J. Biochem. 129, 709–716 (2001)

Characterization of Cytochrome b_5 in the Ascidian *Polyandrocarpa misakiensis* and Budding-Specific Expression[1]

Toshitsugu Yubisui,[2] Fumi Takahashi, Takashige Takabayashi, Shigeki Fujiwara, and Kazuo Kawamura

Laboratory of Cellular and Molecular Biotechnology, Faculty of Science, Kochi University, Akebono-cho, Kochi 780-8520

Received December 7, 2000; accepted February 9, 2001

A cDNA for cytochrome b_5 was cloned from a cDNA library of buds of the ascidian, *Polyandrocarpa misakiensis*, by a hybridization method involving a digoxigenin-labeled cDNA probe of human soluble cytochrome b_5. The nucleotide sequence of the cDNA for the ascidian cytochrome b_5 (Pmb5) consisted of about 1,800 base pairs including 5′- and 3′-noncoding regions, and a coding sequence of 405 base pairs. The amino acid sequence of 135 residues deduced from the coding nucleotide sequence exhibited 54% identity and 76% similarity to chicken cytochrome b_5. A highly conserved amino acid sequence was observed in the amino-terminal domain of 96 residues containing two heme-binding histidine residues. The putative soluble form of the recombinant Pmb5 expressed in *Escherichia coli* was purified to homogeneity by column chromatographies on an anion-exchanger and gel filtration. The purified Pmb5 showed the typical absorption spectrum of cytochrome b_5 with an asymmetric peak at 556 nm and a shoulder at 560 nm upon reduction with NADH and NADH-cytochrome b_5 reductase. The low temperature spectrum of the dithionite-reduced form of the protein contained the split peaks at 551 and 555 nm, this spectrum being very similar to that of mammalian liver cytochrome b_5. Expression of Pmb5 in the ascidian was examined immunohistochemically with a monoclonal antibody against the Pmb5. Apparently high level expression of Pmb5 was found in the developing buds, but the levels of cytochrome b_5 in the parents and juvenile adults were very low. This is the first report on the characterization of Pmb5, and the increased expression of Pmb5 in the ascidian.

Key words: ascidian, budding-specific expression, cDNA, cytochrome b_5, recombinant protein.

To analyze the bud development of the ascidian, *Polyandrocarpa misakiensis* (*P. misakiensis*), we have characterized various proteins and genes for retinoid receptors (*1*, *2*), lectins (*3*, *4*), aldehyde dehydrogenase (*5*), or proteases (*6*), and established a cell lineage derived from the atrial epithelium of the animal (*7*). Beside these studies, we have performed biochemical characterization of the ascidian for further understanding of this animal. Analysis of lipids and fatty acids in the animal revealed that very-long-chain polyunsaturated fatty acids are abundantly present in phospholipids and that an extraordinarily large amount of triacylglycerol (TG) is accumulated in buds of the animal. Such active fatty acid metabolism was supposed to be closely correlated with the activity of cytochrome b_5 in the animal, because cytochrome b_5 in vertebrates is known to participate in the metabolism of fatty acids (*8–17*). This metabolism includes fatty acid desaturation (*8*), fatty acid elongation (*9*), cholesterol biosynthesis (*10*, *11*), some reactions in cytochrome P450-dependent drug metabolism (*12–14*), or steroid hormone biosynthesis (*15–17*).

Therefore, we cloned a cDNA for cytochrome b_5 of the ascidian (Pmb5), and characterized it in this study in relation to the metabolism of fatty acids in the animal. The amino acid sequence (135 residues) deduced from the cDNA of Pmb5 is homologous to those of vertebrate cytochrome b_5s (*18*), which suggests that Pmb5 plays important roles in maintaining the metabolic activities of the ascidian. Very interestingly, Pmb5 was found to be expressed at a high level in the buds, but not in the parents, as determined by whole-mount immunohistochemistry and Western-blotting.

This paper first describes the cloning and characterization of cytochrome b_5 from *P. misakiensis*. The budding-specific expression of Pmb5 seems to be correlated with remarkable accumulation of TG in the growing and developing buds.

MATERIALS AND METHODS

Restriction enzymes, *Hin*dIII, *Xba*I, and *Bsr*GI were purchased from New England Biolabs (Beverly, MA). *Eco*RI, a digoxigenin (DIG) labeling kit for DNA, anti-DIG antibodies conjugated with alkaline phosphatase, nitroblue tetra-

[1] This study was supported in part by a Grant-in-Aid for International Scientific Research (Joint Research) (09044092), and also by the Novo Nordisk Foundation and the Japan Foundation for Applied Enzymology. The nucleotide sequence data in this paper have been deposited in DDBJ under accession number AB042613.

[2] To whom correspondence should be addressed. Tel/Fax: +81-88-844-8313, E-mail: tyubi@cc.kochi-u.ac.jp

zolium, 5-bromo-4-chloro-3-indolyl-phosphate, and blocking reagent for nucleic acid hybridization were obtained from Boehringer Mannheim (Tokyo). Ampicillin and isopropyl-β-D-thiogalactoside (IPTG) were products of Nacalai Tesque (Kyoto). The TA cloning vector was obtained from Invitrogen (Carlsbad, CA). Oligotex dT-30 was purchased from Roche (Tokyo). The nitrocellulose membrane was a product of Bio-Rad Laboratories (Richmond, CA). Other reagents used in this study were all of reagent grade.

Construction of a cDNA Library—Buds of *P. misakiensis* were cut from parental bodies with a razor, and then allowed to develop for one day. Total RNA was extracted from the buds by the acid guanidinium-phenol-chloroform method (*19*), and mRNA was purified using Oligotex dT-30 (Roche). A cDNA library was prepared with the purified mRNA using a ZAP-cDNA^R Synthesis Kit and the Uni-ZAP XR vector (Stratagene, La Jolla, CA). The amplified cDNA library (*4*) was used for screening of the cDNA for Pmb5.

Labeling of cDNA Probe—The cDNA for the soluble form of human cytochrome b_5 (306 bp) (*20*) was cleaved out from expression plasmid pKK223-3 (kindly supplied by Dr. A.W. Steggles of Northeastern Ohio Medical College) by digestion with *Eco*RI and *Hin*dIII, and purified by agarose gel electrophoresis. The cDNA fragment isolated from the gel was labeled with DIG according to the protocol for the reagent kit (Boehringer Mannheim) using Klenow enzyme, and then used for screening of the cDNA for Pmb5.

Screening of a cDNA for Pmb5—A total of 3×10^5 phage clones of the cDNA library described above were screened for cytochrome b_5 cDNA using the DIG-labeled human cytochrome b_5 cDNA. Hybridization was carried out at room temperature (25°C) for 20 h, followed by washing twice with a 2 × SSC (0.3 M sodium chloride and 0.03 M sodium citrate) solution containing 0.1% SDS at room temperature. After washing, the filters were treated with the anti-DIG antibodies conjugated with alkaline phosphatase to detect any positive clones. Phages of putative positive clones were purified by second screening.

