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Structure of a cDNA for *Ciona* Cytochrome b_5 and the Ubiquitous Expression of mRNA in Embryonic Tissues

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A cDNA clone for cytochrome b_5 was isolated from a cDNA library of an ascidian, *Ciona savignyi*, by a plaque hybridization method using a digoxigenin-labeled cDNA for the soluble form of human cytochrome b_5. The cDNA is composed of 5′- and 3′-noncoding sequences, and a 396-base pair coding sequence. The 3′-noncoding sequence contains polyadenylation signal sequences. The amino acid sequence of 132 residues deduced from the nucleotide sequence of the cDNA showed 61% identity and 82% similarity to the cytochrome b_5 of another ascidian species, *Polyandrocarpa misakiensis*, which we previously cloned. The amino-terminal hydrophilic domain of 98 residues contains well-conserved structures around two histidine residues for heme binding. A cDNA expression system was constructed to prepare a putative soluble form of *Ciona* cytochrome b_5. The recombinant soluble cytochrome b_5 showed an asymmetrical absorption spectrum at 560 nm as is shown by mammalian cytochromes b_5 upon reduction with NADH and NADH-cytochrome b_5 reductase. The recombinant *Ciona* cytochrome b_5 is reduced by NADH-cytochrome b_5 reductase with an apparent K_m value of 3.3 μM. This value is similar to that of the cytochrome b_5 of *Polyandrocarpa misakiensis*. The expression of *Ciona* cytochrome b_5 mRNA during development was examined by an *in situ* hybridization method and ubiquitous expression in embryonic tissues was observed. The results indicate that cytochrome b_5 plays important roles in various metabolic processes during development.

Key words: ascidian, cDNA, Cytochrome b_5, expression in embryo, nucleotide sequence, recombinant protein.

Cytochrome b_5, a well-known amphipathic hemoprotein bound on endoplasmic reticulum in cells, participates in many important reactions such as fatty acid desaturation (*1*), fatty acid elongation (*2*), cholesterol biosynthesis (*3*, *4*), steroid hormone biosynthesis (*5–8*), some reactions of P450-dependent drug metabolism (*9*, *10*). The structures of cytochromes b_5 from various mammalian species have been determined (*11–15*). Cytochromes b_5 of yeast (*16*), some higher plants (*17–19*), and nematodes (*20*) have also been studied and shown to have structures and functions similar to those of mammalian species.

There were no available data for ascidian hemoproteins, which are considered to be important in maintaining the metabolism of the animals. However, previously we reported the structure of a cytochrome b_5 cDNA from *Polyandrocarpa misakiensis* and characterized the cDNA and recombinant protein (*21*). The cDNA of *Polyandrocarpa* b_5 is about 1.8 kb in size with long non-coding sequences in both the 5′- and 3′-regions, whereas the cDNA for cytochrome b_5 of *Ciona savignyi* cloned in this study has a relatively short cDNA of about 660 bp. The amino acid sequence deduced from the nucleotide sequence determined in this study showed 61% identity and 82% similarity with the cytochrome b_5 of *P. misakiensis* (*21*). In order to determine whether the structural and biochemical properties of these cDNAs are shared among all ascidians or not, we here prepared a cDNA from cytochrome b_5 of *C. savignyi*, another ascidian, and compared it with that from *P. misakiensis*.

The putative soluble form of the cytochrome b_5 of *C. savignyi* (*Csb5*) showed properties very similar to those of *P. misakiensis*. The expression of *Csb5* mRNA in embryonic tissues was also examined, and the mRNA was found to be expressed ubiquitously in the gastrula, with relatively strong expression observed in the mesenchyme cells, brain and muscle cells in the early tailbud stage. These expression profiles of the cytochrome b_5 mRNA indicate that the cytochrome b_5 is important for supporting the development of the animal.

