Expression and translocation of Drosophila nuclear encoded cytochrome \textit{b}_5 proteins to mitochondria

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Abstract

DNA sequence studies of cytochrome \textit{b}_5 (Cyt-b) genes from \textit{Drosophila melanogaster} and \textit{Drosophila virilis} predict that the Drosophila Cyt-b proteins are extremely hydrophobic and have at least eight potential transmembrane spanning domains. Primary protein sequence analysis also predicts that the Cyt-b proteins have mitochondrial targeting sequences and they contain sites for potential post-translational modification similar to other cytochrome proteins. We report the characterization of the cytochrome \textit{b}_5 proteins from \textit{Drosophila melanogaster} and \textit{Drosophila virilis}. We have used a \textit{Drosophila} cytochrome \textit{b}_5 specific antibody to demonstrate that cytochrome \textit{b}_5 proteins are expressed in muscle-containing tissues in the fly. We also provide evidence that the nuclear encoded cytochrome \textit{b}_5 protein that contains a mitochondrial targeting sequence is translocated to mitochondria. © 2000 Elsevier Science Ltd. All rights reserved.

1. Introduction

Cytochromes are heme binding proteins found in the cells of most aerobic organisms. Cytochrome proteins contain protoheme IX or one of its derivatives and function in electron transport usually as components of a complex reaction pathway that is nearly always associated with a membrane system. There also is a correlation between the concentration of cytochrome proteins and the respiratory activity of cells. For example, one of the highest cellular concentrations of cytochromes identified is in the thoracic muscles of flying insects. Cytochrome \textit{b}_5 in particular is a membrane-bound hemoprotein, which acts as an electron carrier for several membrane bound oxygenases. Two homologous forms of cytochrome \textit{b}_5 have been identified, one found in microsomes and the other in mitochondria (Borgese et al., 1993).

Our laboratory has identified and characterized the cytochrome \textit{b}_5 (Cyt-b) locus of \textit{Drosophila melanogaster} (Levin et al., 1989; Kula et al., 1995). The Cyt-b gene is on the second chromosome at locus 36B adjacent to the sarcomeric myosin heavy chain (sMhc) gene (Rozek and Davidson, 1983; Bernstein et al., 1983). Cyt-b cDNA and genomic clones of \textit{D. melanogaster} have been isolated, sequenced and in order to better understand the structure and function of the Cyt-b protein, we have compared the Cyt-b gene sequences of two evolutionarily diverged Drosophila species (Levin et al., 1989; Kula et al., 1995). The two homologous \textit{Drosophila} Cyt-b genes and proteins show sequence similarity to other \textit{b}-type cytochromes, especially in the N-terminal heme-binding protein domain. These \textit{Drosophila} Cyt-b proteins are extremely hydrophobic and have at least eight potential transmembrane spanning domains. Primary protein sequence analysis also predicts that the Cyt-b proteins have mitochondrial targeting sequences and that they contain sites for potential post-translational modification similar to other cytochrome proteins.

In this communication we use a \textit{Drosophila} Cyt-b specific antibody to demonstrate that the Cyt-b protein is expressed in muscle-containing tissues in the fly. We also provide evidence that the nuclear encoded Cyt-b protein, which contains a mitochondrial targeting sequence, is translocated to mitochondria. These results have important implications for the biological role of this nuclear encoded mitochondrial protein.
2. Materials and methods

2.1. Nucleic acid manipulation, electrophoresis, and isolation

DNA was digested with appropriate restriction endonucleases according to manufacturer specifications and size fractionated on agarose gels following standard methods (Maniatis et al., 1982). DNA fragments were isolated by electroelution into DEAE-ion exchange paper (Dretzen et al., 1981). Vectors were linearized with the appropriate restriction endonucleases and DNA fragments were subcloned following standard procedures (Maniatis et al., 1982).

An 800 bp EcoRI fragment representing the C-terminal half of the D. melanogaster cDNA sequence was cloned into the trp expression vector pATH 3 (Hardy and Strauss, 1988). Transformed cells were induced to express a fusion protein by the addition of β-indoleacrylic acid. Following induction cells were pelleted and lysed for isolation of the insoluble fusion protein according to the method of Hardy and Strauss (1988).