DNA Sequence Analysis—Cycle sequence reactions were performed with cDNAs or PCR products as the template using a Thermo Sequenase Dye Terminator Cycle Sequencing Pre-mix Kit (Amersham Pharmacia Biotech, Tokyo). The DNA sequences of the reaction products were determined with a DNA sequencer 373A (Applied Biosystems, USA), and the obtained sequences were analyzed with homology search programs of BLASTX or BLASTN through the Internet.

Construction of Expression Plasmids for Pmb5—To produce the membrane-bound and putative soluble forms of recombinant Pmb5 in *E. coli*, forward and reverse primers were synthesized based on the cDNA sequence. The forward primer is 5′-GGAATTCATGGCGGAACAACAGACAG-3′, corresponding to the N-terminal sequence of the MAEQQT site including the *Eco*RI sequence (underlined). The reverse primers for the putative soluble and membrane-bound forms are CGCAAGCTTAAGGCTGTTCTTCTTGA-3′ and 5′-CAAGCTTAGTTGGAGATGTAGTAGC-3′ including the *Hin*dIII sequence (underlined), respectively. These sequences correspond to the amino acid sequence of QEEQPstop, 92nd–96th residues, and RYYISNstop, respectively. Using the phage DNA of the positive clone as a template, cDNA fragments for the putative soluble and membrane-bound forms of Pmb5 were amplified by PCR with the primers described above. The PCR products were ligated once to a TA cloning vector, and then the insert DNA was transferred to the *Eco*RI/*Hin*dIII site of the pKK-223-3 vector. Expression of Pmb5 in *E. coli* was judged after culture by observing the pink color of the cell pellet. Culturing of the *E. coli* clone harboring the pKK223-3/Pmb5 was performed as described previously (*21*).

Purification of the Recombinant Pmb5—Preparation of cell homogenates was carried out as described previously (*21*), and Pmb5 was purified by anion-exchange chromatography on a DEAE-Toyopearl column and gel filtration on a Sephacryl S-100 column. The purified Pmb5 was finally concentrated on a small DEAE-Toyopearl column and the Pmb5 eluted from the column was dialyzed against 10 mM KPB (pH 7.5). The purity of the recombinant Pmb5 was examined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) by the method of Laemmli (*22*).

Determination of Absorption Spectra—Absorption spectra of Pmb5 were measured with a Union automatic recording spectrophotometer SM401, and low temperature spectra were obtained under liquid nitrogen. Pyridine-hemochrome of the Pmb5 was prepared according to the method of Falk (*23*), and the spectrum was recorded.

Preparation of a Monoclonal Antibody against Pmb5—The cDNA fragment of the putative soluble Pmb5 was ligated downstream to the glutathione S-transferase (GST) cDNA using the pGEX vector at the *Eco*RI and *Hin*dIII sites. Expression of the GST/Pmb5 fusion protein was induced in *E. coli* BL21 by adding 0.5 mM IPTG. The fusion protein was purified with glutathione-Sepharose. The purified fusion protein was injected into a mouse intraperitoneally every week for one month. Production of the antibodies in the mouse's serum was examined after the last injection. Spleen cells were obtained from the mouse and fused with myeloma cells following the conventional method. Selection and cloning of the hybridoma cells that produce anti-Pmb5 were carried out as described previously (*24*).

Detection of Pmb5 on Whole-Mount Immunohistochemistry—Expression of Pmb5 in the ascidian was examined with the monoclonal antibody by whole-mount immunohistochemistry. Parents and developing buds were fixed in acetic acid:ethanol (1:3) for 30 min at −20°C, and then the fixative was washed off with phosphate-buffered saline (PBS). For immunohistochemistry, the fixed specimens were blocked with 2% skim milk in PBS for 1 h, and then treated with the monoclonal antibody aginst Pmb5 at 4°C overnight. A second antibody against mouse IgG conjugated with horseradish peroxidase (Vector Laboratories, Burlingame, CA) was used to detect Pmb5, and diaminobenzidine was used for color development. Specimens treated as above were embedded in JB-4 plastic medium and then sectioned at 2 μm as described previously (*24*).

RESULTS

Cloning and Nucleotide Sequence Analysis of Pmb5 cDNA—A total of 3×10^5 plaques of the Uni-ZAP cDNA library were screened with the DIG-labeled human cytochrome b_5 cDNA (*20*) as a probe. Among a few positive clones obtained on the first screening, only one clone was identified as a positive one on the second screening. The

phage clone was treated with helper phage to cleave out the pBluescript phagemid from the phage DNAs, and the insert DNA was amplified by PCR using vector primers T7 and T3. The insert DNA was judged to be about 1.8 kb, and the DNA sequence of the clone was determined by the sequencing strategy shown in Fig. 1. The whole sequence determined is shown in Fig. 2. The nucleotide sequence of Pmb5 was then analyzed by means of a homology search with a BLASTX program through the Internet. The amino acid sequence deduced from the cDNA exhibited 54% identity and about 76% similarity with chicken cytochrome b_5.

The coding sequence for the cDNA of Pmb5 consists of 405 bp, and contains two heme-binding His codons. The nucleotide sequence around the initial Met codon, GCAATGG, well fits Kozak's consensus sequence (*25*) recognized in mammalian DNAs. The cDNA does not contain an insertion sequence which will lead to expression of both the solu-