MATERIALS AND METHODS

Yeast extracts and bacto-tryptone were purchased from Difco Laboratories (Detroit, MI). Restriction enzymes, *Hin*dIII, *Xba*I, *Bsr*GI were obtained from New England Biolabs (Beverly, MA). *Eco*RI, a digoxigenin (DIG) DNA labeling kit, anti-DIG antibody conjugated with alkaline phosphatase, nitroblue tetrazolium, 5-bromo-4-chloro-3-indolylphosphate, and the blocking reagent for nucleic acid hybridization were purchased from Boehringer Man-

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nheim Japan (Tokyo). Ampicillin and isopropyl-β-D-thiogalactoside (IPTG) were products of Nacalai Tesque (Kyoto). The TA cloning vector was obtained from Invitrogen Corporation (Carlsbad, CA). A QuickPrep mRNA Micro Purification kit was purchased from Pharmacia Biotech Japan (Tokyo). Nitrocellulose membranes were products of Bio-Rad Laboratories (Richmond, CA). Other reagents used were all of reagent grade.

Construction of a cDNA Library—C. savignyi was kindly supplied by Dr. T. Nishikata of Konan University, Kobe. The tunic was removed, and the tissues were dropped into acid-guanidinium-phenol-chloroform to extract total RNA. RNA was extracted from the entire inside tissues of animals by the method of Chomzynski and Sacchi (*22*), and mRNA was purified using a QuickPrep mRNA Micro Purification Kit. A cDNA library was prepared with the purified mRNA using a ZAP-cDNA^R Synthesis Kit and Uni-ZAP XR vector (Stratagene, La Jolla, CA).

Labeling of a cDNA Probe—The cDNA for the soluble form of human cytochrome b_5 (*Hsb5*, 306 bp) was cleaved from the expression plasmid (kindly supplied by Dr. A.W. Steggles of Northeastern Ohio Medical College) by digestion with *Eco*RI and *Hin*d III, and purified by agarose gel electrophoresis. The cDNA fragment isolated from the gel was labeled with DIG according to the protocol of the reagent kit from Boehringer Mannheim Japan (Tokyo) with Klenow enzyme, and used to screen the cDNA for cytochrome b_5 in the ascidian *C. savignyi* as described previously (*21*).

Screening of a cDNA for Csb5—A total of 1×10^4 phage clones were screened for *Csb5* cDNA using the DIG-labeled *Hsb5* cDNA probe as described previously (*21*). Phages of positive clones were removed from the agar plate and suspended in 500 μl of SM buffer (50 mM Tris-HCl, 100 mM NaCl, 8 mM MgCl2, 0.01% gelatin, pH 7.5), and purified by a second screening.

Construction of an Expression Plasmid for the Putative Soluble form of Csb5—To produce recombinant *Csb5* as a putative soluble form in *E. coli*, forward and reverse primers were synthesized based on the cDNA sequence (forward primer: 5′-GGAATTCATGGCGGAACAACAGACAG-3′; reverse primer: 5′-CGCAAGCTTAAGGCTGTTCTTCTTGG-3′). The forward primer contains an EcoRI sequence at the 5′-terminal side, and the reverse primer contains a HindIII sequence at the 5′-terminal side, to ligate the insert cDNA to the expression vector, pKK223-3 (TaKaRa). The selection of transformants was carried out as described previously (*21*), and the selected transformant was named pKK223-3/*Csb5*. For the large-scale preparation of *Csb5*, the clone harboring pKK223-3/*Csb5* was cultured in 2× YT medium at 37°C for 7–9 h. *Csb5* expression was induced by adding 0.5 mM IPTG to the culture medium.

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

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5'-GAATTCGGCACGAGAATAAACAAAAAAATGGCTGAATGTGAAGAAAAAAAGATTTATCGA      60
                              M  A  E  C  E  E  K  K  I  Y  R        11
   TTGGAAGAAGTTAAAAAGCACAACAATGTTCAATCTGCATGGATTATTATTCATAATAAA     120
    L  E  E  V  K  K  H  N  N  V  Q  S  A  W  I  I  I  H  N  K      31
   GTATATGATTTGACGAAGTTTTTAGAAGAACATCCTGGTGGTGAGGAAGTCCTGTTAGAG     180
    V  Y  D  L  T  K  F  L  E  E  H  P  G  G  E  E  V  L  L  E      51
   CAAGCTGGTCAAGATGCTACCGAATCCTTTGAAGATGTTGGCCATTCTACTGATGCAAGA     240
    Q  A  G  Q  D  A  T  E  S  F  E  D  V  G  H  S  T  D  A  R      71
   GAAATGCAAAAGGATTATTATATTGGTGAACTTCACCCAGATGACCAGTTCACTCAAAAC     300
    E  M  Q  K  D  Y  Y  I  G  E  L  H  P  D  D  Q  F  T  Q  N      91
   CCACGTAGTAAATATGTCACCCTCGGTAGTGATCAAGCTCAAGGAAGTGGACTGAGCAAC     360
    P  R  S  K  Y  V  T  L  G  S  D  Q  A  Q  G  S  G  L  S  N     111
   TGGTTGATTCCTGGACTAGTGGCACTTGGTGTTGCACTAATATATCGATTCTACATGTCT     420
    W  L  I  P  G  L  V  A  L  G  V  A  L  I  Y  R  F  Y  M  S     131
   TCTTAAAGTTCTTCAAAGCTTCAATTCAATAATGTGTGCTGCAATCATGATATCAATTGT     480
    S  *                                                           132
   ATAATGTATATGTAACAACTTTATTCCGATTTCTGACCAAGTTTTTCTATTTTTACAGTA     540
   ACAATGTAACAATGTAAACATAATATGTTCATTTTGTCAGTACAAACTTACTTAATTCAG     600

