Structural Organization of Nuclear Gene for Subunit Vb of Mouse Mitochondrial Cytochrome c Oxidase*

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We have recently isolated a cDNA for nuclear encoded subunit Vb of mouse cytochrome c oxidase by screening mouse bone marrow and kidney cDNA libraries. In the present study, this cDNA was used as a probe to screen a mouse genomic library and isolate the complete gene encoding subunit Vb. Southern blot hybridization of mouse genomic DNA with the cDNA probe suggested the occurrence of multiple genes including many retroinserts. Restriction analysis followed by Southern blot hybridization of genomic clones was used to identify the putative retroinserts from the intron containing genes. Of the 10 initial genomic clones isolated, one clone (MG3) showing the most complex hybridization pattern was found to contain the complete gene for subunit Vb. The DNA sequence analysis show that the subunit Vb gene contains four exons of 149, 73, 99, and 189 bases interrupted by three relatively small introns of 520, 165, and 648 nucleotides in a gene spanning about 2.5 kilobase pairs. As determined by a combination of primer extension and S1 protection analyses, the major transcription start site appears to be located 49 nucleotides upstream of the translation initiation codon. The ability of the 5′ upstream DNA to initiate transcription was studied using the eukaryotic chloramphenicol acetyltransferase (CAT) expression plasmids in NIH 3T3 cells. Using this system we observed that a segment of the gene spanning nucleotides −574 to +45 can drive the transcription of CAT gene in an orientation dependent manner. The upstream region of subunit Vb gene lacks the TATA and CAAT elements, although it contains several GC rich elements and a pyrimidine rich stretch around the transcription start site.

Cytochrome c oxidase (EC 1.9.3.1), the terminal oxidase of the mitochondrial electron transport chain, is an oligomeric complex containing seven to nine subunits in the lower eu-

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2 The abbreviations used are: cytox, cytochrome c oxidase; bp, base pair; kb, kilobase pair(s); CAT, chloramphenicol acetyltransferase; PIPES, 1,4-piperazinediethanesulfonic acid.

largest subunits of the complex involved in the catalytic function are encoded by the mitochondrial genomes and the remaining smaller subunits are coded by the nuclear genes and targeted to mitochondria by a post-translational mechanism (2, 3). Although the precise functions of the nuclear encoded subunits remain unknown, a number of these sub-

EXPERIMENTAL PROCEDURES

Materials—Almost all chemicals and biochemicals used were of reagent grade purchased from Fisher Scientific (Pittsburgh, PA) or from Fluka Chemical Corp. (Ronkonkoma, NY). All of the restriction enzymes and T7 DNA ligase were purchased from New England Biolabs, Inc. (Beverly, MA). Calf intestinal alkaline phosphatase was from Boehringer Mannheim. S1 nuclease was from Life Science Technologies Inc. (Gaithersberg, MD). [32P]dCTP (6000 Ci/mmol), [γ-32P]ATP (9000 Ci/mmol), [35S]dATP (>600 Ci/mmol), and [3H]dGTP (>600 Ci/mmol), and [3H]dGTP (>600 Ci/mmol)
chloramphenicol (57 mCi/mmol) were purchased from Amersham Corp. A mouse genomic DNA library in phage λ EMBL-3 vector was purchased from Clontech Laboratories, Inc. (Palo Alto, CA). Avian myeloblastosis viral reverse transcriptase, RNasin, pGEM plasmids for cloning, and CAT plasmids for transient expression were purchased from Promega Corporation (Madison, WI). Nytran membranes for Southern and Northern blot analysis were purchased from Schleicher & Schuell Inc. A mixed primer based DNA labeling and T7 polymerase based DNA sequencing kits were purchased from Pharmacia LKB Biotechnology Inc.

