolutionary process, the cytochrome c-type promoter sequences have been inserted into the 5'-upstream of *COX17* to express its function related with cellular respiration. Further study is required to substantiate this idea. Since mice have been used extensively as experimental animals (i.e., mouse models for diseases of copper metabolism (Kodama and Murata, 1999) and mitochondrial function (Inoue *et al.*, 2000)), the promoter region analysis of the mouse *COX17* gene would be useful in producing transgenic mice and for developing further insight into perturbations of the Cox17p-Sco1p (or Sco2p) pathway in disease, such as fatal infantile cardioencephalomyopathy (Papadopoulou *et al.*, 1999; Jaksch *et al.*, 2000).

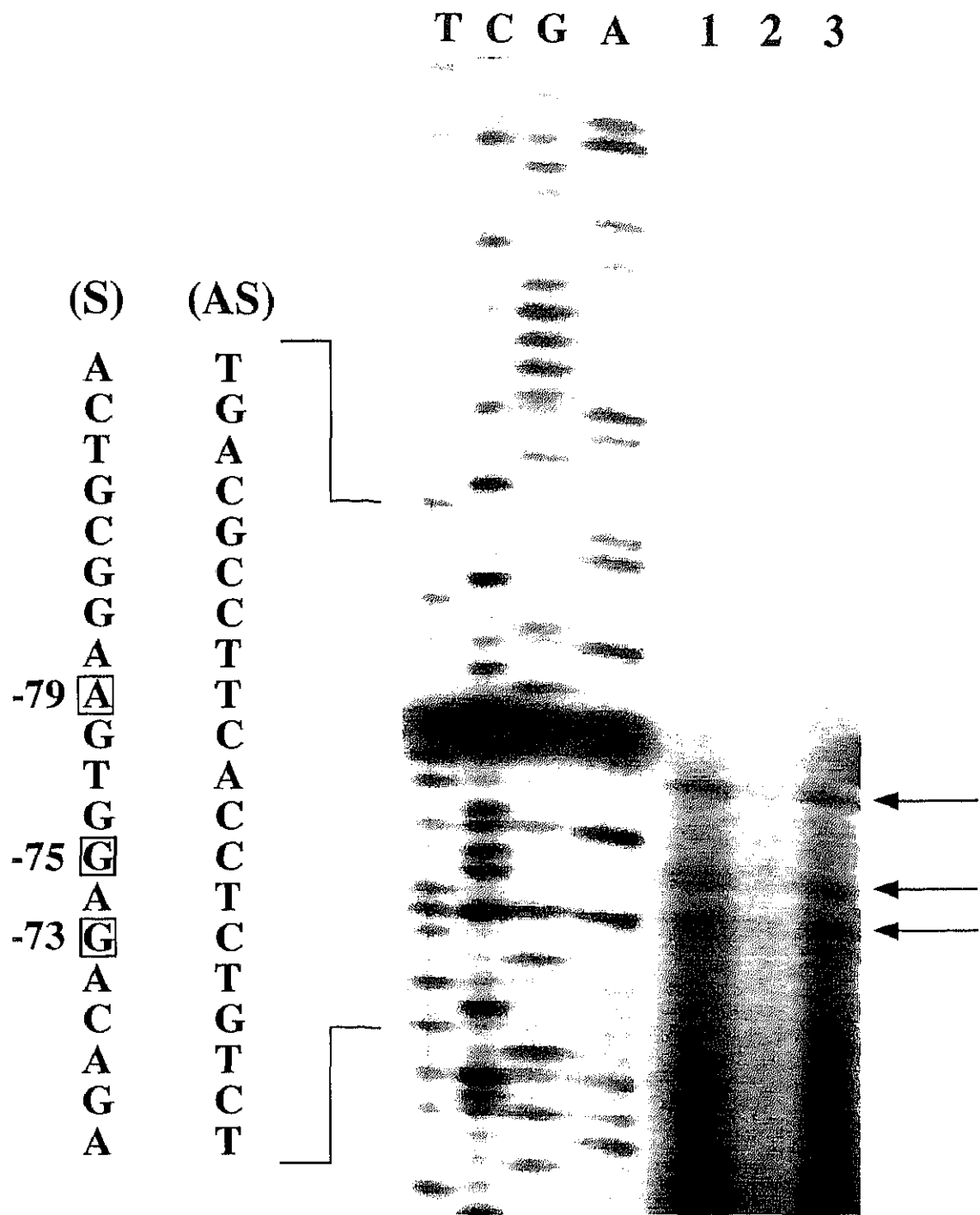
**Table III-1. Oligonucleotides used for EMSAs\***