   TTGGAATACCATATTTTGTTGAGATTTAATGTTTTAATAAATTGAATAAACAGTAAAAAA     660

   AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAACAAAAAAAAAAAAAAAAAACTCGAG-3'  716
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Fig. 1. **Nucleotide sequence of the *Csb5* cDNA and the amino acid sequence deduced from the base sequence.** A Kozak's consensus sequence around the initial Met codon is underlined, heme-binding motifs containing His are shown by dotted lines, the stop codon is indicated by an asterisk, and the polyadenylation signals are indicated by waves. The heme-binding His residues are expressed in boldface.

Polyacrylamide Gel Electrophoresis—The purity of the recombinant cytochrome b_5 was examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) in the presence of dithiothreitol by the method of Laemmli (*23*).

Spectrophotometric Determinations—Absorption spectra of cytochromes b_5 were measured with a Union automatic recording spectrophotometer SM401 (Union Giken, Osaka); low temperature spectra of the dithionite-reduced form of various cytochromes b_5 were recorded under liquid nitrogen. Determinations of cytochrome b_5 reducing activities were also carried out with the same apparatus.

In Situ Hybridization—The spatial expression of cytochrome b_5 mRNA in embryonic tissues of *C. intestinalis* was examined by *in situ* hybridization using a DIG-labeled RNA probe by the method of Nagatomo *et al.* (*24*). The DIG-labeled RNA probe was prepared from a cytochrome b_5-encoding cDNA clone obtained from the *C. intestinalis* EST project (*25*). Preparation of a *C. savignyi* embryo was not successful during this study for unknown reasons. Therefore, a *C. intestinalis* embryo was used in this experiment.

RESULTS AND DISCUSSION

Isolation of a cDNA for Csb5—A cDNA for Csb5 was cloned from the cDNA library of *C. savignyi* by screening a total of 1×10^4 plaques by hybridization with a DIG-labeled human cytochrome b_5 cDNA fragment. Only one positive clone was obtained by the screening, and the phage was purified by a second screening. The conditions for hybridization with a human probe were almost the same as described previously (*21*). Hybridization of the DIG-labeled probe and also washing of the probe after

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Rat                 ------AEQSDKDVKYYTLEEIQKHKDSKSTWVILHHKVYDLTKFLEEHPGGEEVLREQA
Rabbit              -----MAAQSDKDVKYYTLEEIKKHNHSKSTWLILHHKVYDLTKFLEEHPGGEEVLREQA
Human               ------AEQSDEAVKYYTLEEIQKHNHSKSTWLILHHKVYDLTKFLEEHPGGEEVLREQA
Bovine              -----MAEESSKAVKYYTLEEIQKHNNSKSTWLILHYKVYDLTKFLEEHPGGEEVLREQA
Chicken             MVGSSEAGGEAWRGRYYRLEEVQKHNNSQSTWIIVHHRIYDITKFLDEHPGGEEVLREQA
P.misakiensis       ---MAEQQTEQTEKRIIRYEEVKQHNSIKSAWNVIHNKVYDVTKFLEDHPGGEEVLLEQA
C.savignyi          -------MAECEEKKIYRLEEVKKHNNVQSAWIIIHNKVYDLTKFLEEHPGGEEVLLEQA
                             .    :    **:::*:  :*:* ::* ::**:****::******** ***