Isolation and Sequencing of Genomic DNA—A library of adult DBA 23 mouse liver genomic DNA in phage EMBL-3 vector was screened with \(^3^P\) labeled DNA probe as described by Maniatis et al. (22). *Escherichia coli* NM383 cells were infected with about 5 \(\times\) 10\(^8\) plaque-forming units and plated on a 136-mm LB plate. About 10\(^6\) plaques from 20 plates were lifted on the Nytran membranes and hybridized with the labeled cDNA for subunit Vb described recently from this laboratory (21). Positive clones were isolated by repeated screening, and the DNA from resultant clones were isolated on a preparative scale by plate lysis followed by adsorption to Lambda Sorb, an affinity matrix from Promega Biotec Corp., and phenol extraction. The genomic DNA inserts (8–14 kb) from the resultant DNA preparations were purified by partial digestion with Sall alone or Sall and BamHI and cloned in pGEM 3z or 7z plasmids. Restriction digests of these clones were subcloned to generate smaller and overlapping inserts of 0.8–1.5 kb for further characterization. Miniprep double-stranded DNA was sequenced by the dideoxy chain termination method of Sanger et al. (23), using the T7 polymerase based sequencing kit. A mixed primer based DNA labeling and \(3^P\)-labeled subunit Vb cDNA probe and isolated 10 positive clones were subcloned in pGEM 32 and 72 plasmids. Restriction digests of these plasmids were subcloned to generate smaller and overlapping inserts of the 187 nucleotides long S1 probe. 5' end-labeled primer (100 ng) was annealed to 50 pg of total RNA isolated from mouse liver in 0.2 M KCl, 1 mM EDTA, 10 mM Tris-Cl (pH 8.0), and 2 units/\(\mu\)l of RNasin in a total volume of 16 ul and incubated in a water bath at 68°C for 20 min. The water bath was steadily cooled down to 37°C over a period of 3 h as described before (25). The reaction mixture was adjusted to contain 75 mM Tris-Cl (pH 8.0), 10 mM MgCl\(_2\), 10 mM dithiothreitol, 70 mM KCl, 800 \(\mu\)M each of dATP, dTTP, dCTP, and dGTP, and 1 unit/\(\mu\)l of avian myeloblastosis virus reverse transcriptase in a total volume of 32 \(\mu\)l. The reaction was carried out at 42°C for 2 h and stopped by adding 5 \(\mu\)l EDTA. The reaction products were extracted with phenol-chloroform, precipitated with ethanol, denatured by heating at 80°C for 5 min in 80% formamide, and analyzed on a 6% polyacrylamide, 8 M urea sequencing gel. A sequencing ladder of the genomic DNA containing the 5' region of the gene was generated by using the same 23-mer oligonucleotide and used as the size marker.

**S1 Nuclease Protection**—The same primer used for the primer extension analysis was used to generate a 187 bp genomic DNA fragment of about 15 ng. This 5' end-labeled primer (100 ng) was annealed to 15 \(\mu\)g of alkali denatured DNA from a genomic subclone containing the first 1-kb region of the gene and extended using the Klenow fragment of DNA polymerase I (22). The double-stranded DNA thus generated was digested with Accl, end-labeled, and hybridized with S1 nuclease at 37°C for 30 min essentially as described before (26). The S1-resistant fragments were denatured and resolved on a 6% sequencing gel as described for the primer extension analysis.