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Sp1	5'- TCCGGAG <u>AGGCGGGACT</u> TCC -3' AGGCCTCTCCGCCCTGAAGG
Mut. Sp1	5'- TCCGGAGAT <b>T</b> AGGGACTTCC -3' AGGCCTCT <b>AAT</b> CCCTGAAGG
NRF-1	5'- TTCTTTT <u>TGCGCACGCGCTT</u> CTTAG -3' AAGAAAACGCGTGCGCGAAGAATC
Mut. NRF-1	5'- TTCTTTTGT <b>AAACGAAT</b> TTCTTAG -3' AAGAAAACATTTGCTTAAAGAATC

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\* Sequences corresponding to *cis*-elements are underlined and mutated nucleotides are shown to bold letters.



**Fig. III-1. Identification of the transcription initiation sites of the mouse *COX17* gene.** Results of the primer extension with mouse AtT-20 (1), NIH3T3 (2) and heart (3) RNA and sequencing reactions. A PCR-based double-stranded DNA sequencing reaction was run in parallel with the primer extension product on a 6% sequencing gel. From left to right, the lanes of the gel are T, C, G, A, and the primer extension products.



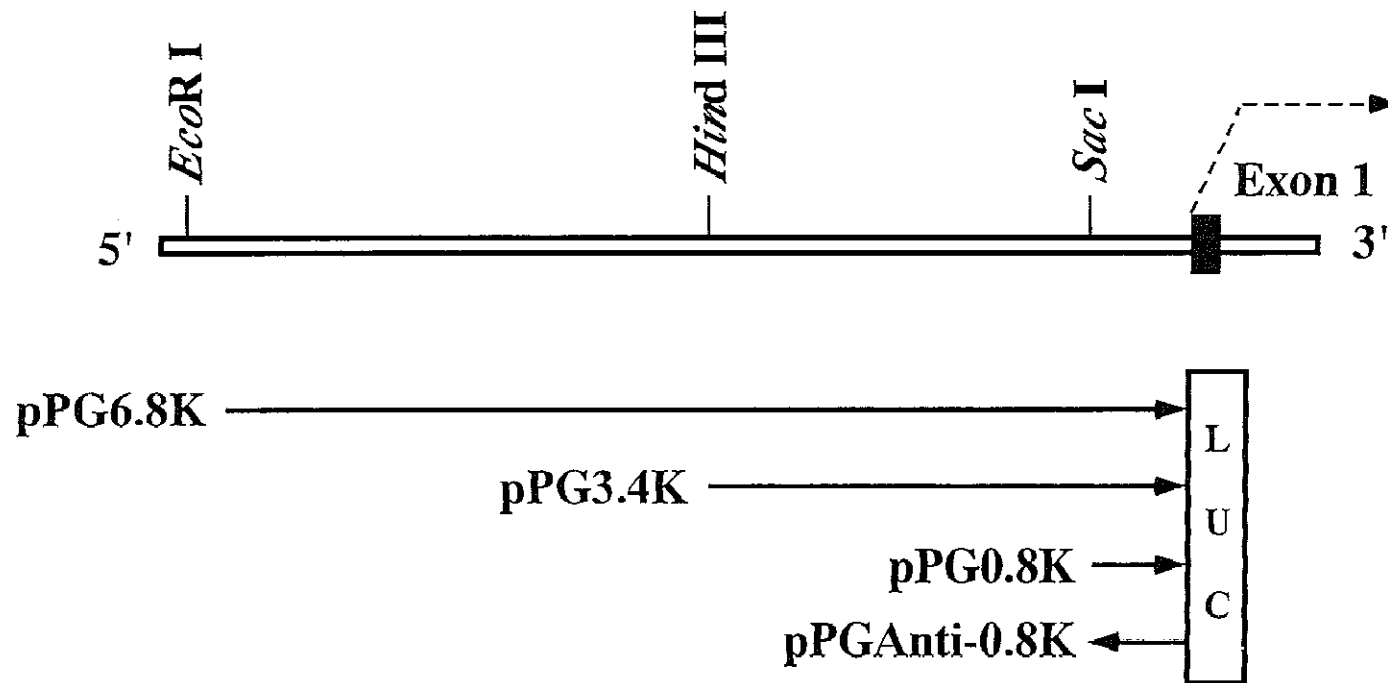
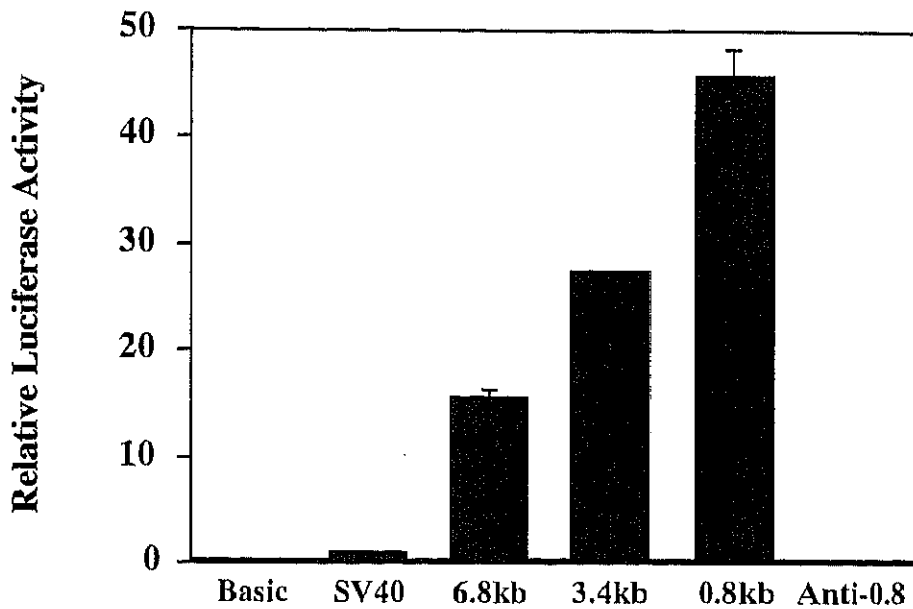
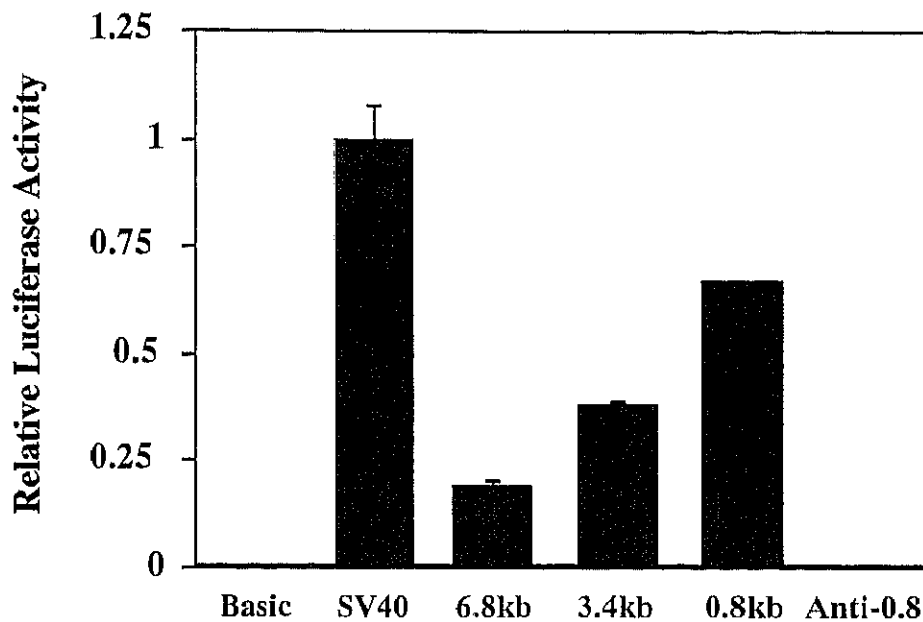


Fig. III-2. Schematic view of the constructs used to analyze the *COX17* promoter upstream fragment.



**Fig. III-3. The activity of the *COX17* promoter in transiently transfected AtT-20 cells.** Triplicate wells of AtT-20 cells were transiently transfected with the reporter vectors indicated. Luciferase activity was normalized to co-transfected  $\beta$ -Gal activity and the normalized values were then compared to the value for a positive control reporter vector transfected at the same time. The data shown is the average of at least seven independent experiments; error bars show the SEM.



