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Developmental expression of nuclear genes that encode mitochondrial proteins: Insect cytochromes *c*

(organelle biogenesis/polysome immunoadsorption/myogenesis)

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ABSTRACT **To investigate tissue-specific developmental regulation of mitochondrial biogenesis, we studied the expression of the *Manduca sexta* (tobacco hornworm moth) thoracic muscle cytochrome *c* gene during adult eclosion and used this information to obtain a cDNA clone for this gene, which in turn was used to isolate the corresponding *Drosophila melanogaster* gene. Over the 3 days prior to adult *Manduca* emergence, mitochondrial inner membranes become progressively more electron dense and lamellar, and, while there is no accumulation of apocytochrome *c*, the amount of the holoprotein increases 40-fold per insect thorax. As determined by *in vitro* translation and blot hybridization analysis, the major thoracic muscle cytochrome *c* gene is primarily regulated at the transcriptional level, with cytochrome *c* mRNA increasing from <0.01% to 0.04% of total poly(A)$^+$ RNA and declining to an undetectable level by day 2 after eclosion. Furthermore, the ratio of cytochrome *c* to the other cytochromes remains the same at all times, indicating that these components of the respiratory chain follow coordinated developmental programs. By using polysome immunoadsorption, a poly(A)$^+$ RNA population of ≥95% cytochrome *c* mRNA was isolated from thoracic muscle tissue and was used to construct a cDNA library, which was screened by hybrid selection/translation. We report the sequence of one of those clones, pMSc750, and its use to isolate the major thoracic muscle cytochrome *c* gene of *Drosophila*.**

Most of the information presently available on the biogenesis of mitochondria has been obtained with *Saccharomyces cerevisiae* (see review in ref. 1). Mitochondria reproduce by fission and provide only a small number of organelle-specific polypeptides. Therefore, the nucleus maintains a preeminent biosynthetic role coding for practically all of the 300–400 different proteins that exist in the mitochondrion. Cytochrome *c* is one of these nuclear-encoded proteins (2). The apoprotein is synthesized on free polysomes (3, 4) and converted to the holoprotein after binding to and passage through the outer mitochondrial membrane. Even though a substantial amount of knowledge has been and is being obtained on the transcriptional regulation of the two yeast cytochrome *c* genes (5–7), almost nothing is known about the nature of the cellular signals that coordinate expression of the nuclear and mitochondrial genomes.

Drosophila offers many obvious advantages for the study of regulatory pathways that affect mitochondrial biogenesis and tissue-specific expression in higher eukaryotes. This report examines the relation between cytochrome *c* gene expression and mitochondrial biogenesis during the period of adult eclosion in the tobacco hornworm moth and presents the isolation and sequence of both a *Manduca* cDNA clone and a *Drosophila* genomic clone for the major thoracic muscle transcript in both species. Like other eukaryotes, *Drosophila* has multiple cytochrome *c* genes of limited homology, allowing an examination of the relations between tissue-specific expression and the regulation of different genes in the same gene family.

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MATERIALS AND METHODS

Manduca sexta pupae, kindly provided by A. H. Baumhover, were grown in a specially designed incubator at 26°C and 82% relative humidity. The first 24 hr after pupation is defined as day 0, and animals generally emerged late on day 17. The plasmids pYeCYC1(0.6) (8), pRC4 (9), and pDMA4 (act57A) (10) were the kind gifts of B. D. Hall, R. Scarpulla, and E. Fyrberg, respectively. *Drosophila melanogaster* (Canton-S) flies were generously provided by The Mid-America Drosophila Stock Center. Cytochrome *c* holoprotein was isolated from adult moths (11), and the apoprotein was prepared (12). Mitochondria from *Manduca* thoracic tissue (13, 14) were assayed for cytochrome content as described by both Williams (15) and Vanneste (16). The total amounts of cytochrome *c* apo- and holoprotein were determined by HPLC. Rabbit anti-cytochrome *c* antisera for both the holo- and apoprotein, affinity-purified anti-holoprotein antibodies, and protein A-Sepharose-purified anti-holoprotein IgG were prepared as described (17).