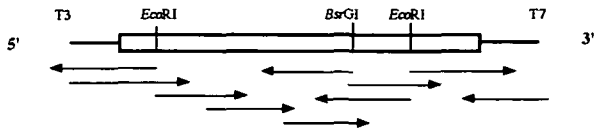

Fig. 1. **Restriction map and sequence strategy for the Pmb5 cDNA.** Arrows indicate the direction for sequencing and the length. T3 and T7 indicate the vector primers.

```
5'  GGC ACG AGG GGT GAC TGC CGG AAA CCA GGT AAA ATT AAT GCA GTT TTA TAC GCG   54
    TAA ATT TCC TCA TAT TGG ACC TGC TAT GCT TTA CGC AAC AAC AAT TTT ACG ATC  108
    GCG GCA GAG CAT GAA TTC CCA AGA CTG CAG AGA TGA GGA GTG AGG TTT ATA AAT  162
    CAT TTT CTG TTC TAC TGC CTT CAA GAT TAA TTT TTC ACT CTT ATC TTA CCG GTA  216
    TCT GTT ATC AGA AGT TTA GTC GCC CTT GGA CTA TAA TAG GCT TAG TGT GTG TTC  270
    CAC TTT TAC CTG CAT ATA AGT ATT GCA ATG GCG GAA CAA CAG ACA GAA CAA ACC  324
                                      M   A   E   Q   Q   T   E   Q   T   (9)

    GAA AAA CGG ATT ATA CGT TAT GAA GAA GTC AAG CAG CAT AAC AGC ATA AAA TCA  378
     E   K   R   I   I   R   Y   E   E   V   K   Q   H   N   S   I   K   S (27)

    GCA TGG AAT GTG ATT CAT AAT AAG GTC TAT GAC GTC ACA AAA TTT TTG GAA GAC  432
     A   W   N   V   I   H   N   K   V   Y   D   V   T   K   F   L   E   D (45)

    CAC CCT GGT GGT GAA GAA GTT CTT TTG GAA CAA GCT GGC AAG AAT GCT ACA GAG  486
     H   P   G   G   E   E   V   L   L   E   Q   A   G   K   N   A   T   E (62)

    GCA TTT GAG GAT GTT GGT CAC TCA TCA GAT GCT AGA TCA CTC GCT GAA GAG CAT  540
     A   F   E   D   V   G   H   S   S   D   A   R   S   L   A   E   E   H (80)

    TTG ATT GGA GAG CTT CAT CCT GAT GAT CAT TTT CAA GAA GAA CAA CCT CAA TTC  594
     L   I   G   E   L   H   P   D   D   H   F   Q   E   E   Q   P   Q   F (98)

    GTC ACA ACA CAC GAA AGC ATG GCT GAA ACA AGT TCA TGG AGT AAT TGG GTC ATA  648
     V   T   T   H   E   S   M   A   E   T   S   S   W   S   N   W   V   I (116)

    CCA GCC ATT GTC GCA CTT GCA GTT GCC CTT GTT TAC CGC TAC TAT ATA TCC AAT  702
     P   A   I   V   A   L   A   V   A   L   V   Y   R   Y   Y   I   S   N (134)

    TAA AAT GCA GTT AAC ACC GAT CAA CCC ACT TCG TCG ACT TAT AGT AAT ATT AAT  756
     *
    AAG GAT GCA AAA TTT TTG TGG CAA TGG AGT GCG ATA AAT AGC GAA CTT TCT TAT  810
    TGC AAG CAA TTT GGA GAA TTT TTA ATA TAT ACC AGT AAT AAT TAA TCT AAT CAT  864
    CTT CAC GCA CAG GGT AAT ACT ATG ATT GCT TTT ACT GTG TGA ATA ATT CGG ATG  918
    TAG ACT TGT TTG TTC CGG TAC TGC TAT TTC ATT TTA TGG ACA CTG CTT TGA ATC  972
    TAT TTG CAT TTG CCC ATA TAC GGT TGA GGA AAG CGT AGG CCT ATG TAT ATA ATG 1026
    CTG GAG TTT TTG TCT TCA AGT TAT CCT GAT AGC ATA ATT TTG ACG GAA GTG TGA 1080
    ATT GTA ATA TGC CAT ATG CGA TAG AAT TAT GTA CAA TAG TTT AAC AGC CAT GTC 1134
    CGC GCC GCT ATG TAT GAC TTA ATT ATA GCA ATT ACA TTA AAA CAA GAA AAG TAC 1188
    GGG CTG TAA AGT TAT AGT AGA CTG ACT AAA GTT ACT TAG AGA AAA TTT TGA ATT 1242
    TTG CTT CAT TGT TAG TTG TCT ACT TTG AAC TTT ACG GCC TTT AAA TTT ATG TGT 1296
    CTC TGT TGT TGC GTG AAA AGA TAA TTC TGC TGT TTG TGT CAC ATA GCC TAC CTG 1350
    TCA CTT AAA TAA TGC GAA TTC TTG TTC TTT TCC AAA TTG TAA AAA TAA AAT TAA 1404
    ATG TGG TAT TAG TTT AAT TTT TCG TAT GGC GCC TGT AGT CGC TTG CGA GCA TCT 1458
    TGG AAT ATT TAT CAA CTC TTT TGG AAG CAA GCC TTT CCA AAT CAC ACA GAA ATG 1512
    GTT GGT AAA TTG TAG CAC ACA CGA GGC CAG GCA GGT GAG TGA TTT GGG TGT GAA 1566
    ATG ATA ATG TGC TCT TTT TTT TCT TTA ACT TTA TTT TTT GGC ATT ACA GGT ACG 1620
    GGC AAA GTC TTG CCT CTG TCA AGT CTG TAA AAG TTA CAA GGG ACG ACT ACA TGC 1674
    CTG CCT GCA TTG TAT TTA TTT TGG CTG CTA TGA AAG GTA GGA TTT CAT AAT TAT 1728
    CAA TCC ATT TTT ACA TCG GAC GCT TTG GNA CCA CGT GAN GGC GGG GCC CGG TAC 1782
    AAG GCT CGC C                                                         1792
3'
```

Fig. 2. **Nucleotide sequence of Pmb5 cDNA.** The entire nucleotide sequence of Pmb5 is shown together with the amino acid sequence deduced from the nucleotide sequence. The sites of the restriction enzymes, *Eco*RI (GAATTC) and *Bsr*GI (TGTACA), and the sequencing primers are indicated by single underlines. The underlining from 221–240 and 865–884 indicates the primers for sequencing. The conserved sequences around the putative heme-binding His are shown by double underlining. The putative heme-binding His is denoted by bold letters. The stop codon is indicated by an asterisk. The nucleotide sequence around the putative initial Met, GCAATGG, fits the Kozak's consensus sequence.

ble and membrane-bound forms, as described by Steggles *et al.* (*20*, *26*, *27*) for human, rabbit, or porcine cytochrome b_5. The amino acid sequence deduced from the Pmb5 DNA was compared with those of other cytochrome b_5s, and highly conserved sequences were found in Pmb5 around the heme-binding His, as shown in Fig. 3. The membrane-binding domain of Pmb5 also contains the conserved amino acid sequence, but the hinge region between the two do-mains has relatively varied residues. The C-terminal peptide of about 10 amino acids is very similar to those of mammalian cytochrome b_5s except that Glu before the C-terminus is absent in Pmb5, as shown in Fig. 3. This result suggests that the C-terminal peptide of Pmb5 also plays a role to destine the protein on the ER membrane as described for rat cytochrome b_5 (*28*). Figure 4 shows the hydropathy profile of Pmb5 determined by the method of Kyte and Doolittle (*29*), the profile being very similar to those of mammalian cytochrome b_5s.

Phylogenetic Tree of Cytochrome b_5s—A phylogenetic tree of cytochrome b_5s from various species was constructed by the method of Fitch and Margoliash (*30*), as shown in Fig. 5. The full-length amino acid sequences for various cytochrome b_5s were obtained from the SwissProt and GenBank databases, and compared with that of the ascidian cytochrome b_5s. Pmb5 together with *Ciona* b_5 (Yubisui, T.; DDBJ accession number AB042613 for Pmb5 and AB042616 for *Ciona* b_5) formed a separate branch from those of mammalian and insect cytochrome b_5s, and also from those of plant cytochrome b_5s. Pmb5 exhibited 62% identity and 83% similarity to the cytochrome b_5 of another ascidian species, *Ciona savignyi*. These results indicate that cytochrome b_5 is a widespread protein in various species, and has a relatively conserved structure in various species to play important roles in maintaining metabolic processes of cells.