**Fig. III-4. The activity of the *COX17* promoter in transiently transfected NIH3T3 cells.** Triplicate wells of NIH3T3 cells were transiently transfected with the reporter vectors indicated. Luciferase activity was normalized to co-transfected  $\beta$ -Gal activity and the normalized values were then compared to the value for a positive control reporter vector transfected at the same time. The data shown is the average of at least seven independent experiments; error bars show the SEM.

CA repeat

-874 aatggttgat ttgaagcaat ggagctctac tgaggggggtt acactttcag tatcagttac catgtcattt acttatccaa aacacacaca cacacacaca

GA repeat

-774 cacacacaca cacacacaca cacacacaga gagagagaga gagagagaga gagagagaga gagaaacaga gacagagaga cagagacagc coatcacagt

-674 tgttgaaagt agaaaaaaaa cttatcttct ttcattgacta ctgtgtcatg atatattatc tgaaaattta gcatatatgc cggagagaaa taggaggtag

NRF-2 NRF-2L

-574 ggggcctttg tatctgtatc tgcagaatgt ctacaaagca ggaaggaaga aagaccataa aatatcccag ggtggaacc ggatctgcta ctgcttgtcc

NGFI-B NRF-2 NRF-2

-474 agtacccaaaa ggtcagccct gaaaatacga atacagttag cattatactg gactgaatgg gtcatatagg aatatatgta tgcattgtata catataagca

NRF-2 NRF-2L NRF-2 NRF-2L NRF-2L

-374 tgcaataaca attagggagg aaaaggccat gaatttgaag gatagtggg aatgggtatg tatatgtaag gatttgtaga aaggaaagg aaaggga

CRE

-274 tgatgtaatt aaattacaat ctcaaaaaaca aaacaaaaac aaaaaccatt ctgggtgacc ttttaacctt tgcgccagcg ctgacgccac gcccttggag

NRF-1 NRF-2 GC box (SP1) NRF-2

-174 cgaggccttg taccacttct tttgcgcacg cgcttcttag acttacttcc gtccggag gag gcgggg cttc ctgtccccct tcgattgact gcggaagtgg

MRE MRE NF-kB +1 NRF-2

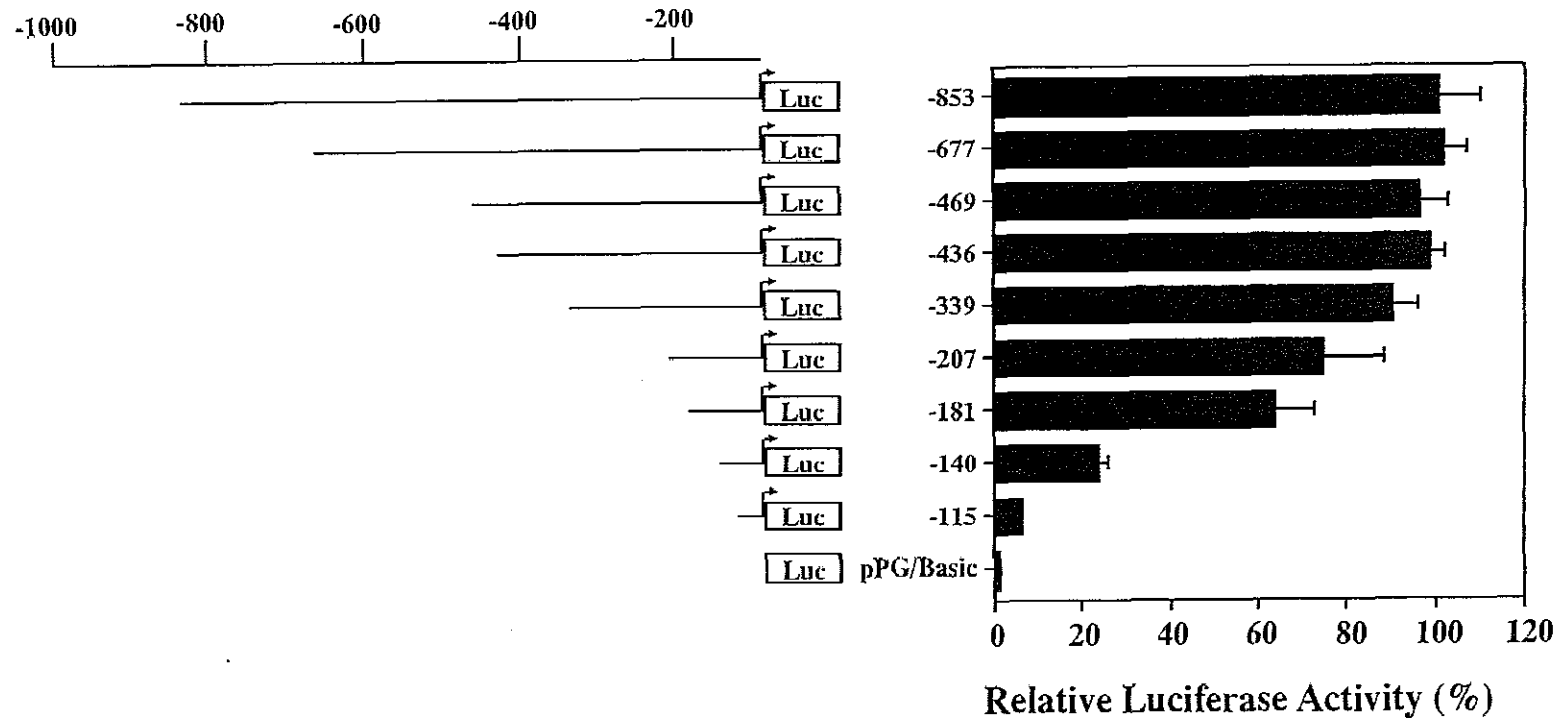
-74 agacagacgg ctcagggtag tcggagtttg ggagctttgc gcgtgtgcgc tcatatgttc tttcgcctgg **aaagATGCCG** G GACTGGCGG CCGCTAGCCC