Nucleic Acid Isolations and Blots. Poly(A)$^+$ RNA was isolated by using guanidinium thiocyanate (18), banding in cesium chloride (10), and chromatographing on oligo(dT)-cellulose (19). Glyoxalated-RNA (5 μg) was electrophoresed through 1.4% agarose, blotted, and hybridized as described by Thomas (20). Restriction fragments were isolated from agarose gels by electrophoresis into DE 81 paper (Whatman) (21). Nick-translations were performed as previously described (22) and ^{32}P-labeled RNA probes were prepared by run-off transcription (23).

High molecular weight DNA was prepared as described (10). Southern blot (24) hybridizations with either DNA or RNA probes were for 24 hr at 23°C (in the case of heterologous probes) and at 42°C (for homologous probes) in 50% formamide/5× SSPE buffer (1× SSPE = 0.18 M NaCl/0.01 M NaH_2PO_4/0.001 M EDTA, pH 7.4)/0.2% $NaDodSO_4$/5× Denhardt's solution (25) containing 250 μg of calf thymus DNA per ml and, if cDNA or RNA probes were used, 10 μg of poly(A) per ml.

Library Preparation and Screening. First- and second-strand cDNAs were prepared (26), and libraries were constructed by C-tailing, annealing to G-tailed pBR322, and transforming *E. coli* HB101 (27). The cDNA library was screened as described by Grunstein and Hogness (28). The genomic library screened (29) was a λ Charon 4/Canton-S

Abbreviation: bp, base pairs.

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embryonic isolate (30), the kind gift of T. Maniatis and D. Goldberg.

Restriction Mapping, Subcloning, and DNA Sequencing. Bacteriophage and subclone restriction maps were derived by conventional single/double restriction enzyme digests and were mapped in detail (31). Gene fragments were subcloned into pSP64 (Promega Biotec, Madison, WI). DNA sequences of ^{32}P-end-labeled fragments were derived by sequencing both strands, using a modification (32) of the chemical degradation procedure (33) that allowed reading to ≈550 base pairs (bp) from a single-labeled end.

Hybridization Selection/Translation and Polysome Immunoadsorption. Optimized *in vitro* translations were performed by using mRNA-dependent rabbit reticulocyte lysates (34) for 60 min at 37°C in 25-μl reaction volumes. Hybridization selection was carried out by a modification of the technique of Ricciardi *et al.* (35). NaDodSO$_4$/polyacrylamide slab gels were run by using 15% separating and 5% stacking gels (36). For analysis of total translational products, 1 μl of lysate was used per lane; for immunoprecipitation analysis, the precipitate from 24 μl of starting lysate was loaded. Immunoprecipitations were performed with *Staphylococcus* A cells (37, 38). Immunoadsorption was performed with polysomes (39) isolated from the dissected thoracic muscles of over 1000 *Manduca* pupae, the tissue having been thoroughly washed with Robb's medium (40) prior to homogenization.

RESULTS AND DISCUSSION

Initial attempts to isolate *Drosophila* cytochrome *c* genes by using the heterologous hybridization probes that contain the yeast *iso-1* cytochrome *c* gene (8) and the rat somatic cytochrome *c* gene (9) failed because of insufficient nucleotide homology. To circumvent this problem, it was decided to construct a cDNA library from a cytochrome *c*-enriched poly(A)$^+$ RNA population. Unfortunately, cytochrome *c* is normally expressed at very low levels in higher eukaryotic cells. However, Chan and Margoliash (41) reported that, from 3 days prior to eclosion, the amount of cytochrome *c* in a saturniid moth thoracic muscle increases dramatically to reach, in the adult, a level (per gram of muscle) about 10 times that in horse heart and that this resulted from *de novo* synthesis of the apoprotein. *Manduca* was selected for large-scale polysome isolation because, in contrast to *Drosophila*, homogeneous preparations of thoracic muscle and the cytochrome *c* required for the production of antibodies can be readily obtained in quantity. The level of specific mRNA was followed by *in vitro* translation and immunoprecipitation to determine the optimal time for cytochrome *c* polysome isolation. This also allowed a study of how mitochondrial biogenesis, cytochrome *c* gene expression, and thoracic muscle formation are correlated.