Purification and Properties of Pmb5—Expression of the membrane-bound forms of Pmb5 was performed as well as that of the putative soluble form. Sufficient expression of apo-protein for the membrane-bound form of Pmb5 was detected on Western blotting with the specific monoclonal antibody (data not shown). However, production of the holohemoprotein was unsuccessful under various conditions. Changing the culture temperature for *E. coli* from 37°C to 30°C or 25°C, and lowering the IPTG concentration from 0.5 to 0.05 mM did not lead to any improvement of the result. Transformation of some different *E. coli* strains was also unsuccessful. Contrary to this, expression of the putative soluble form of Pmb5 was well induced by IPTG. Therefore, extraction and purification of the putative soluble form of Pmb5 were carried out as described under "MATERIALS AND METHODS," and the purified Pmb5 was characterized.

The purity of the recombinant Pmb5 was examined by SDS-PAGE, as shown in Fig. 6A. The Pmb5 gave an appar-

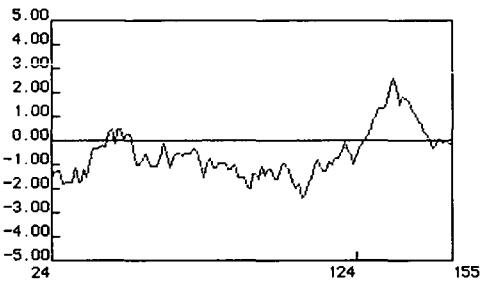

Fig. 4. **Hydropathy profile of Pmb5.** A hydropathy profile of the full length Pmb5 was prepared by the method of Kyte and Doolittle (*29*). Calculation was performed with a window size of 13 amino acid residues. The upper scale on the ordinate shows the hydrophobisity.

```
Rabbit      MAAQSDKD-----VKYYTLEEIKKHNHSKSTWLILHHKVYDLTKFLEEHPGGEEVLREQA
Human       MAEQSDEA-----VKYYTLEEIQKHNHSKSTWLILHHKVYDLTKFLEEHPGGEEVLREQA
Horse       -AEQSDKA-----VKYYTLEEIKKHNHSKSTWLILHHKVYDLTKFLEDHPGGEEVLREQA
Rat         MAEQSDKD-----VKYYTLEEIQKHKDSKSTWVILHHKVYDLTKFLEEHPGGEEVLREQA
Swine       MAEQSDKA-----VKYYTLEEIQKHNNSKSTWLILHHKVYDLTKFLEEHPGGEEVLREQA
Bovine      MAEESSKA-----VKYYTLEEIQKHNNSKSTWLILHYKVYDLTKFLEEHPGGEEVLREQA
Chick       MVGSSEAGGEAWRGRYYRLEEVQKHNNSQSTWIIVHHRIYDITKFLDEHPGGEEVLREQA
Pm          MAEQQTEQT---EKRIIRYEEVKQHNSIKSAWNVIHNKVYDVTKFLEDHPGGEEVLLEQA
             . ..         :    **:::*:  :*:* ::* ::**:*****::******** ***

Rabbit      GGDATENFEDVGHSTDARELSKTFIIGELHPDDRSKLSKPMETLITTVDSNSSWWTNWVI
Human       GGDATENFEDVGHSTDAREMSKTFIIGELHPDDRPKLNKPPETLITTIDSSSSWWTNWVI
Horse       GGDATENFEDIGHSTDARELSKTFIIGELHPDDRSKIAKPVETLITTVDSNSSWWTNWVI
Rat         GGDATENFEDVGHSTDARELSKTYIIGELHPDDRSKIAKPSETLITTVESNSSWWTNWVI
Swine       GGDATENFEDVGHSTDARELSKTFIIGELHPDDRSKIAKPSETLITTVESNSSWWTNWVI
Bovine      GGDATENFEDVGHSTDARELSKTFIIGELHPDDRSKITKPSESIITTIDSNPSWWTNWLI
Chick       GGDATENFEDVGHSTDARALSETFIIGELHPDDRPKLQKPAETLITTVQSNSSSWSNWVI
Pm          GKNATEAFEDVGHSSDARSLAEEHLIGELHPDDHFQEEQPQFVTTHESMAETSSWSNWVI
            * :*** ***:***:*** ::: .:*********: :  :*         :..* *:**:*

Rabbit      PAISALIVALMYRLYMADD
Human       PAISAVAVALMYRLYMAED
Horse       PAISAVVVALMYRIYTAED
Rat         PAISALVVALMYRLYMAED
Swine       PAISALVVSLMYHFYTSEN
Bovine      PAISALFVALIYHLYTSEN
Chick       PAIAAIIVALMYRSYMSE-
Pm          PAIVALAVALVYRYYISN-
            *** *: *:*:*: * ::
```

Fig. 3. **Alignment of the amino acid sequence of Pmb5 with those of vertebrate cytochrome b_5s.** Alignment was obtained with an Internet program of CLUTALW. Identical amino acids are indicated by asterisks, and similar amino acids shown by :. Heme-binding His are indicated by boldface.

ent single band corresponding to about 18 kDa in size, judging from the size markers, which is greatly different from the calculated molecular weight of 11,163 based on the amino acid sequence. Human cytochrome b_5 (MW = 11,137) and rat cytochrome b_5 (MW = 11,097) also moved more slowly than expected from their molecular weights (lanes 3 and 4), while the rat membrane-bound cytochrome b_5 (MW = 15,223) moved reasonably (lane 5). The N-terminal amino acid sequence of 10 residues of the recombinant protein was determined to be identical with that deduced from the nucleotide sequence. Determination of the molecular mass of Pmb5 by mass-spectroscopy indicated a mass of 11,036.6 (Fig. 6B), which corresponds exactly to that without the initial methionine. Therefore, the apparent large molecular weight observed on SDS-PAGE was concluded to be an artifact.

Figure 7A shows the absorption spectra of the purified Pmb5. The absorption spectrum of the oxidized form of Pmb5 had an absorption peak at 412 nm in the Soret region, and the reduced form showed absorption peaks at 423, 517, and 556–560 nm. The typical asymmetric peak at 556–560 nm was identical to those of mammalian cytochrome b_5s. Molecular extinction coefficients for the difference between the reduced and oxidized forms were determined by preparing the pyridine hemochrome (*23*), as shown in Table I. They were determined to be 120 mM^{-1} cm^{-1} at 424 nm and 19 mM^{-1} cm^{-1} at 556 nm. These spectral properties are summarized in Table I, and are very similar to those of mammalian cytochrome b_5s (*31–33*). Figure 7B shows the low temperature spectra of the dithionite-reduced form of the Pmb5, absorption peaks being observed at 422, 514, 525, 532, 551, and 555 nm. The positions and shapes of the split peaks at 551 and 555 nm are very similar to those of other b_5s (*34*, *35*).