M P G L A A A S P

27 TGCCCCGCCC GAGGCTCAGG AGAAGAAGCC ACTGAAGCCC TGCTGTGCCT GCCCGGAAAC CAAGAAGGCG CGTGATGCCG Ggtcagtgcgta gccc----

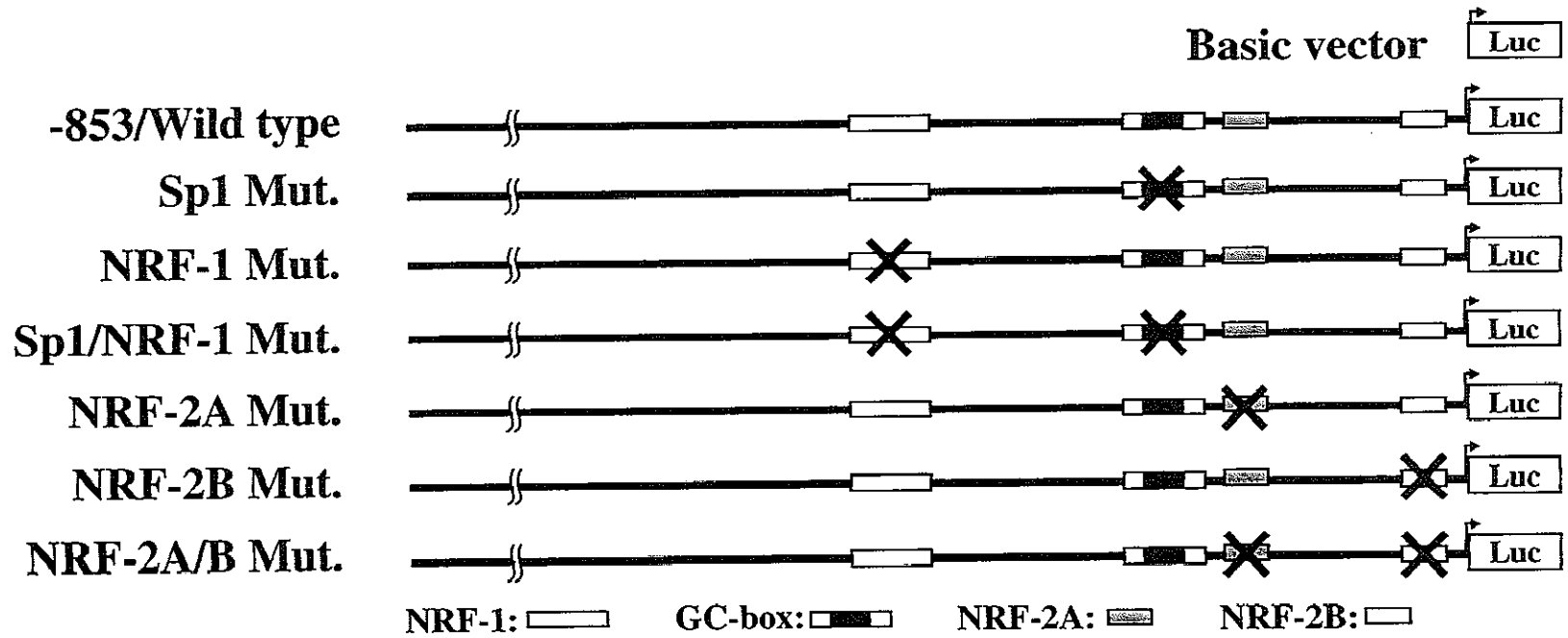
A P P E A Q E K K P L K P C C A C P E T K K A R D A