Rat                 GGDATENFEDVGHSTDARELSKTYIIGELHPDDRSKIA-KPSETLITTVESNSSWWTNWV
Rabbit              GGDATENFEDVGHSTDARELSKTFIIGELHPDDRSKLS-KPMETLITTVDSNSSWWTNWV
Human               GGDATENFEDVGHSTDAREMSKTFIIGELHPDDRPKLN-KPPETLITTIDSSSSWWTNWV
Bovine              GGDATENFEDVGHSTDARELSKTFIIGELHPDDRSKIT-KPSESIITTIDSNPSWWTNWL
Chicken             GGDATENFEDVGHSTDARALSETFIIGELHPDDRPKLQ-KPAETLITTVQSNSSSWSNWV
P.misakiensis       GKNATEAFEDVGHSSDARSLAEEHLIGELHPDDHFQEE-QPQFVTTHESMAETSSWSNWV
C.savignyi          GQDATESFEDVGHSTDAREMQKDYYIGELHPDDQFTQNPRSKYVTLGSDQAQGSGLSNWL
                    * :*** *******:*** : : . ********:     :.         :. *  :**:

Rat                 IPAISALVVALMYRLYMAED-----
Rabbit              IPAISALIVALMYRLYMADDAPAQQ
Human               IPAISAVAVALMYRLYMAED-----
Bovine              IPAISALFVALIYHLYTSEN-----
Chicken             IPAIAAIIVALMYRSYMSE------
P.misakiensis       IPAIVALAVALVYRYYISN------
C.savignyi          IPGLVALGVALIYRFYMSS------
                        **.: *: ***:*: * :.
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Fig. 2. **Comparison of the *Csb5* amino acid sequence with those of various species.** The amino acid sequence of *Csb5* was compared with those of *Pmb5* and also with mammalian cytochromes b_5. The amino acid sequences of cytochromes b_5 from rat, rabbit, human and bovine were from the SwissProt database (P00173, Poo169, P00167, P00171); and that of *Pmb5* was from a previous paper (*21*).

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Cib5                MSECEEKKVFRLEEVKKHNNVQSAWIVVHNKIYDVTKFLEEHPGGEEVLLEQAGQDATES
Csb5                MAECEEKKIYRLEEVKKHNNVQSAWIIIHNKVYDLTKFLEEHPGGEEVLLEQAGQDATES
                    *:******::****************::***:**:*************************

Cib5                FEDVGHSSDAREMQKDYYIGELHPDDQFKENSRSKYVTLGNEESQASALSNWVIPGLVAL
Csb5                FEDVGHSTDAREMQKDYYIGELHPDDQFTQNPRSKYVTLGSDQAQGSGLSNWLIPGLVAL
                    *******:********************.:*.********.:::*.*.****:*******

Cib5                GVALIYRFYMST
Csb5                GVALIYRFYMSS
                    ***********:
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Fig. 3. **Sequence homology of *Csb5* with that of *C. intestinalis* b_5.** The amino acid sequence of *Csb5* deduced from the DNA sequence analyzed in this study was compared with the amino acid sequence deduced from the nucleotide sequence found in the database of the *C. intestinalis* EST project (*25*). The ClustalW program was used for sequence alignment.

hybridization were successful below 42°C. The cDNA was composed of 654 bp, including an open reading frame of 396 base pairs and a 3′-non-coding region of about 230 bp, that contained two tandemly repeated polyadenylation signals followed by a poly (A) sequence as shown in Fig. 1. The 5′-non-coding sequence was very short, 27 bp, and it seems that cDNA synthesis was incomplete. The AATGG underlined in Fig. 1 fits the Kozak's consensus

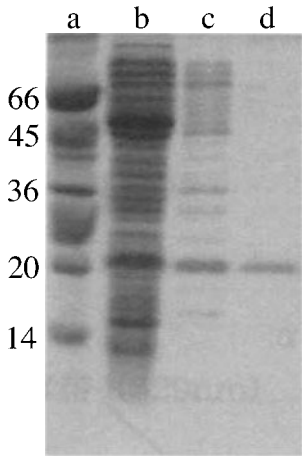

Fig. 4. **SDS-PAGE of *Csb5*.** Purity of the recombinant *Csb5* was examined by SDS-PAGE. Lane (a) size markers, (b) crude extract, (c) fraction purified by anion exchanger, (d) *Csb5* purified by gel filtration.