2.2. Polyclonal antibody production and immunoreactions

Fusion protein was mixed in a 1:1 emulsion with Freund’s complete adjuvant and injected into rabbits. Antibodies were purified by the method of McKinney and Parkinson (1987). Proteins immobilized on nitrocellulose were blocked with 5% non-fat dry milk in 5% BSA, 10 mM Tris-Cl pH 8.0, 150 mM NaCl (TBS). Primary antibody reactions were incubated overnight. Secondary antibodies were peroxidase conjugated goat anti-rabbit IgG. Positive reactions were visualized colorometrically.

2.3. Drosophila protein preparations and subcellular fractionation

Various protein preparations from Drosophila were used to identify and isolate the Cyt-b protein. The subcellular fractionation procedure used in this communication has been adapted from several methods which are successful using insect models (Gnagey et al., 1987; Alzari et al., 1985; Tupper and Tedeschi, 1969). Total fly preparations consisted of whole adult D. melanogaster preparations clonalized from the C-terminal half of the D. melanogaster cDNA sequence was cloned into the transcription vector pGEM-3Z (Promega). Transcription of Cyt-b RNA was carried out at 37°C for 2 h with the addition of rATP, homogenized at 0°C in 0.25 M sucrose, 50 mM sodium phosphate pH 7.5. Cellular debris was removed by centrifugation at 1000 g for 10 min at 4°C and the supernatant was respun under the same conditions to further pellet eye pigments and nuclei. Mitochondria require higher speeds and longer times of centrifugation, and were pelleted from this supernatant at 15,000 g for 1 h at 4°C. The non-mitochondrial fraction (supernatant) was removed and centrifuged at 100,000 g for 1 h at 4°C to pellet microsomes. The microsomal pellets were resuspended in 500 μl 50 mM NaPi buffer, pH 7.5. The mitochondrial pellets were fractionated as follows. Mitochondria were resuspended in 1 ml 50mM NaPi and were combined with an equal volume of 0.5 M NaCl and incubated at 0°C for 1 h. The resuspended mitochondria were centrifuged at 100,000 g for 45 min at 4°C. The final supernatant represented the high salt soluble peripheral mitochondrial proteins, and the final pellet, resuspended in NaPi, contained the insoluble integral membrane mitochondrial proteins.

Aliquots of protein that were positive when tested with the anti-Cyt-b polyclonal were also tested in succinate dehydrogenase assays to verify their mitochondrial origin. Spectrophotometric measurements were completed to monitor the presence of heme in these fractions. All fractionation pellets were used to test specificity of antibody. We did not detect cytochrome b in blots of cytoplasmic, microsomal and nuclear proteins.

2.4. Electrophoresis of proteins and western blotting

Proteins were separated on SDS-polyacrylamide denaturing gels (Laemmli, 1970). After electrophoresis, the proteins were transferred to nitrocellulose by electroblotting. The protein blots were blocked for 3 h to overnight in 5% BSA dissolved in 10 mM Tris pH 8.0, 150 mM NaCl, 0.05% Tween-20 (TBST). Primary antibodies were diluted in TBST containing 0.1% BSA and incubated with the blots for 3 h at room temperature. After three washes in TBST, the secondary antibody was added (1:7500 dilution of horseradish peroxidase conjugated goat anti-rabbit or anti-mouse (Cappel) in TBST containing 0.1% BSA) and incubated with the blots for 3 h at room temperature. After three washes in TBST, the secondary antibody was added (1:7500 dilution of horseradish peroxidase conjugated goat anti-rabbit or anti-mouse (Cappel) in TBST containing 0.1% BSA) and incubated with the blots for 3 h at room temperature. After three washes in TBST, the secondary antibody was added (1:7500 dilution of horseradish peroxidase conjugated goat anti-rabbit or anti-mouse (Cappel) in TBST containing 0.1% BSA) and incubated with the blots for 3 h at room temperature. After three washes in TBST, the secondary antibody was added (1:7500 dilution of horseradish peroxidase conjugated goat anti-rabbit or anti-mouse (Cappel) in TBST containing 0.1% BSA) and incubated with the blots for 3 h at room temperature. After three washes in TBST, the secondary antibody was added (1:7500 dilution of horseradish peroxidase conjugated goat anti-rabbit or anti-mouse (Cappel) in TBST containing 0.1% BSA) and incubated with the blots for 3 h at room temperature.
rCTP, rGTP, rUTP and T7 RNA polymerase (Boehringer Mannheim). The DNA template was removed with DNase I (Boehringer Mannheim) and the reaction mix was extracted and precipitated. The in vitro transcribed Cyt-b RNA was resuspended in 30 μl sterile ddH₂O. The in vitro translation (IVT) of Cyt-b was carried out in rabbit reticulocyte lysate with or without the addition of canine pancreatic microsomal membranes according to the manufacturer (Promega) at 30°C for 1 h.