Ultrastructural Development and Cytochrome Content of *Manduca* Thoracic Muscle. Following five instars of larval growth, *Manduca* enters the pupal stage in which the puparium is formed and histolysis of larval tissues commences. At 26°C, the transition from this stage to adult eclosion requires approximately 18 days, during which time the adult tissues are fully formed from cells released from the imaginal discs. Electron micrographs (Fig. 1) of thoracic muscle show that this tissue changes primarily in terms of the structure and organization of the interfibrillar space. Longitudinal sections taken several days prior to and on the day of adult eclosion (Fig. 1) all display myofibrils interspersed by columnar mitochondria. The section from an emerged adult (Fig. 1*b*) illustrates the ultrastructure of the mature muscle with well-aligned sarcomeres, 3 μm in length, divided by continuous and homogeneous Z lines. Large cisternae, or double dyads (42), can be seen to be regularly spaced with two per sarcomere and to be located midway between the H and Z bands. Mitochondria are tightly packed within the interfibrillar space and are oriented parallel to the fiber axis. The organelle also appears to be extremely electron dense, with numerous, highly lamellar cristae. On the day of eclosion (day 17), the ultrastructure is very similar to that of the fully mature muscle (day 19). In contrast, 3 days preemergence (day 14), the interfibrillar space is much less regular, with various numbers of mitochondria between myofibrils and a much-less-organized T-system. Mitochondria prepared at daily intervals throughout the eclosion period displayed essentially unchanged ratios of cytochrome concentrations. The mean value for the concentration ratio of cytochromes $[aa_3]/2[c]$ was 0.81 ± 0.08 when calculated according to Williams (15) and 1.10 ± 0.16 when calculated according to Vanneste (16). The corresponding $[c]/[c_1]$ ratios were 0.56 ± 0.22 and 1.15 ± 0.22, while the $[c]/[b]$ ratios were 0.65 ± 0.12 and 1.27 ± 0.21. Over the same period, the total amount of holocytochrome *c* increased 40-fold to about 6 nmol per *Manduca* thorax, while at no time was there an appreciable accumulation of the apocytochrome *c*.

***In Vitro* Translations of Developmentally Staged Poly(A)$^+$ RNA.** Thoracic tissue from developmentally staged moths was dissected, and poly(A)$^+$ RNA was isolated for each 24-hr period to determine when cytoplasmic cytochrome *c* mRNA concentration was maximal. The RNA was translated *in vitro*, and immunoprecipitation of cytochrome *c* was performed. Since cytochrome *c* is a small protein with relatively few antigenic binding domains, steps were taken to optimize the specificity and the titer of the antibody. Rabbit anti-holocytochrome *c* affinity-purified and protein A-Sepharose-purified IgG populations were initially used. However, these consistently coprecipitated several species besides apocytochrome *c*, one of 9.7 kDa, complicating the in-

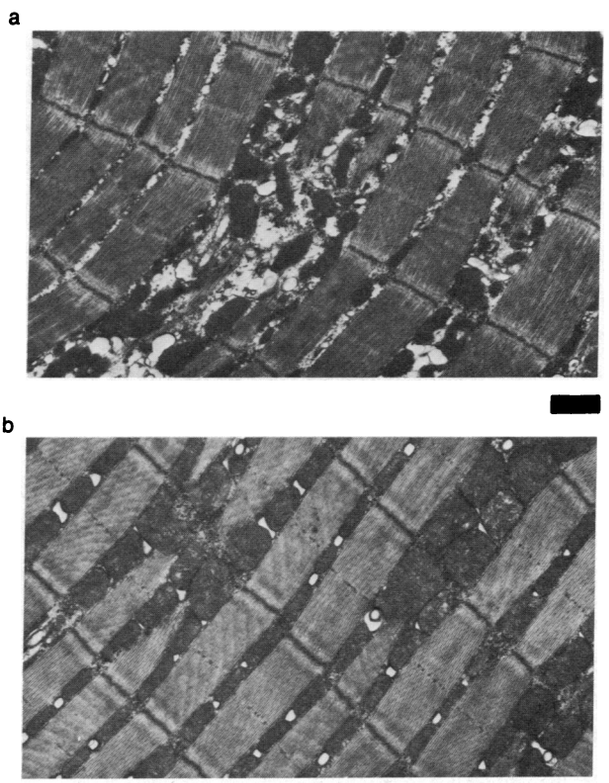

FIG. 1. Electron micrographs of developing *Manduca* dorsal flight muscle. Longitudinal sections, viewed and photographed on a JEOL 100 CX TEM, are of tissue isolated at various developmental stages. The days examined were: day 14, 3 days prior to eclosion (*a*); and day 17, the day of eclosion (*b*).