The kinetic properties of the purified Pmb5 were studied by using crude extracts of *P. misakiensis* as the enzyme solution, and the Michaelis constant of the enzyme against the Pmb5 was determined to be 5 μM, this value being similar to those (4–10 μM) of mammalian cytochrome b_5s (*21*, *36*).

Detection of Pmb5 in the Tissues of the Ascidian—Expression of Pmb5 in the ascidian was examined by whole-mount immunohistochemistry with a specific monoclonal antibody. Figure 8B shows the specificity of the monoclonal antibody. The monoclonal antibody prepared in this study did not cross-react with any other cytochrome b_5s tested. As

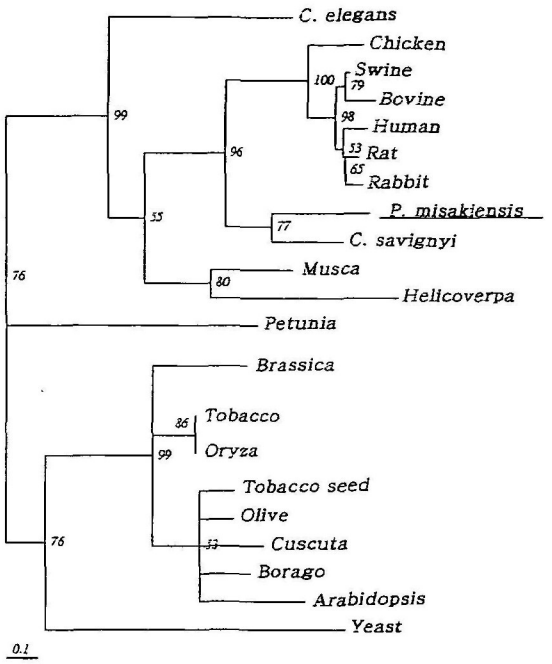

Fig. 5. **Phylogenetic tree of cytochrome b_5s.** A phylogenetic tree was constructed by the method of Fitch and Margoliash (*30*) from the amino acid sequences of cytochrome b_5s from animals and plants, and Pmb5. Bootstrap values are indicated at the branching points. The amino acid sequences of cytochrome b_5s for chicken, swine, bovine, human, rat, rabbit, tobacco, tobacco seed, *Arabidopsis*, and yeast were obtained from the SwissProt database (P00174, P00172, P00171, P00167, P00173, P00169, P49098, P49099, PP119353, and P40312), and those for *C. elegans*, *Musca*, *Helicoverpa*, *Brassica*, *Cuscuta*, *Borago*, *Oryza*, Olive, and *Petunia* were obtained from the GenBank database (AF003141, L38464, AF061105, M87514, L22209, U79011, X75670, CAA04703, and G7331156).

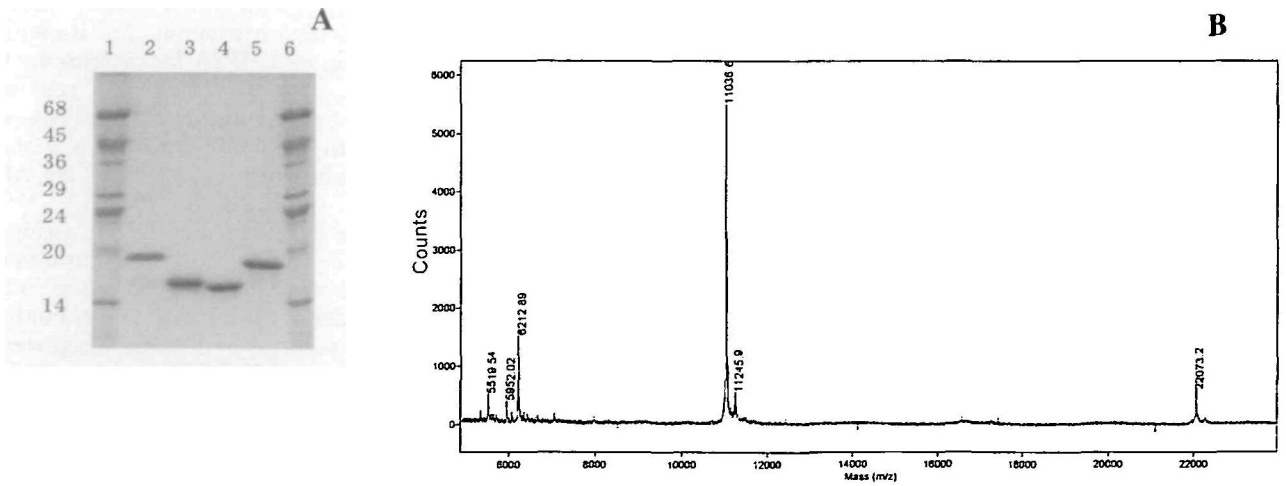

Fig. 6. **Analysis of the recombinant Pmb5.** A: The purity of the recombinant Pmb5 was examined by SDS-PAGE. Lanes 1 and 6, molecular weight standard markers; 2, putative soluble form of Pmb5; 3, human soluble cytochrome b_5; 4, rat soluble b_5; 5, rat membrane-bound cytochrome b_5. B: Determination of the molecular mass of Pmb5 by mass-spectroscopy. Protein mass calibration was performed with thioredoxin (11,674.4), lysozyme (14,310.4), and apomyoglobin (16,953.8) as standard.

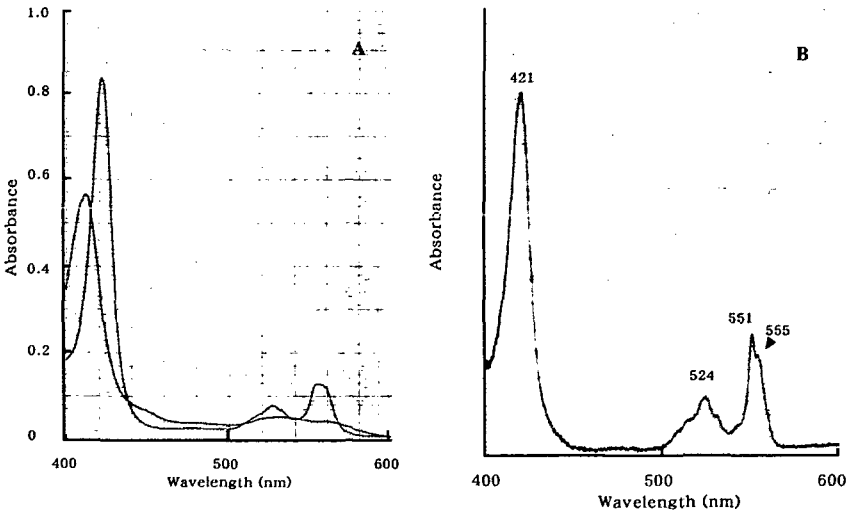

Fig. 7. **Absorption spectra of Pmb5.** A: Absorption spectra of the putative recombinant soluble Pmb5 were measured in 50 mM Tris-HCl buffer (pH 7.6). The reduced form of the Pmb5 was prepared by adding NADH and human NADH-cytochrome b_5 reductase, or by adding a few grains of sodium dithionite. B: Low temperature spectrum of the reduced form of Pmb5. The low temperature spectrum of Pmb5 was measured under liquid nitrogen. The reduced form was prepared by adding sodium dithionite.