2.6. Immunocytochemical localization of Cyt-b

Pupal cases from 75 h old pupae were removed and the thoraces were dissected and placed in 4% formaldehyde/PBS for overnight fixation. The tissues were washed in PBS for 2 h following fixation. Adult thoraces were then dehydrated through a graded series of ethanol solutions (25%, 50%, 75%, 90%, 95%, and 100%), embedded in LR White resin (Polysciences, Inc., Warrington, Pennsylvania) and sectioned. The thoraces were blocked in filtered 5% normal goat serum/TBS for 15 min at room temperature. The sections were then washed in TBS and incubated with a 1:1000 dilution of the anti-Cyt-b polyclonal antibody made in a 1% normal goat serum/TBS solution overnight at 4°C. The adult sections were washed in TBS and incubated with gold conjugated anti-rabbit antibody at 1:20 dilution for 4 h at room temperature. All antibody incubations were carried out in a humidified chamber. All solutions were filter sterilized before use, and all antibody sources were spun in a microcentrifuge for 5 min prior to use. Sections were counterstained with 2% uranyl acetate and lead citrate.

3. Results

3.1. Identification of Drosophila Cyt-b proteins

An antibody specific for Cyt-b proteins was generated in the following manner. The Cytochrome-b₅ proteins from two Drosophila species are predicted to be over 75% identical (Kula et al., 1995), therefore we cloned a portion of the D. melanogaster cDNA (Levin et al., 1989) corresponding to the C-terminal half of the putative protein into the expression vector pATH 3 (Hardy and Strauss, 1988). This portion of the putative protein was used as an immunogen because of its high predicted antigenicity. The C-terminus of the putative Cyt-b protein is also the region that shares the least overall amino acid sequence homology with other cytochrome proteins, and an antibody directed against this epitope could potentially eliminate cross-reactivity with other cytochrome proteins. The clone was expressed in E. coli and the fusion protein was gel purified and used as an antigen.

The specificity of the Cyt-b antibody was demonstrated in the following manner. In vitro synthesized Cyt-b mRNA was translated in vitro in the presence of S<sup>35</sup>-methionine. The radio-labeled IVT reaction was run on 10% SDS-PAGE gel, transferred to nitrocellulose and reacted with the Cyt-b antibody. Following an immunodetection reaction, the blot was exposed to autoradiographic film. The results are shown in Fig. 1 where the antibody detects a single 47 kDa protein (lane 1). In the autoradiograph (lane 2) we see that the most abundant protein detected is also 47 kDa. This is the correct molecular weight for a primary translation product of the Drosophila nuclear encoded cytochrome-b₅ gene (Kula et al., 1995).

Cyt-b proteins were identified in protein extracts of total flies as follows. Total fly proteins were isolated from both D. melanogaster and D. virilis adults and resolved on denaturing gels, blotted and reacted with the antibody. The results are shown in Fig. 2. Lanes 1 and 2 represent coomassie blue stained total D. melanogaster.
Fig. 2. Identification of the putative Cyt-b protein in vivo. Total adult D. melanogaster and D. virilis proteins were homogenized and boiled in 1X Laemmli sample buffer (Laemmli, 1970) and separated through 10% SDS-PAGE. Duplicate preparations were then transferred to nitrocellulose for immunoreactions. Lanes 1 and 3, D. melanogaster total adult protein; lanes 2 and 4, D. virilis total proteins. Lanes 1 and 2 were stained with Coomassie blue, and lanes 3 and 4 were reacted with the anti-Cyt-b antibody.

and D. virilis adult proteins. Lanes 3 and 4 are the immunoblot reactions after transfer of the proteins to nitrocellulose. Two proteins are detected in both species of Drosophila which are 63 kDa and 66 kDa in molecular weight. Since the two proteins detected in total protein extracts differ by 3kDa in molecular weight, this suggests that we may be detecting a membrane bound (66 kDa) and a translocated form (63 kDa) of the cytochrome b5 protein. Our previous nucleic acid study (Kula et al., 1995) predicted that the primary translation product of the Cyt-b gene had a mitochondrial targeting sequence, which was approximately 3 kDa.