**Fig. III-5. The nucleotide sequence of the 5'-flanking region of the mouse *COX17*.** The transcription start site is designated as +1. The sequence is numbered with the first nucleotide of the translation initiation codon as +1. The translation initiation triplet (ATG) is highlighted in bold. Underlines show potential transcription factor recognition sites. The arrow indicates the primer region used for primer extension analysis, and the terminal positions of the primer extension are shown by arrowheads.

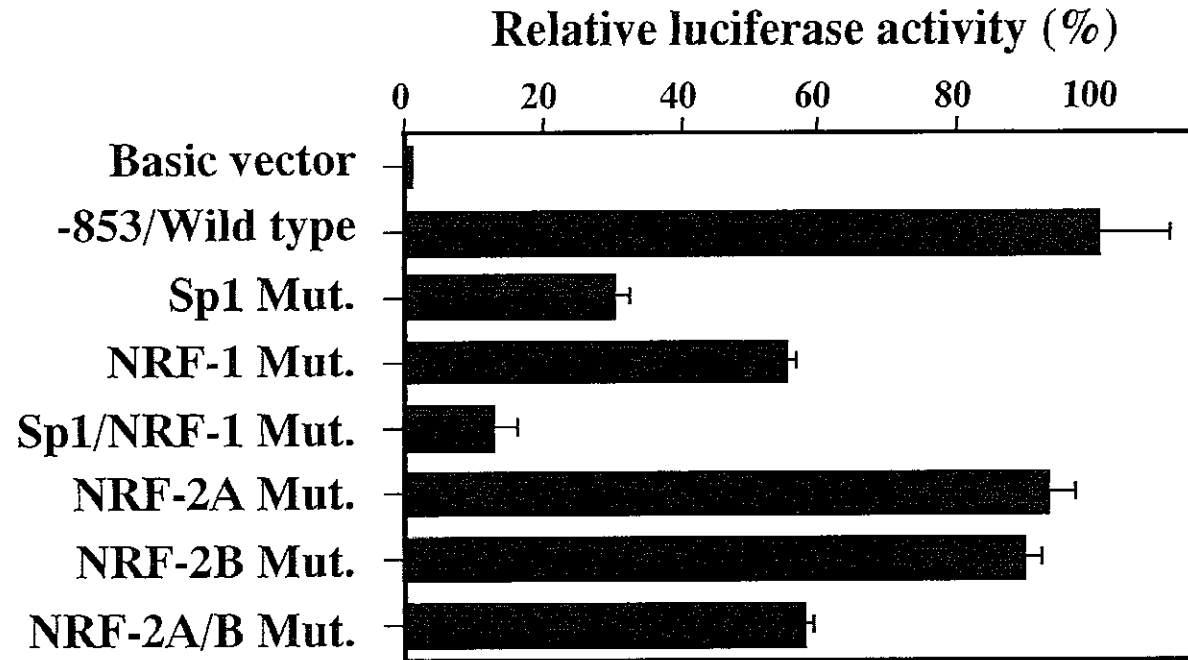


**Fig. III-6. Promoter activity of the 5'-flanking region of the mouse *COX17* gene.** Schematic representation of a series of 5' deletion constructs (left). Triplicate wells of AtT-20 cells were transiently transfected with these plasmids. Luciferase activity was normalized to cotransfected  $\beta$ -Gal activity and these normalized values were then compared to the most active construct (-853/Wild type, right). The data shown are averages of at least three independent experiments; error bars show the SDM.