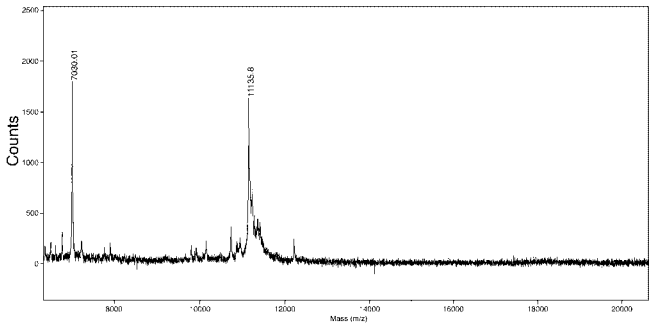

Fig. 5. **Determination of the molecular mass of *Csb5* by mass-spectroscopy.** Protein mass calibration was performed with thioredoxin (11,674.4), lysozyme (14,310.4), and apomyoglobin (16,953.8).

sequence well (*26*). We, therefore, assumed the middle ATG to be the initial codon for the cytochrome b_5 of *C. savignyi*. The coding sequence contained two His codons for heme-binding, and the amino acid sequences around the heme-binding sequences were well conserved as compared with mammalian cytochromes b_5 (*11*) as well as that of *P. misakiensis* (*21*) as shown in Fig. 2. The amino acid sequence deduced from the nucleotide sequence of Csb5 showed 62% identity and 78% similarity to that of chicken, and 61% identity and 82% similarity to that of *P. misakiensis* (*21*). *Csb5* contains one Cys on the amino-terminal side, which is rare in the structure of animal cytochromes b_5. In many plant cytochromes b_5, however, one Cys is usually present in the amino-terminal domain (*17–19*). The structural significance of the Cys residue in *Csb5* and plant cytochromes b_5 is not clear at present, but probably is not directly related to function, because the amino-terminal portion is on the opposite side of the heme-binding domain (*27*). *Csb5* lacks Glu or Asp residues in the carboxyl-terminus as in the case of *Pmb5* (*21*), and those residues have been suggested to be important for the binding of mammalian cytochrome b_5 to the endoplasmic reticulum (*28*). The intracellular localization of *Csb5* and *Pmb5* remains undetermined.

Homology of Csb5 with C. intestinalis b_5—Recently, the *C. intestinalis* EST project analyzed many DNA clones (*25*), and the DNA for cytochrome b_5 is also contained in the database. The DNA sequence for cytochrome b_5 was obtained from the database, and the amino acid sequence deduced from the nucleotide sequence was compared with that of *Csb5* as shown in Fig. 3. *C. intestinalis* b_5 was found to show 86% identity and 96% similarity with the sequence of *Csb5*, while the *Csb5* amino acid sequence showed 61% identity and 82% similarity with the sequence of *Pmb5* (*21*). This seems consistent in that *C. intestinalis* is evolutionarily closer to *C. savignyi* than to *P. misakiensis*.

Expression and Purification of a Recombinant Soluble Csb5—The amino acid sequence of *Csb5* (Fig. 2) deduced from its DNA sequence (Fig. 1) suggests the expression of the membrane-bound form of *Csb5* in animal cells. In this