TABLE I. **Molecular extinction coefficients of Pmb5 and cytochrome b_5s from other species.**

Cytochrome b_5s from	Reduced-oxidized		Reference
	$\varepsilon_{424\ nm}$	$\varepsilon_{556\ nm}$	
	(mM^{-1} cm^{-1})		
P. misakiensis	120[a]	19	this study
Human erythrocytes	124	19.3	(*31*)
Steer liver microsomes	100	—	(*32*)
Hen liver microsomes	121	19	(*33*)

[a]Molecular extinction coefficients of Pmb5 were determined by the pyridine-hemochrome method (*23*). Purified Pmb5 (10–20 μM) was mixed with 0.2 ml of pyridine and 0.2 N NaOH in a total volume of 1.0 ml. Extinction coefficients were determined for 3 determinations.

shown in Fig. 8C, Pmb5 was apparently detected on Western-blotting in homogenates of the growing buds (0-day developing), and 1- and 2-day developing buds, but was not detectable in the parents. The maximum level was observed in 1- and 2-day developing buds. The bands in lanes 4–7 were considered to be of the membrane-bound Pmb5, judging from the single species expressed in the tissues and from the DNA sequence of Pmb5. Figure 8D shows the immunohistochemical detection of Pmb5. Pmb5 was most strongly stained in the mesenchymal cells and then significant staining was observed in the atrial epithelium in 1- and 2-day-developing buds (panels b and c). Apparent staining was not observed in the epidermis (panels b and c). However, the staining of Pmb5 in the parent tissues was very weak (panel a), and in the 7-day-developing buds (panel d), Staining was observed in few of the mesenchymal cells in the atrial epithelium. The Pmb5 expression detected on Western-blotting coincides well with that on immunohistochemistry. These results reveal novel expression of cytochrome b_5 as an inducible hemoprotein in the ascidian, which is clearly different from the vertebrate cytochrome b_5, which is known as a housekeeping protein, not an inducible protein.

DISCUSSION

In the previous study to characterize the ascidian, *P. misakiensis*, we found that the animal's tissues contain abundant very-long-chain polyunsaturated fatty acids, and an extraordinarily large amount of TG is accumulated in buds of the ascidian (Yubisui, T., the manuscript submitted elsewhere). As cytochrome b_5 is well known to participate in the fatty acid metabolism in vertebrates (*8–17*), in this study we investigated the cytochrome b_5 of the ascidian in relation to the accumulation of TG. We first succeeded in the cloning of a cDNA for cytochrome b_5 in an ascidian. As the biochemical characterization of ascidians has not been well performed, especially of the redox hemoproteins, the results of this study will well contribute to understanding of the biochemical metabolism in ascidians.

The cDNA for Pmb5 obtained in this study codes the well-conserved amino acid sequence as compared with those of mammalian cytochrome b_5s. The conserved structure was observed in the amino-terminal hydrophilic region of about 96 residues, especially in the heme-binding domain. There are two heme-binding His boxes in Pmb5, TKFLxxHPGGEEVL and ATExFEDVGHSxDAR, as observed in various mammalian cytochrome b_5s. These heme-binding His-motifs are also conserved in other cytochrome b_5s from yeast (*37*), and higher plants (*35*, *38*, *39*). The membrane-binding region of Pmb5 also contains similar residues to those of mammalian cytochrome b_5s. Recently, we succeeded in the cloning of a cDNA for cytochrome b_5 from another ascidian, *C. savignyi*. The amino acid sequence deduced from the *Ciona* cytochrome b_5 cDNA sequence exhibited 62% identity and 83% similarity to that of Pmb5 (Yubisui, T., unpublished results, DDBJ AC No. AB-042616).

In the phylogenetic tree shown in Fig. 5, the ascidian cytochrome b_5s form an independent branch from those of mammals and insects. The structure of Pmb5 seems to be relatively divergent (62% identity) from that of the another ascidian, *C. savignyi*. The occurrence of these cytochrome b_5s in various species indicates the important roles of cytochrome b_5 in cell functions. Analysis of the unique multifunctional properties of cytochrome b_5s is becoming an important target of protein chemistry.

We tried to express the membrane-bound form of Pmb5 in this study, but were unsuccessful for an unknown reason. Expression of apo-protein for the membrane-bound form was sufficient (data not shown), but that of holopro-