**Assays for Transcription Promoter Activity**—The Sall-Aval segment of the gene containing nucleotide number -574 to +45 relative to the mRNA cap site was tested for transcription promoter activity using expression plasmids containing the bacterial CAT reporter gene. The two plasmids used in this study have a common pUC19 backbone and intact CAT coding sequence. One of them named pCAT-basic lacks both promoter and enhancer elements, whereas the second plasmid named pCAT-enhancer contains an SV40 enhancer element, but no promoter (Promega Corporation). For cloning the Sall-Aval segment in the sense orientation, the Aval end was blunted by gap filling with dNTPs and T4 polymerase (22), and the 638-bp DNA was released by digesting with Sall. This fragment contains the region +45 to -574 of the gene, and a 19 nucleotide long polylinker sequence at the upstream end 5' to the cap site. The pCAT-basic and pCAT-enhancer plasmids were first cut with XbaI, and the protruding ends were blunted by gap filling. The plasmid was subsequently cut with Sall and ligated to the 638-bp DNA modified as described above. For cloning the Sall-Aval insert in the reverse orientation, the pCAT-basic plasmid was first cut with HindIII, blunt-ended by gap filling, and the modified 638-bp DNA was subsequently cloned in the blunt ended HindIII and Sall sites. Using this method three different plasmid constructs were generated, each containing the cDNA insert in the same orientation as the S1 fragment. One insertion was generated using pCAT-Bs (basic, sense orientation), pCAT-Br (basic, reverse orientation), and pCAT-Es (enhancer containing sense orientation).

The promoter activities of the plasmid constructs were tested by transfecting the DNA to NIH 3T3 cells. The cells were maintained in 75-cm\(^2\) flasks using Dulbecco's modified Eagle's high glucose medium supplemented with 2 mM glutamine, 50 \(\mu\)g/ml gentamycin, and 10% heat-inactivated fetal bovine serum. For transfection experiments, the cells were seeded at a density of 8 \(\times\) 10\(^5\) cells/10 cm dish and transfected 24 h later with 15 \(\mu\)g of DNA using the calcium phosphate precipitation method (27). After 48 h incubation, the cells were harvested, and the cell extracts were assayed for protein content, and CAT activity. CAT assays were performed using \(\[^{14}C\] chloramphenicol as the substrate and 25–50 \(\mu\)g of cellular protein as the enzyme source as described by Gorman et al. (28). Acretylized forms of chloramphenicol were separated by thin layer chromatography on Silica gel plates and viewed by autoradiography. The extent of conversion was quantitated by scanning the x-ray films through an LKB Ultrascan-XL (Pharmacia).

**RESULTS AND DISCUSSION**

**Strategy for the Isolation of Cytochrome Oxidase Vb Gene**—The complexity of cytox Vb gene in the mouse genome was determined by Southern hybridization of restriction enzyme digested genomic DNA with \(^3^P\)-labeled cDNA probe. As shown in Fig. 1A, the cDNA hybridized with multiple bands of 2–10 kb from mouse genomic DNA digested with EcoRI, HindIII, and BamHI, suggesting the existence of multiple genes. A previous study on the Northern blot analysis of RNA, and sequencing of cDNA, however, failed to detect any significant tissue specific heterogeneity in mouse subunit Vb (21). The results of Southern hybridization thus suggested the possible occurrence of pseudogenes as predicted for some of the cytochrome oxidase genes and also ribosomal protein genes (1, 2, 18, 19, 29–31).

Initially, we screened about 10\(^8\) plaque-forming units of mouse liver genomic DNA library in EMBL-3 vector using \(^3^P\)-labeled subunit Vb cDNA probe and isolated 10 positive clones. Inserts of 8–14 kb were extensively analyzed by restriction mapping, and the fragments hybridizing with the subunit Vb cDNA probe were subcloned in pGEM 3z and 7z plasmids for sequencing. Although not presented here, the first two clones we sequenced turned out to be the fully processed retroposon type of pseudogenes. We therefore decided to search for the intron-containing gene using a restriction mapping strategy. As shown in Fig. 2, the genomic DNA...
The complexities of cytox Vb gene in the mouse genome. Ten μg of each genomic DNA from mouse Ehrlich ascites cells was digested to completion with the appropriate restriction enzymes, resolved by electrophoresis on a 0.8% agarose gel, transferred to Nytran membrane, and probed with 32P-labeled DNA probes as described under "Experimental Procedures." A shows the patterns of hybridization with 32P-labeled cytox Vb cDNA probe. Lane 1, HindIII; lane 2, BamHI; and lane 3, EcoRI. B shows the hybridization profile with 32P-labeled intron 1-specific Neo I-SphI fragment (see Fig. 3) of the gene. Lane 1, BamHI; lane 2, EcoRI; and lane 3, XbaI. HindIII-digested λ DNA was run alongside as molecular weight markers.