A

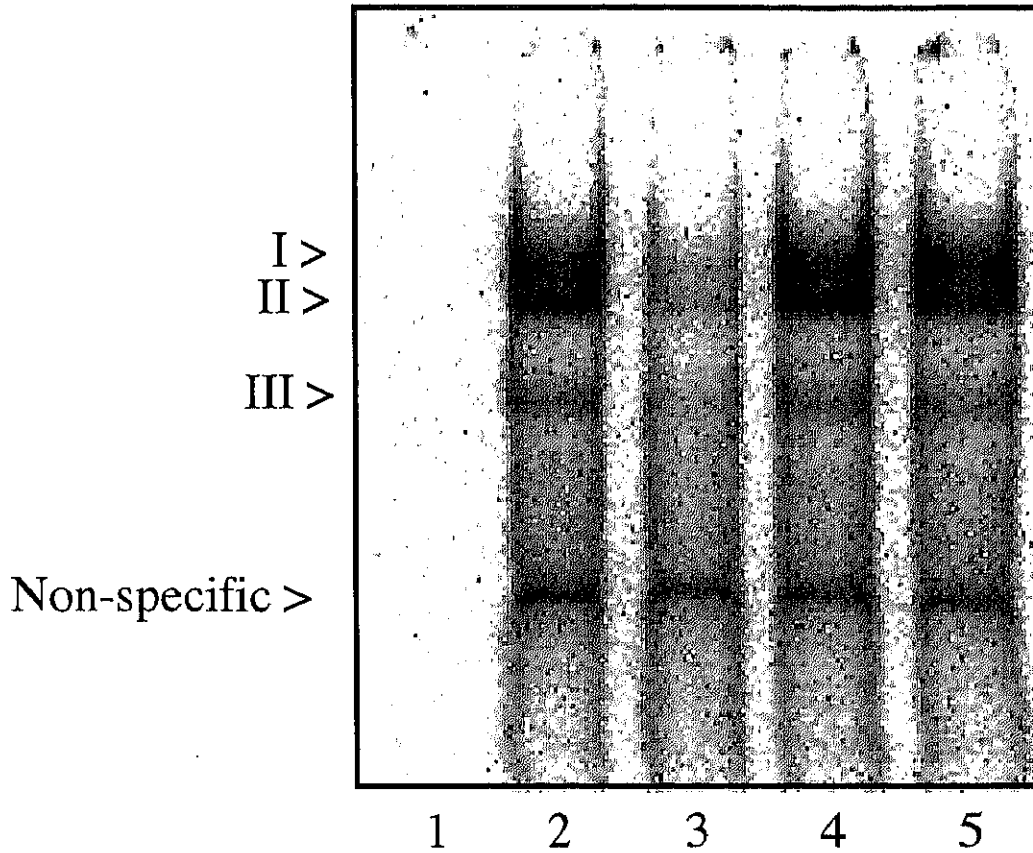


(Fig. III-7)