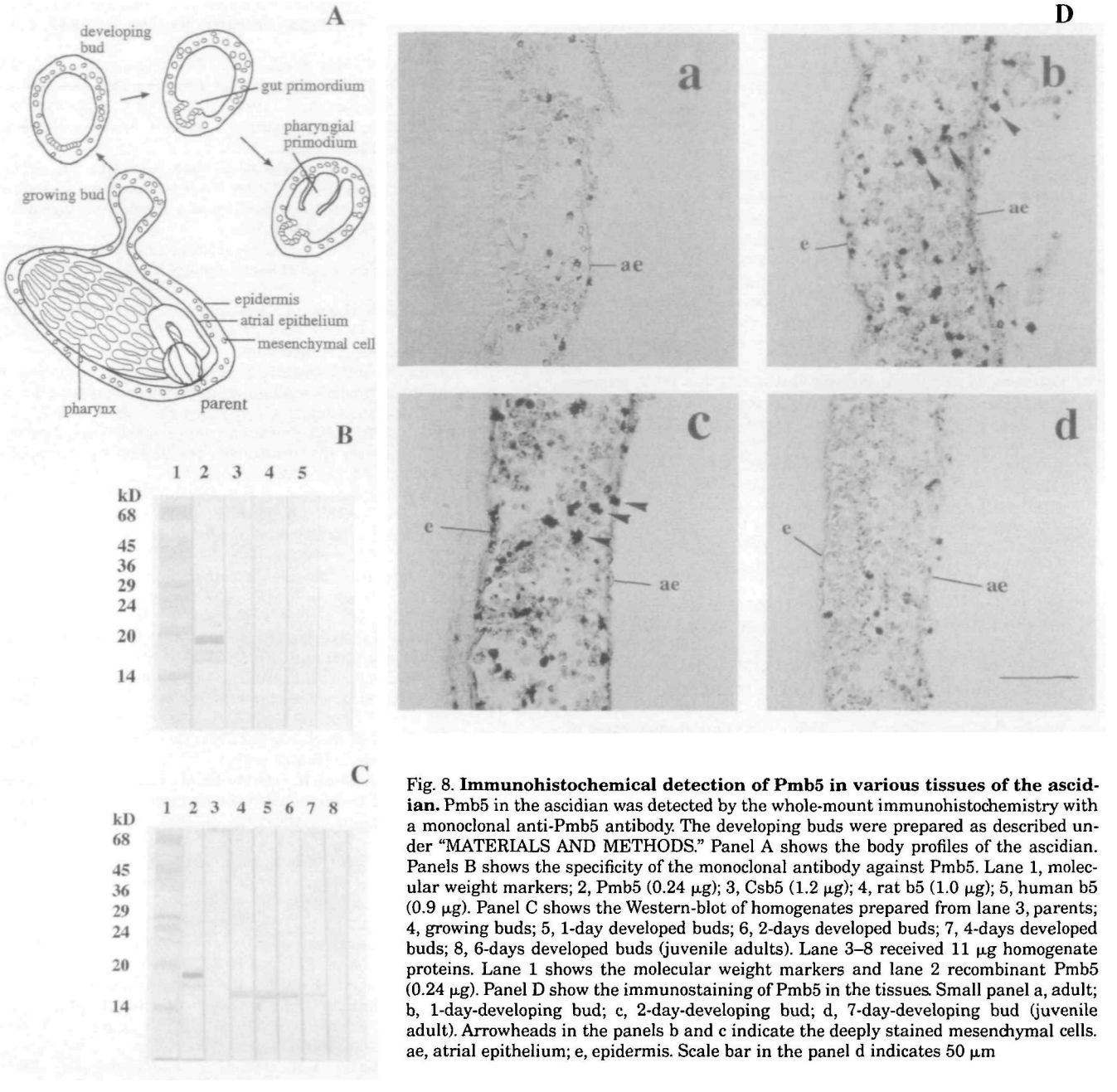

Fig. 8. **Immunohistochemical detection of Pmb5 in various tissues of the ascidian.** Pmb5 in the ascidian was detected by the whole-mount immunohistochemistry with a monoclonal anti-Pmb5 antibody. The developing buds were prepared as described under "MATERIALS AND METHODS." Panel A shows the body profiles of the ascidian. Panels B shows the specificity of the monoclonal antibody against Pmb5. Lane 1, molecular weight markers; 2, Pmb5 (0.24 μg); 3, Csb5 (1.2 μg); 4, rat b5 (1.0 μg); 5, human b5 (0.9 μg). Panel C shows the Western-blot of homogenates prepared from lane 3, parents; 4, growing buds; 5, 1-day developed buds; 6, 2-days developed buds; 7, 4-days developed buds; 8, 6-days developed buds (juvenile adults). Lane 3–8 received 11 μg homogenate proteins. Lane 1 shows the molecular weight markers and lane 2 recombinant Pmb5 (0.24 μg). Panel D show the immunostaining of Pmb5 in the tissues. Small panel a, adult; b, 1-day-developing bud; c, 2-day-developing bud; d, 7-day-developing bud (juvenile adult). Arrowheads in the panels b and c indicate the deeply stained mesenchymal cells. ae, atrial epithelium; e, epidermis. Scale bar in the panel d indicates 50 μm

tein was not. The culture and induction conditions were varied to express the membrane-bound form, but without success. Using the same expression vector, we obtained high level expression of the putative soluble form of Pmb5, and also the membrane-bound form of human cytochrome b_5. Therefore, we expressed the putative soluble form of Pmb5 and characterized it in this study, although there is no evidence of the existence of the soluble form of Pmb5 *in vivo*. Western-blot analysis of homogenates of the ascidian gave only a single band, as shown in Fig. 8C. These results apparently indicate a single Pmb5 species in the ascidian, and the Pmb5 detected seems to be the membrane-bound form considering the cDNA sequence.

The apparently high level expression of Pmb5 in the developing buds is a very interesting finding compared with the low level in the parent (Fig. 8), as cytochrome b_5 in vertebrates has been known as a housekeeping protein. Thus, this paper first described the apparently elevated expression of cytochrome b_5 during bud development in an ascidian. Another interesting finding is that the pattern of the elevated expression of Pmb5 in buds coincides well with the pattern of accumulation of triacylglycerol in buds, but not in the parents of the ascidian (Yubisui, T., manuscript submitted elsewhere). These results suggest that Pmb5 is deeply correlated with the metabolism of fatty acids and the development of buds in the ascidian. Analysis of the structure of the regulatory region in the Pmb5 gene will provide new insights into the regulatory mechanism of the expression of Pmb5 in the ascidian.

The authors wish to thank Dr. Tomohiko Suzuki of Kochi University for the kind construction of the phylogenetic tree of cyto-

chrome b_5s, and Dr. A.W. Steggles of Northeastern Ohio Medical College for the generous gift of an expression system of human cytochrome b_5. Thanks are also due to Ms. Sayo Kataoka of the Immunology Division, Medical Research Center, Kochi Medical School, for her assistance in the mass analysis.