Restriction analysis of mouse genomic clones. Cytox Vb genomic DNA clones and cDNA were digested with NcoI and DdeI and probed by the Southern blot hybridization with 32P-labeled NcoI-DdeI fragment of cDNA by Southern blot hybridization. The choice of these two restriction enzymes was based on our previous observations that the mouse cytox Vb cDNA contains a unique NcoI site close to the 5' end and a DdeI site 300 nucleotides downstream toward the 3' end. The Southern blot in Fig. 2 shows the restriction patterns of four different genomic clones and the Vb cDNA digested with NcoI and DdeI. It is seen that two of the genomic clones contain a 300-bp fragment similar to the fragment from the cDNA digest suggesting the possible absence of introns in these genomic clones. Although not shown the remaining five genomic clones showed a similar 300-bp NcoI-DdeI fragment. The genomic clone designated as MG9 yielded a single band of 500 nucleotide, suggesting that it may be a partially processed gene, whereas clone MG3 exhibited the most complex pattern with fragments of 500 and 750 bp (see Fig. 2). The latter clone was further characterized and found to harbor the complete subunit Vb gene. Hybridization of mouse genomic DNA blots with a probe specific for the first intron of the gene yielded single bands (see Fig. 1B), suggesting that there may be a single copy of the complete intron containing gene in the mouse genome. Our results, however, do not rule out the possible existence of an evolutionarily divergent Vb gene in the mouse genome.

Structural features of cytox Vb gene. As shown in Fig. 3, subunit Vb gene contains one site each for SacI, BglI, and Accl, two sites for NcoI and AcoI, and four sites for HindII in a stretch of about 2.5-kb DNA. Restriction fragments of 0.5-1.5-kb DNA were subcloned in pGEM 32 or 72 plasmids for sequencing. Over 80% of the gene was sequenced in both directions using the strategy shown in Fig. 3.

The complete nucleotide sequence of the subunit Vb gene has been presented in Fig. 4. The gene contains four exons of 149, 73, 99, and 189 bp, which are interrupted by introns of 520, 165, and 648 nucleotides in the 5' to 3' direction. The first intron starts 10 nucleotides downstream from the N terminus of the mature protein. The remaining two introns also occur within the protein coding region of the gene. The intron/exon boundary sequences conform to published consensus sequence (32). The intron splice phase is type 1 (interrupting the codons) for the first and third introns, and the second intron is type 0, since it does not interrupt the codon. As seen from Fig. 4, each splice donor begins with a GT and each acceptor site ends with an AG, preceded by a poly pyrimidine stretch. Furthermore, the exon sequences are identical to the corresponding cDNA sequence (21), except for a single substitution each in the 3'-untranslated region compared with mouse bone marrow cDNA at position 1838 (T instead of C) and mouse kidney cDNA at position 1825 (C instead of T). It is also seen that exon 1 codes for the 5'-untranslated region.

| S | A | G | C | V | N | A | C | N | A | C | L | A | N | I | S | H | H | H |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |

Fig. 3. The restriction map of cytox Vb gene and the strategy for sequencing. The arrows indicate the direction of sequencing and the regions covered by each sequencing run. The closed boxes indicate the relative locations and sizes of exons 1–4.
The hydropathy profile of mouse cyt c Vb protein with respect to exon-intron organization has been presented in Fig. 5. As previously shown for this subunit from bovine heart (4), the protein shows clusters of hydrophobic domains as expected of a subunit extrinsic to the membrane. Although the intron positions coincide with the transition regions, the functional implications of these domains remain unclear at this time. It is also seen that the N-terminal signal sequence shows an unusual biphasic nature with the second half of the signal showing hydrophilicity. Furthermore the cyt c Vb precursor sequence contains Thr at position −4, Ala at position −7, and Arg at position −9, similar to the "conserved three-amino acid" motif predicted for mitochondrial proteins undergoing a two-step proteolytic processing (33). The relative location of the hydroxylated hydrophobic (although weakly), and the basic amino acid residues in the cyt c Vb protein, however, is shifted by one residue as compared with the predicted locations in other proteins (33). In view of models on cytox subunit topology (2, 4), suggesting that subunit Vb is an ectopic protein facing the cytosolic side of the membrane, it remains to be seen if the putative two step processing signal has any role in the transport of the protein to cytoplasmic side of the inner membrane.