We also note that the molecular weights of the detected proteins differ from the predicted molecular weight of 47 kDa for a primary translation product of the D. melanogaster Cyt-b gene (Kula et al., 1995; Fig. 1). The difference in molecular weight of a primary translation product and the proteins detected in whole adult extracts is a strong indication that the Cyt-b protein is post-translationally modified. There are several examples of other cytochrome b5 proteins which have been identified in vivo with greater apparent molecular weights than those predicted from sequence determination (Spatz and Strittmatter, 1971; Tzagoloff, 1982; Kleinberg et al., 1989). It has been shown in some cases that the migration discrepancy is due to post-translational modification of the cytochrome b5 proteins. Post-translational modification of cytochrome b5 proteins and other proteins that are targeted to membranes is common.

3.2. Subcellular localization of Cyt-b proteins

The results presented thus far indicate that the Cyt-b protein potentially contains a mitochondrial targeting sequence, which is represented by a stretch of 35 amino acids at the N-terminus (Kula et al., 1995). To investigate this possibility we fractionated total Drosophila proteins into subcellular components. The protocol used was an adaptation of others previously shown to successfully fractionate proteins from Drosophila preparations (Gnagey et al., 1987; Alziari et al., 1985; Tupper and Tedeschi, 1969). Particular attention was paid to the mitochondrial enriched protein fraction, which could be further separated into peripheral and integral membrane fractions. The proteins directed to the outer mitochondrial membrane via the transit signal that are essentially “in line” for subsequent translocation constitute the peripheral fraction. These proteins are held at the mitochondria by electrostatic forces that can be disrupted with salt. Proteins that have been translocated make up the integral membrane fraction, and are assumed to no longer possess transit signal sequences. Such membrane associated proteins may be solubilized with detergents. Proteins were fractionated as described in Materials and methods and these fractions were used to further analyze the Cyt-b protein in vivo.

Protein preparations from adult D. melanogaster and D. virilis subcellular fractions were run on SDS-PAGE gels and blotted for immunodetection. Fig. 3 shows a coomassie stained gel where the difference in protein composition of the various subcellular fractions is apparent. Lane 1 represents D. melanogaster total adult protein; lane 2 contains total mitochondrial enriched proteins; lane 3 contains the peripheral mitochondrial proteins; and lane 4 represents the integral membrane fraction of mitochondrial proteins. D. melanogaster and D. virilis total mitochondrial, peripheral mitochondrial and integral membrane proteins were electrophoresed,
Protein distribution in subcellular fractions. Total adult protein preparations were separated into various subcellular fractions. Shown is the Coomassie blue stained 10% gel after electrophoresis of the D. melanogaster proteins in each fraction before immunoblotting. Lane 1, total adult fly proteins; lane 2, total mitochondrial enriched proteins; lane 3, peripheral mitochondrial proteins; lane 4 integral membrane mitochondrial proteins.

Blotted to nitrocellulose and incubated with the Cyt-b antibody, Fig. 4 shows the results. Two Cyt-b proteins are detected with apparent molecular weights of 63 kDa and 66 kDa in the total mitochondrial enriched fraction (lanes 1 and 4). The proteins detected are the same molecular weight as proteins detected in total adult extracts (Fig. 2). In the peripheral mitochondrial protein fractions (lanes 2 and 5), we detect a single protein which comigrates with the 66 kDa molecular weight form detected in the total mitochondrial enriched fraction. In the integral membrane protein fraction (lanes 3 and 6), we detect a single protein which comigrates with the 63 kDa protein detected in the total mitochondrial enriched fraction.

We interpret this result in the following manner. The two proteins detected with the Cyt-b antibody differ in molecular weight within the range of a targeting signal (approximately 3 kDa, Kula et al., 1995). The 66 kDa protein detected in the peripheral mitochondrial fraction represents a membrane bound form of Cyt-b with an intact mitochondrial targeting sequence. This protein is probably associated with the outer membrane of the mitochondria and has not been translocated. The 63 kDa protein detected represents an integral membrane protein that is a translocated form of the peripheral protein and represents Cyt-b after cleavage of the targeting sequence. This result supports our previous predictions concerning the Cyt-b protein derived from our nucleic acids study (Kula et al., 1995).