REFERENCES

1. Hisata, K., Fujiwara, S., Tsuchida, Y., Ohashi, M., and Kawamura, K. (1998) Expression and function of a retinoic acid receptor in budding ascidians. *Dev. Genes Evol.* **208**, 537–546
2. Kamimura, M., Fujiwara, S., Kawamura, K., and Yubisui, T. (2000) Functional retinoid receptors in budding ascidians. *Dev. Growth Differ.* **42**, 1–8
3. Suzuki, T., Takagi, T., Furukohri, T., Kawamura, K., and Nakauchi, M. (1990) A calcium-dependent galactose-binding lectin from the tunicate *Polyandrocarpa misakiensis*. *J. Biol. Chem.* **265**, 1274–1281
4. Shimada, M., Fujiwara, S., and Kawamura, K. (1995) Expression of genes for two C-type lectins during budding of the ascidian *Polyandrocarpa misakiensis*. *Roux's Arch. Dev. Biol.* **204**, 406–411
5. Harafuji, N., Miyashita, S., Kawamura, K., and Fujiwara, S. (1996) Molecular cloning and expression of aldehyde dehydrogenases in the budding ascidian *Polyandrocarpa misakiensis*. *Mem. Fac. Sci. Kochi Univ. Ser. D (Biol.)* **16/17**, 7–16
6. Ohashi, M., Kawamura, K., Fujii, N., Yubisui, T., and Fujiwara, S. (1999) A retinoic acid-inducible molecular protease in budding ascidians. *Dev. Biol.* **214**, 38–45
7. Kawamura, K. and Fujiwara, S. (1995) Establishment of cell lines from multipotent epithelial sheet in the budding tunicate, *Polyandrocarpa misakiensis*. *Cell Struct. Funct.* **20**, 97–106
8. Oshino, N., Imai, Y., and Sato, R. (1971) A function of cytochrome b_5 in fatty acid desaturation by rat liver microsomes. *J. Biochem.* **69**, 155–167
9. Keyes, S.R. and Cinti, D.L. (1980) Biochemical properties of cytochrome b_5-dependent microsomal fatty acid elongation and identification of products. *J. Biol. Chem.* **255**, 11357–11364
10. Reddy, V.V.R., Kupfer, D., and Capsi, E. (1977) Mechanism of C-5 double bond introduction in the biosynthesis of cholesterol by rat liver microsomes: evidence for the participation of microsomal cytochrome b_5. *J. Biol. Chem.* **252**, 2797–2801
11. Fukushima, H., Grinstead, G.F., and Gaylor, L. (1981) Total enzymic synthesis of cholesterol from lanosterol. Cytochrome b_5-dependent 4-methyl sterol oxidase. *J. Biol. Chem.* **256**, 4822–4826
12. Hildebrandt, A. and Estabrook, R.W. (1971) Evidence for the participation of cytochrome b_5 in hepatic microsomal mixed-function oxidation reactions. *Arch. Biochem. Biophys.* **143**, 66–79
13. Onoda, M. and Hall, P.F. (1982) Cytochrome b_5 stimulates purified testicular microsomal cytochrome P-450 (C_{21} side-chain cleavage). *Biochem. Biophys. Res. Commun.* **108**, 454–460
14. Shinzawa, K., Kominami, S., and Takemori, S. (1985) Studies on cytochrome P-450 (P45017α, lyase) from guinea pig adrenal microsomes. Dual function of a single enzyme and effect of cytochrome b_5. *Biochim. Biophys. Acta* **833**, 151–160
15. Sakai, Y., Yanase, T., Takayanagi, R., Nakao, R., Nishi, Y., Haji, M., and Nawata, H. (1993) High expression of cytochrome b_5 in adrenocortical adenomas from patient with Cushing's syndrome associated with high secretion of adrenal androgens. *J. Clin. Endocrinol. Metabol.* **76**, 1286–1290
16. Katagiri, M., Kagawa, N., and Waterman, M.R. (1995) The role of cytochrome b_5 in the biosynthesis of androgen by human P450c17. *Arch. Biochem. Biophys.* **317**, 343–347
17. Yanase, T., Sasano, H., Yubisui, T., Sakai, Y., Takayanagi, R., and Nawata, H. (1998) Immunohistochemical study of cytochrome b_5 in human adrenal gland and in adrenocortical adenomas from patient with Cushing's syndrome. *Endocrine J.* **45**, 89–95
18. Ozols, J. (1989) Structure of cytochrome b_5 and its topology in the microsomal membrane. *Biochim. Biophys. Acta* **997**, 121–130
19. Chomezymski, P. and Sacchi, N. (1987) Preparation of RNA from tissues by AGPC method. *Anal. Biochem.* **162**, 158–159
20. Yoo, M. and Steggles, A.W. (1988) The complete nucleotide sequence of human liver cytochrome b_5 mRNA. *Biochem. Biophys. Res. Commun.* **156**, 576–580
21. Shirabe, K., Nagai, T., Yubisui, T., and Takeshita, M. (1998) Electrostatic interaction between NADH-cytochrome b_5 reductase and cytochrome b_5 studied by site-directed mutagenesis. *Biochim. Biophys. Acta* **1384**, 16–22
22. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685
23. Falk, J.E. (1964) Haems. I. Determination as pyridine haemochromes in *Porphyrins and Metalloporphyrins*, pp. 181–188, Elsevier Publishing, Amsterdam
24. Kawamura, K. and Fujiwara, S. (1994) Transdifferentiation of pigmented multipotent epithelium during morphallactic development of budding tunicates. *Int. J. Dev. Biol.* **38**, 369–377
25. Kozak, M. (1986) Point mutations define a sequence flanking the initiator codon that modulates translation by eukaryotic ribosomes. *Cell* **44**, 283–292
26. Giordano, S.J. and Steggles, A.W. (1993) Differential expression of the mRNAs for the soluble and membrane-bound forms of rabbit cytochrome b_5. *Biochim. Biophys. Acta* **1172**, 95–100
27. VanDerMark, P.K. and Steggles, A.W. (1997) The isolation and characterization of the soluble and membrane-bound porcine cytochrome b_5. *Biochem. Biophys. Res. Commun.* **240**, 80–83
28. Mitoma, J. and Ito, A. (1992) The carboxy-terminal 10 amino acid residues of cytochrome b_5 are necessary for its targeting to the endoplasmic reticulum. *EMBO J.* **11**, 4197–4203
29. Kyte, J. and Doolittle, R.F. (1982) A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157**, 105–132
30. Fitch, W.M. and Margoliash, E. (1967) Construction of phylogenetic trees. *Science* **155**, 279–284
31. Yubisui, T., Murakami, K., Takeshita, M., and Takano, T. (1988) Purification by hydrophobic chromatography of soluble cytochrome b_5 of human erythrocytes. *Biochim. Biophys. Acta* **936**, 447–451
32. Strittmatter, P. and Velick, S.F. (1956) A microsomal cytochrome reductase specific for diphosphopyridine nucleotide. *J. Biol. Chem.* **221**, 277–286
33. Ohnishi, T., Imai, Y., and Yamasaki, I. (1975) The oxidation-reduction characteristics of cytochrome b_5 in hen liver microsomes. *Arch. Biochem. Biophys.* **167**, 488–495
34. Yu, Y., Yamasaki, H., Kita, K., and Takamiya, S. (1996) Purification and molecular characterization of a novel b_5-type cytochrome of the parasitic nematode, *Ascaris suum*. *Arch. Biochem. Biophys.* **328**, 165–172
35. Bonnerot, C., Galle, A.M., Jolliot, A., and Kader, J.C. (1985) Purification and properties of plant cytochrome b_5. *Biochem. J.* **226**, 331–334
36. Yubisui, T. and Takeshita, M. (1982) Purification and properties of soluble NADH-cytochrome b_5 reductase of rabbit erythrocytes. *J. Biochem.* **91**, 1467–1477
37. Truans, G., Epinat, J.C., Rougeulle, C., Cullin, C., and Pompon, D. (1994) Cloning and characterization of a yeast cytochrome b_5-containing gene which suppress ketoconazole hypersensitivity in a NADPH-P450 reductase-deficient strain. *Gene* **149**, 123–127
38. Kearns, E.V., Keck, P., and Somerville, C.R. (1992) Primary structure of cytochrome b_5 from cauliflower (*Brassica oleracea* L.) deduced from peptide and cDNA sequences. *Plant Physiol.* **99**, 1254–1257
39. Smith, M.A., Stobart, A.K., Shewry, P.R., and Napier, J.A. (1994) Tobacco cytochrome b_5: cDNA isolation, expression analysis and in vitro targeting. *Plant Mol. Biol.* **25**, 527–537