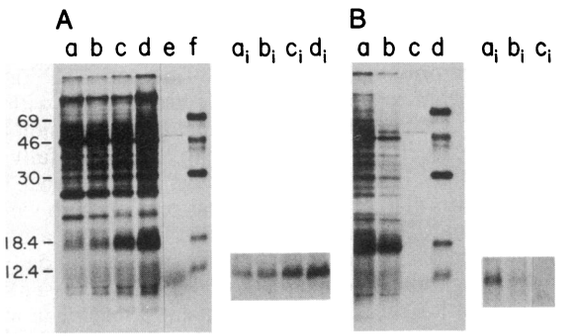

FIG. 2. *In vitro* translation products of total *Manduca* poly(A)$^+$ RNA isolated during the eclosion period. (*A*) Prior to eclosion. Lanes: a–d, days 14–17, respectively; e, without added RNA; f, molecular size standards (shown in kDa); a_i–d_i, immunoprecipitates of lanes a–d. (*B*) After eclosion. Lanes: a, day 18; b, day 19; c, without added RNA; d, molecular size standards; a_i–c_i, immunoprecipitates of lanes a–c.

terpretation. Therefore, chemically prepared *Manduca* apoprotein was used as antigen in the preparation of antiserum, and this antiserum immunoprecipitated only one polypeptide, which comigrated with the chemically made apoprotein (Fig. 2). The maximal amount of apocytochrome *c* produced in the reticulocyte translational assay occurs on day 17, representing an approximately 5-fold increase over day 14 and an 8-fold increase over day 19. These comparative values, generated from integration of densitometer tracings of the $NaDodSO_4$/PAGE fluorographs, agree remarkably well with values obtained with the blot hybridizations described below. Thus, it appears that the heterologous translation system faithfully uses the available cytochrome *c* mRNA present at the various developmental periods and that there is no accumulation of this apoprotein. Therefore, transcriptional regulation of the cytochrome *c* gene is the primary control mechanism for the developmental appearance of the holoprotein.

Isolation of *Manduca* Cytochrome *c* mRNA and cDNA Library Construction. More than 1,000 carefully staged day 17 pupae were used to isolate cytochrome *c*-enriched poly(A)$^+$ RNA by polysome immunoadsorption. The level of purification attained with purified antibody was remarkable. Globin, present in the reticulocyte lysate, excluded cytochrome *c* from the region of the gel where it normally migrates so that it appeared as a compressed band under the rabbit globin (Fig. 3*A*, lanes c and d). However, immunoprecipitation of the translational products yielded a polypeptide species comigrating with apocytochrome *c* (Fig. 3*A*, lanes c_i and d_i). As Fig. 3 dramatically illustrates, this procedure yielded nearly pure poly(A)$^+$ RNA for cytochrome *c*, even though the concentration in day 17 thoracic muscle was only about 0.04%.