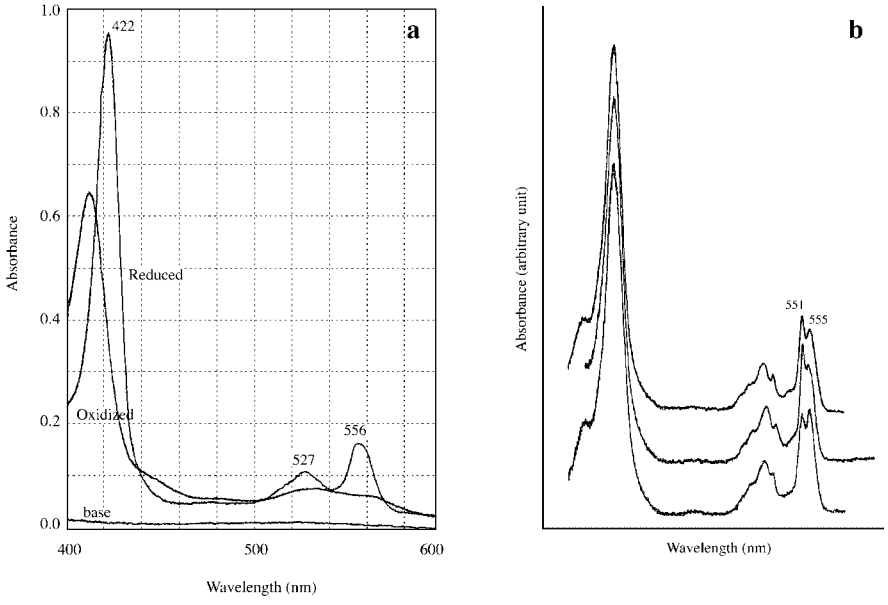

Fig. 6. **Absorption spectra of purified *Csb5*.** (a) The absorption spectra of the purified *Csb5* were measured in 0.05 M Tris-HCl buffer (pH 8) at room temperature. The cytochrome was reduced with dithionite. (b) Low-temperature spectra of the dithionite-reduced form of cytochromes b_5 were measured at liquid nitrogen temperature. The spectra of *Csb5*, *Pmb5*, and *Hsb5* are shown from bottom to top.

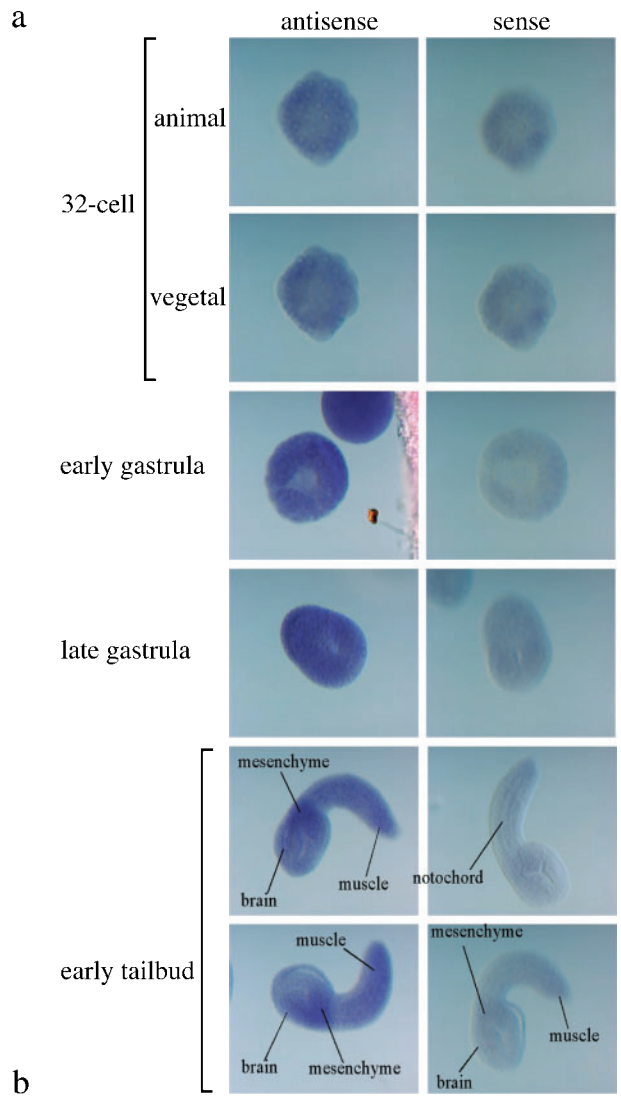

Fig. 7. **Expression of *Ciona* b_5 mRNA in various tissues during the development.** The expression of *Ciona* b_5 mRNA was examined by *in situ* hybridization using the RNA of *Ciona* b_5 as a probe as described in Methods. Left) Staining with DIG-labeled antisense *Ciona* b_5 RNA; right) staining with the DIG-labeled sense *Ciona* b_5 RNA.

study, *in vitro* expression of the membrane-bound form of the recombinant *Csb5* was tried under various conditions, but was unsuccessful. Westernblot analysis with antibody raised against *Csb5* indicated that a small amount of apocytochrome b_5 was expressed in inclusion bodies. The addition of hemin to the culture medium of *E. coli*, however, did not result in the expression of the membrane bound form of cytochrome b_5. Therefore, the characterization of the *Csb5* protein was carried out with the putative soluble form of *Csb5,* although we do not have any evidence that cells of *C. savignyi* express the soluble form of *Csb5*.