We have also run controls to demonstrate the specificity of this antibody for proteins associated with mitochondria. In these experiments we have incubated the antibody with blots of cytoplasmic, microsomal and nuclear proteins. We do not detect any cross reaction of the antibody with proteins in these fractions. We have also conducted additional experiments to verify the homogeneity of the subcellular fractionation procedure used in the above experiments. These experiments have included assays for the presence of succinate dehydrogenase, a mitochondrial specific enzyme, and spectrophotometry to monitor for the presence of heme. All preparations, which contained Cyt-b protein as determi-
ined by immunodetection also, tested positive for succinate dehydrogenase and heme (not shown). Finally, we have used 2-D protein gel electrophoresis to verify that the pI of the protein detected in immunoblots is within the range of the predicted PI of the Cyt-b protein (Kula et al., 1995).

3.3. Immunological localization of Cyt-b by transmission electron microscopy

Cytochrome b$_{5}$ was first identified in the microsomal fraction of rat liver and later was shown to be associated with the membranes of the mitochondria (Fowler et al., 1976). The results presented indicate that the nuclear encoded form of Drosophila cytochrome b$_{5}$ is localized to the mitochondria. To further investigate if Cyt-b is translocated to the mitochondria, the protein was localized immunocytochemically in adult fly thorax tissue sections. Flies prior to eclosion were embedded, sectioned and incubated with the anti-Cyt-b antibody. Following this incubation a secondary antibody, which was anti-rabbit conjugated with 10 nm colloidal gold, was added to the incubation. The sections were then stained for electron microscopy. The results are shown in Fig. 5. In all of the panels, the thoracic myofibrils can be seen interspersed with the giant mitochondria common to Drosophila muscle (Bernstein et al., 1983). Fig. 5a shows a section of adult thorax first incubated with pre-immune serum and then the secondary antibody. As can be seen, there is low background. Fig. 5b shows a section incubated with anti-Cyt-b at a dilution of 1:1000. The gold particles are predominantly localized to the mitochondria. Fig. 5c is a higher magnification to show more precisely the mitochondrial localization of Cyt-b. The fixation procedure used in the immunodetection experiments partially destroys the epitope in these sections resulting in a low level of reaction with the antibody (Crough et al., 1994). For comparison Fig. 5d shows a section of larval muscle, which has been reacted with a Drosophila myosin heavy chain antibody and illustrates the extent of a positive reaction with an abundant muscle protein.

4. Discussion

Invertebrates have special forms of b-type cytochromes, which function as electron carriers and terminal oxidases (Lemberg and Barrett, 1973). Muscles, which move rapidly such as those in the flight muscles of birds or the thoracic flight muscles of insects, have a high content of cytochromes and cytochrome oxidases. The rapidly contracting insect muscle involved in flight increases oxygen consumption nearly six fold, and this flight muscle is one of the most metabolically active tissues known (Weis-Fogh, 1961). The mitochondria of flight muscles are giant and densely packed (Lemberg and Barrett, 1973), and there is a strong correlation between mitochondria content and muscle activity.

![Fig. 5. Immunodetection of Cyt-b proteins in thoracic flight muscle. Panel A. Transmission electron microscopy of D. melanogaster adult thoracic muscle section reacted with pre-immune serum. Magnification — 50 000x. Panel B. Immunocytochemical localization of D. melanogaster Cyt-b. Shown is a 75 h pupal thorax section reacted with the anti-Cyt-b antibody at a dilution of 1:1000. Magnification — 60 000X. Panel C. Increased magnification of EM localization of Cyt-b. The immunocytochemical reaction shown in Panel B is enlarged to show in more detail the localization of the Cyt-b protein. Magnification — 100 000x. Panel D. Immunocytochemical localization of myosin heavy chain. A third instar larval section reacted with a D. melanogaster anti-Mhc antibody (Crough et al., 1994). The muscle is the large crescent shaped material. Mitochondria are rounded organelles along the concave portion of the muscle.](image-url)
The cytochrome b₅ proteins in particular constitute a group of b-type cytochromes represented by many protein forms that in turn have various cellular functions. Most recently in the rat, it was found that two genes encode for two homologous integral membrane forms of cytochrome b₅, one of which is post-translationally targeted to the mitochondrial membrane, the other to the endoplasmic reticulum (Borgese et al., 1993). These are considered the most common forms of cytochrome b₅ proteins. Thus, cytochrome b₅ donates its electrons to a variety of acceptors involved in different cellular metabolic functions. Cytochrome b₅ can also accept electrons from some cytochrome P450 reductases, and participate with some P450s in cellular detoxification (Hodgson and Rose, 1991). The various protein forms of cytochrome b₅ with different cellular targets suggest that these proteins may perform different functions depending on their subcellular localizations.