The transcription start site(s) of cyt c Vb gene was determined using a combination of primer extension and S1 nuclease protection analysis. The same 23-mer synthetic oligonucleotide containing the anti-sense sequence of the translated region (see Fig. 4) was used as the primer for the RNA-dependent extension and also for preparing the S1 probe as described under "Experimental Procedures." As shown in Fig. 6 (lane 2), primer extension against mouse liver RNA template shows a major and longest product corresponding to a C residue located 49 nucleotides upstream from the translation initiation site. It is also seen from lanes 9 and 10 that the major and also the largest fragment resistant to S1 digestion is of identical size. These results show that the major transcription of cyt c Vb gene begins at 49 nucleotides upstream of the translation start site. This position, the putative mRNA cap site has been designated as +1 nucleotide of the gene. The results of primer extension and the S1 analysis also suggest the possible occurrence of minor transcription initiations at about +9 and +20 nucleotides of the gene.

Characteristics of the 5′-Upstream Sequences—The sequence upstream of the transcription initiation site revealed several structural features which might be important in transcription regulation. As shown for a number of housekeeping genes and genes coding for some of the mitochondrial proteins in addition to the entire presequence, and three and one-third amino acids of the mature protein. Exon 4 codes for the C-terminal 36 and 2/3 amino acids in addition to the 79 nucleotide long 3′-untranslated region. Based on comparison with the cDNA sequence, the polyadenylation site appears to be at nucleotide 1848, preceded by the polyadenylation signal sequence at positions 1817 to 1822.

**Fig. 4.** Complete sequence of mouse cyt c Vb gene. The nucleotide residues are numbered beginning with the transcription start site, the putative mRNA cap site. The transcription start site and the direction of transcription have also been marked with a solid arrow. The dotted arrow indicates the predicted N terminus of mature Vb subunit based on the bovine sequence (60). The polyadenylation site (residue 1848) has been marked with an asterisk. The intron regions have been indicated by a dotted line underneath the nucleotide sequence. The sequences showing complete or partial homologies to known transcription factor binding sites have been indicated. Also underlined is the position of the 23-mer anti-sense primer used for generating the S1 probe and also for the primer extension analysis.
(34-37), the cytochrome Oxidase Subunit Vb gene lacks the TATA and CAAT sequences which act in concert as the transcription promoter in a wide variety of cells from lower eukaryotes and animal cells. The sequences around the major transcription start site are rich in pyrimidine residues and show part homology to the "initiator" sequence (PyPyCAPyPyPyPyPy) found in a mouse liver RNA (39). In the vicinity of GC boxes we found a number of GC-rich sequences showing partial or complete homology to the transcription factor SP1 binding sites (39-41). The sequence GCCGGCCGG at positions -133 to -141 and partly inverted sequence CGCCCC at positions -145 to -150 are some of the examples. In addition there is one enhancer core like sequence GTGGAAG (positions -288 to -294), which is nearly identical to the consensus sequence GTGGAAAG (42). In the vicinity of GC boxes we found sequence TGACTGA at positions -156 to -162, which closely resembles the GCN4 or AP-1 transcription factor binding sequence TGAG(C)TCA (43, 44). In addition, there is a sequence CAGCTAGCGG on the opposite strand at positions -169 to -178 that is homologous to the AP-4 binding site of the SV40 late promoter (45). A similar stretch of sequence has recently been shown to occur at about 123 nucleotides upstream of the major transcription start site of rat cytochrome subunit IV gene (18). An octanucleotide sequence TTCGTGGGT partially overlapping the putative enhancer core sequence is located at positions -293 to -300. This sequence is reminiscent of the UAS2 element (TNPyTTYGGT) which is known to be present in the upstream regions of Yeast nuclear genes coding for mitochondrial proteins (46-49) and implicated in binding transcription factor Hap 2/Hap 3. Similar sequences are detected in nuclear genes coding for cyto subunit IV (20), subunit IV (18, 19), and also other mitochondrial heme proteins in animal cells (50, 51).