A transformant harboring an expression plasmid for soluble *Csb5*, pKK223–3/*Csb5* was cultured in 2× YT medium at 37°C, and the expression of the *Csb5* protein was induced by adding 0.5 mM IPTG for about 8 h. Preparation of crude extract and purification of *Csb5* were carried out as described previously (*21*).

The purity of the *Csb5* was examined by SDS-PAGE as shown in Fig. 4. After chromatography on a DEAE-Toyopearl and gel filtration column, the purified *Csb5* showed electrophoretically a single band at around 20 kDa, although the molecular weight calculated from the amino acid sequence is 11,270. A similar discrepancy was observed for *Pmb5* (*21*) between the calculated molecular weight and the apparent molecular weight estimated from the mobility in SDS-PAGE. The molecular mass of *Csb5* determined by mass-spectroscopy is 11,135.8, which corresponds exactly to the deduced sequence without the initial Met as shown in Fig. 5.

Absorption Spectra of Csb5—The absorption spectra of the putative soluble *Csb5* are shown in Fig. 6a. The dithionite-reduced form shows absorption peaks at 422, 527, and 556 nm with a shoulder at 560 nm. The asymmetric absorption maximum at 556–560 nm is very similar to that of *Pmb5* (*21*), and also to those of mammalian cytochromes b_5 (*29*). The low temperature spectrum of the dithionite-reduced form of the *Csb5* is also shown in Fig. 6b, in comparison with those of *Pmb5* and *Hsb5*. *Csb5* shows split peaks at 551 and 555 nm as well as the spectra of *Pmb5* (*21*) and *Hsb5*. However, very interestingly, the ratio of the heights at 551 nm and 555 nm is apparently different from that of the heights of *Pmb5* (*21*) and *Hsb5* as shown in Fig. 6b. For *Csb5*, the peak at 555 nm is higher than that at 551 nm, while, contrary to this, the heights of the peak at 551 of *Pmb5* and *Hsb5* are clearly higher than those of the 555 nm peak. The shape of the low temperature spectrum of *Csb5* is similar to that of plant cytochrome b_5 (*19*), which also contains a Cys residue in the amino-terminal region. The molecular basis for the difference in the shapes of the low temperature spectra is not clear at present, but is an interesting problem to be solved.

Kinetic Properties of the Csb5—The apparent K_m value of NADH-cytochrome b_5 reductase for the *Csb5* was determined to be 3.3 μM from the Lineweaver-Burk plot using the crude extract of *C. savignyi* as the enzyme. This value is similar to the K_m values of *Pmb5* (5 μM) (*21*) and mammalian b_5 (4–13 μM) (*30*, *31*).

Expression of Ciona b5 mRNA in Embryonic Tissues—The spatial expression of *Ciona* b_5 mRNA in embryonic tissues during development of *C. intestinalis* was examined by *in situ* hybridization using a DIG-labeled *Ciona* cytochrome b_5 RNA probe. As shown in Fig. 7a, *Ciona* b_5 mRNA expression was observed from the 32-cell stage. Expression was observed in all blastomeres, suggesting that the *Ciona* b_5 mRNA encodes a housekeeping protein, as is the case for mammalian b_5s. However, as shown in Fig. 7b, strong expression in a few cell types, such as the brain, mesenchyme and muscle cells, suggests a specific role of *Ciona* b_5 during embryogenesis and/or high metabolic activity of these cell types. Previously, we suggested the role(s) of *Pmb5* in *P. misakiensis*, which propagates through the asexual reproduction by budding, in relation to fatty acid metabolism (*21*). In the case of *Csb5* from *C. savignyi*, which propagates through sexual reproduction, however, the role(s) of *Csb5* may be different from that (those) of *Pmb5*. In the tissues of developing rats, NADH-cytochrome b_5 reductase is activated in brain microsomes (*32*). Therefore, oxidation-reduction activities may be important to promote the development of these tissues in

ascidians. To reveal the role(s) of *Pmb5* and *Csb5* in ascidians, further investigations are required.

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