The Cyt-b in Drosophila is highly conserved both in nucleic acid content and proximity to Mhc (Levin et al., 1989; Kula et al., 1995). This gene encodes a unique protein expressed throughout development that represents the putative form of cytochrome b₅. The Cyt-b locus which we have characterized represents a nuclear encoded form of cytochrome b₅ in Drosophila. This protein is the first cytochrome b₅ to be identified in Drosophila, and is an example of a nuclear encoded protein that is post-translationally localized to the mitochondria of insect muscle. Other investigations have identified a 43 kDa-cytochrome b protein, which is encoded by the Drosophila melanogaster mitochondrial genome (Clary and Wolstenholme, 1985; Garesse, 1988).

Cytochrome proteins are encoded by both nuclear and organellar DNA (Hennig and Neupert, 1981). Most, but not all, of the nuclear encoded mitochondrial specific polypeptides contain N-terminal target sequences, which direct post-translational localization (Colman and Robinson, 1986). Hemoproteins destined for the mitochondria are translated as apoproteins on free polysomes and converted to holoprotein forms after crossing the outer membrane and associating with heme in the mitochondrial intermembrane space (Hennig and Neupert, 1981). Assembly of the mitochondrial inner membrane involves the insertion of the large number of nuclear encoded subunits in association with those synthesized by the mitochondria.

The D. melanogaster and D. virilis proteins share similar potential mitochondrial transit peptide sequences (Gavel and von Heijne, 1990; Kula et al., 1995) suggesting that these proteins are post-translationally transported to their sites of function. Absence of a true endoplasmic reticulum localization signal (Mitoma and Ito, 1992) further suggests that these proteins are not microsomal b-type cytochromes, although recent work has implicated that an extremely hydrophobic C-terminus could in itself allow insertion into the microsomal membrane (D’Arrigo et al., 1993).

The Cyt-b antibody detects two distinct proteins that are larger than the predicted molecular weight of a primary translation product. Such molecular weight anomalies have been seen by others in studies of microsomal b₅s from rabbit liver (Spatz and Strittmatter, 1971) and of mitochondrial b₅s from yeast, human, and beef heart mitochondria (Tzagoloff, 1982). For example, Kleinberg et al. (1989) document human cytochrome b₅ ranging in molecular weights from predicted sizes of 78, 74 and 77 kDa to observed sizes of 93, 115 and 110 kDa, respectively. A similar molecular weight anomaly is seen in the range of 15–20 kDa for Drosophila Cyt-b protein and this suggests that the protein may be post-translationally modified. Many potential post-translational modifications are predicted to occur in the C-terminal two-thirds of the sequence (Kula et al., 1995). Such changes could serve to ensure or cooperate in the association of Cyt-b with a membrane. Glycosylation, phosphorylation, and myristoylation have all been implicated in protein localization to membranes, anchorage in those membranes, protein-protein interactions, signal transduction, and increased hydrophobicity. Craig and Wallace (1993) have documented the phosphorylation of cytochrome c. Koshy et al. (1992) have also shown the phosphorylation of cytochrome P450s results in retarded electrophoretic mobilities on SDS-PAGE. Myristoylation of proteins has been implicated in protein localization to membranes, anchorage in those membranes, protein-protein interactions, signal transduction and overall increased protein hydrophobicity (Strittmatter et al., 1993; Pietrini et al., 1992; Utsumi et al., 1988; Pillai and Baltimore, 1987).