**B**

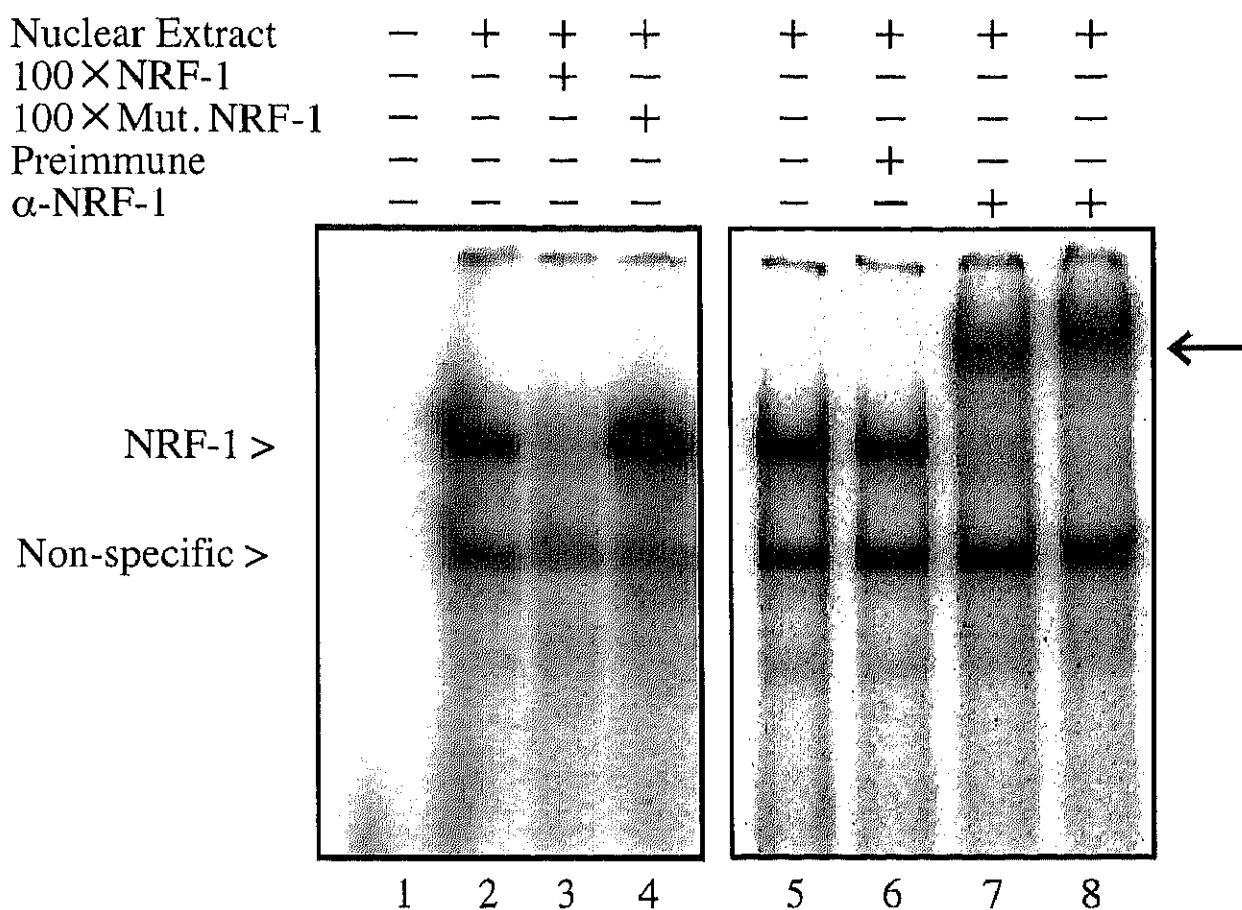
**Fig. III-7. Function of NRF-1, NRF-2 and Sp1 binding sites in the promoter activity of the 5'-flanking region of the mouse *COX17* gene.** (A) Schematic representation of the constructed reporter plasmids. (B) NIH3T3 cells were transfected with the indicated plasmids, and luciferase assays were performed. Luciferase activity was normalized to cotransfected  $\beta$ -Gal activity and these normalized values were then compared to the most active construct (-853/Wild type). The data shown are averages of at least three independent experiments; error bars show the SDM.

Nuclear extract	-	+	+	+	+
100 × Sp1	-	-	+	-	-
100 × Mut. Sp1	-	-	-	+	-
200 × Mut. Sp1	-	-	-	-	+



**Fig. III-8. EMSA with Sp1 element of the mouse *COX17* promoter.** EMSA was performed with 20 fmol of labeled GC-box-containing fragment as a probe. The DNA/protein complexes were numbered (I-III). Lane 1, free probe (without nuclear extract); lanes 2-5 all contained 20  $\mu$ g of protein from NIH3T3 nuclear extracts. Competition analysis was performed in the presence of a 100-fold molar excess of unlabeled wild type probe (lane 3), unlabeled mutant Sp1-containing probe (lane 4), and a 200-fold molar excess of unlabeled mutant Sp1-containing probe (lane 5).





**Fig. III-9. EMSA with NRF-1 element of the mouse *COX17* promoter.** EMSA was performed with 15 fmol of labeled NRF-1 site-containing fragment as a probe. The specific DNA/NRF-1 complex and non-specific band were detected, respectively. Lane 1, free probe (without nuclear extract); lanes 2-8 all contained 20  $\mu$ g of protein from AtT-20 nuclear extracts. Competition analysis was performed in the presence of a 100-fold molar excess of unlabeled wild type probe (lane 3) and unlabeled mutant NRF-1 containing probe (lane 4). Supershift analysis was performed in the presence of normal rabbit serum (lane 6) and anti-NRF-1 antiserum ( $\alpha$ -NRF-1) (1  $\mu$ l, lane 7; 2  $\mu$ l, lane 8). The arrow indicates the shift of the complexes by  $\alpha$ -NRF-1.