Promoter Activity of the 5'-Flanking Sequence—The ability of the 5'-flanking region of cytochrome Oxidase Subunit Vb gene to promote transcription initiation was examined using the CAT expression plasmids. The 638-bp DNA containing the genomic sequence +45 to -574 with respect to the cap site and 19 nucleotide region of the polynucleotide at the 5' end was cloned in the sense or anti-sense orientation in pCAT-basic and pCAT-enhancer plasmids (see "Experimental Procedures") and used for transfecting NIH 3T3 cells. As shown in Fig. 7, the 638-bp fragment introduced in the sense orientation in the promoter/enhancer-less plasmid (pCAT-Bs) directed the expression of CAT to the extent of 35% conversion of the input substrate. When this fragment was placed in the sense orientation downstream of SV40 enhancer plasmid (pCAT-Es) the expression is increased significantly, yielding about 56% conversion (see Fig.
However, the same fragment cloned in the anti-sense orientation was unable to drive the transcription of CAT gene to any significant level. The failure to drive the CAT activity in the anti-sense orientation was not due to an aberrant translation initiation, since the insertion of the 638-bp DNA did not introduce any additional ATG codons. Furthermore, although not shown the DNA sequence from the polylinker region, including the 19 nucleotides attached to the cytox Vb upstream sequence, was unable to promote the transcription in either directions suggesting that the observed activity is indeed due to the upstream genomic DNA sequence. Thus, as shown for a number of transcription promoter elements the cytox Vb element used in these experiments is directional in nature.

The ability of the 619-bp upstream DNA to drive the transcription of CAT gene in transient expression studies (Fig. 7) in an orientation-dependent manner suggests that this region houses the putative transcription promoter elements. These results along with the results of Southern blot analysis presented in Fig. 1B provide direct proof that the subunit Vb gene contains typical TATA and CAAT elements (20). As seen from Fig. 4, the gene lacks the classical TATA and CAAT consensus motifs. The presence of these structures and the occurrence of GC-rich boxes are the characteristics of transcription promoters of housekeeping genes which are transcribed in most tissues, although similar features are found in some genes expressed in a tissue-specific manner (52–55). Genes with such features typically have several transcription start sites. In keeping with these observations, the results of primer extension and S1 protection experiments (Fig. 6) show multiple transcription start sites for cytox Vb gene as well. In many respects the promoter region of the subunit Vb gene resembles the TATA less subunit IV gene which contains typical TATA and CAAT elements (20).

Although the promoter element(s) responsible to drive the transcription in the transient expression assay system has not yet been fine mapped, the 180-nucleotide region upstream of the cap site might be critical for the promoter function. The presence of the putative SP1, AP1, and AP4 binding sites within this region may be of special significance for transcription initiation. Some of the GC-rich elements can also function as enhancer elements in presence of transcription activator factors such as SP1 or ETF (56, 57) and as repressor elements in the presence of inhibitory factors such as GCF (58).

In summary this paper describes the isolation and characterization of mouse cytochrome oxidase subunit Vb gene with 5'-flanking sequences capable of promoting transcription initiation in NIH 3T3 fibroblast cells. Furthermore, the relatively simple restriction analysis strategy used in this study might be helpful in the identification and isolation of intron containing functional genes for other cytox subunits from higher animals.

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