Glycosylation represents one of the important co-translational and post-translational modifications of proteins. Glycoproteins exist both as soluble and membrane-bound forms (Sharon, 1985). Many groups have documented the glycosylation of cytochrome and mitochondrially localized proteins. Various sources of cytochrome P450s are shown to be glycosylated (Kochs et al., 1992; Sethumadhavan et al., 1991; Ohashi et al., 1992; Shimozawa et al., 1993; Szczesna-Skorupa and Kemper, 1989). Kleinberg et al. (1989) also showed the molecular weight variability of human cytochrome b₅s from different cell types ranged from predicted values of 74 kDa to observed values as high as 115 kDa due to glycosylation. Glycosylation is believed to play a major role in the membrane association of structural proteins (Sakaguchi et al., 1992; Yu and Yu, 1980). Evidence also suggests that glycosylation may protect proteins from proteolysis (Post et al., 1991). Glycosylation has also been shown to play a role in the association of proteins with membrane systems (Ohashi et al., 1992; Knoller et al., 1991; Szczesna-Skorupa and Kemper, 1989; Katz et al., 1977).
The predicted sequence analysis suggests sites within the *Drosophila* Cyt-b protein that may serve for post-translational modifications. Such modifications are predicted to occur in the C-terminal portion of the sequence. Such changes could ensure or cooperate in the association of Cyt-b with a membrane. Glycosylation, phosphorylation and myristoylation have all been implicated in protein localization to membranes, anchorage in those membranes, protein-protein interactions, signal transduction and increased hydrophobicity (Craig and Wallace, 1993; Pietrini et al., 1992; Pyerin and Taniguchi, 1989). We have completed several preliminary experiments to determine if the Cyt-b protein is post-translationally modified. At present, the results of this work are inconclusive. We also note that the difference in molecular weight of the two proteins could also result from differential modification of the primary translation product (Hahne et al., 1994).

Transmission electron microscopy demonstrates the localization of the Cyt-b protein to mitochondria in muscle tissue. It is also apparent from these experiments that Cyt-b is directed to the membrane system of the mitochondria. Upon close inspection of the pattern of staining in the mitochondria, it can be seen that the Cyt-b protein is specifically located in the inner membrane that protrudes into the matrix forming the cristae of the mitochondria (Fig. 5). This is the expected location of a Cyt-b protein involved in the electron transport of the mitochondria. Upon close inspection of the pattern of staining in the mitochondria, it can be seen that the Cyt-b protein is specifically located in the inner membrane that protrudes into the matrix forming the cristae of the mitochondria (Fig. 5). This is the expected location of a Cyt-b protein involved in the electron transport of the mitochondrial respiratory chain.

Throughout evolution it is believed that most, but not all, essential genetic information of the mitochondrial ancestor has been transferred to the nuclear genome (Brennicke et al., 1993). Cytochrome b proteins are often found as domains of larger multicenter redox systems, suggesting that these proteins arose early in evolution and were used for the construction of more complicated proteins (Borgese et al., 1993). The nuclear encoded Cyt-b protein of *Drosophila* does not resemble its mitochondrially encoded counterpart and thus does not appear to be a true duplication or gene transfer product. Instead, the nuclear Cyt-b may represent a redundancy. Such a situation may protect the fly from the aging effects on mtDNA and translation, or may simply provide more energy producing machinery for highly active tissues such as flight muscles. Conversely, the nuclear encoded protein may function in an entirely different system such as that involved in the metabolism of naturally occurring compounds or xenobiotic detoxification. At the subcellular level insect monoxygenases (including cytochrome P450) are found in both the endoplasmic reticulum and the mitochondria (Hodgson and Rose, 1991). Cyt-b could be functioning in concert with the monoxygenase system to process oxidants produced in highly active tissues like muscle. Cytochrome b5 and P450 have also been documented to be involved in oxidation of xenobiotics such as insecticides and plant metabolites in a variety of insects (Hodgson and Rose, 1991). It has also been suggested that there exists a state of co-evolution between nuclear and mitochondrial components of biochemical complexes, namely cytochrome c and b (Ma et al., 1993). It is interesting to note that two Cyt-c genes have previously been identified in *D. melanogaster* which are genetically linked to the Cyt-b locus (Limbach and Wu, 1985) raising the possibility that these loci may represent a coordinately expressed domain in the fly genome.

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