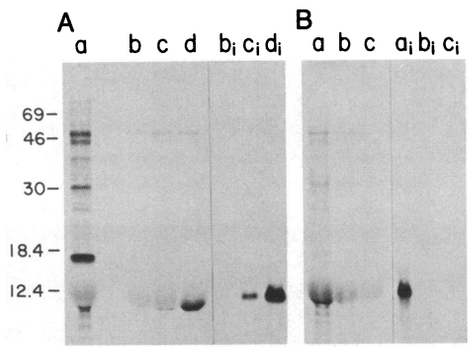

FIG. 3. *In vitro* translations of polysomal immunoadsorbed and hybrid-selected *Manduca* poly(A)$^+$ RNA. (*A*) Thoracic muscle polysomes isolated from moth pupae on day 17 were further purified by polysome immunoadsorption (39). Poly(A)$^+$ RNA was selected on oligo (dT)-cellulose and used for *in vitro* translation. Lanes: a, total day 17 poly(A)$^+$ RNA; b, control without added RNA; c, polysome-immunoadsorbed RNA; d, same as lane c except that 5 times the amount of immunoadsorbed RNA was added per assay; b_i–d_i, immunoprecipitates of material in lanes b–d. (*B*) Hybridization selection and *in vitro* translation of poly(A)$^+$ RNA isolated from moths on day 17 were performed with various plasmid DNAs immobilized on nitrocellulose (35). Lanes: a, pMS*c*750; b, pBR322; c, nitrocellulose control without added DNA; a_i–c_i, immunoprecipitates of material in lanes a–c.

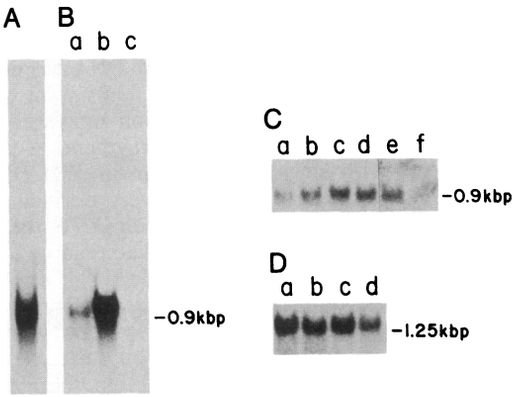

FIG. 4. RNA blot analysis of the developmental expression of the moth and fly major thoracic muscle cytochrome *c* transcript. Total poly(A)$^+$ RNA was isolated from various developmental periods and blotted onto nitrocellulose. (*A*) Day 17 RNA hybridized with single-stranded cDNA synthesized directly from the polysome-immunoadsorbed RNA. (*B*) Expression of cytochrome *c* during larval and adult development hybridized with nick-translated pMS*c*750. Lanes contain RNAs isolated from the fifth instar *Manduca* larvae (lane a), day 17 *Manduca* pupae (lane b), and newly eclosed *Drosophila* adults (lane c). (*C*) Expression of *Manduca* cytochrome *c* RNA during the eclosion period probed with nick-translated pMS*c*750. Lanes a–f, respectively, contain day 14–19 RNAs. (*D*) Expression of *Manduca* actin hybridized with pDMA4 (act57A). Lanes a–d, respectively, contain day 14–17 RNAs.

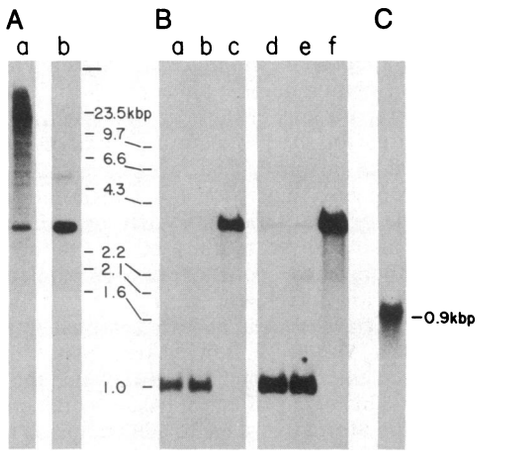

FIG. 5. DNA and RNA blot analysis of the major *Drosophila* cytochrome *c* gene and its transcript. (*A*) Genomic DNA (10 μg) was digested with *Eco*RI , electrophoresed on 1% agarose gels, blotted onto nitrocellulose, and hybridized with an RNA probe prepared by run-off transcription from *Eco*RI-cleaved pMS*c*750 (lane a) or with the nick-translated *Hin*dIII insert of pDMc01 (lane b). (*B*) Phage DNA (2 μg) was digested with *Hin*dIII (lanes a and d), *Eco*RI (lanes c and f) or *Eco*RI/*Hin*dIII (lanes b and e), electrophoresed through 1% agarose, blotted, and hybridized with the pMS*c*750 RNA probe (lanes a–c) or the pDMc01 RNA probe (lanes d–f). (*C*) Poly(A)$^+$ RNA was prepared from newly eclosed *Drosophila* flies, blotted, and hybridized with the nick-translated *Hin*dIII insert of pDMc01.

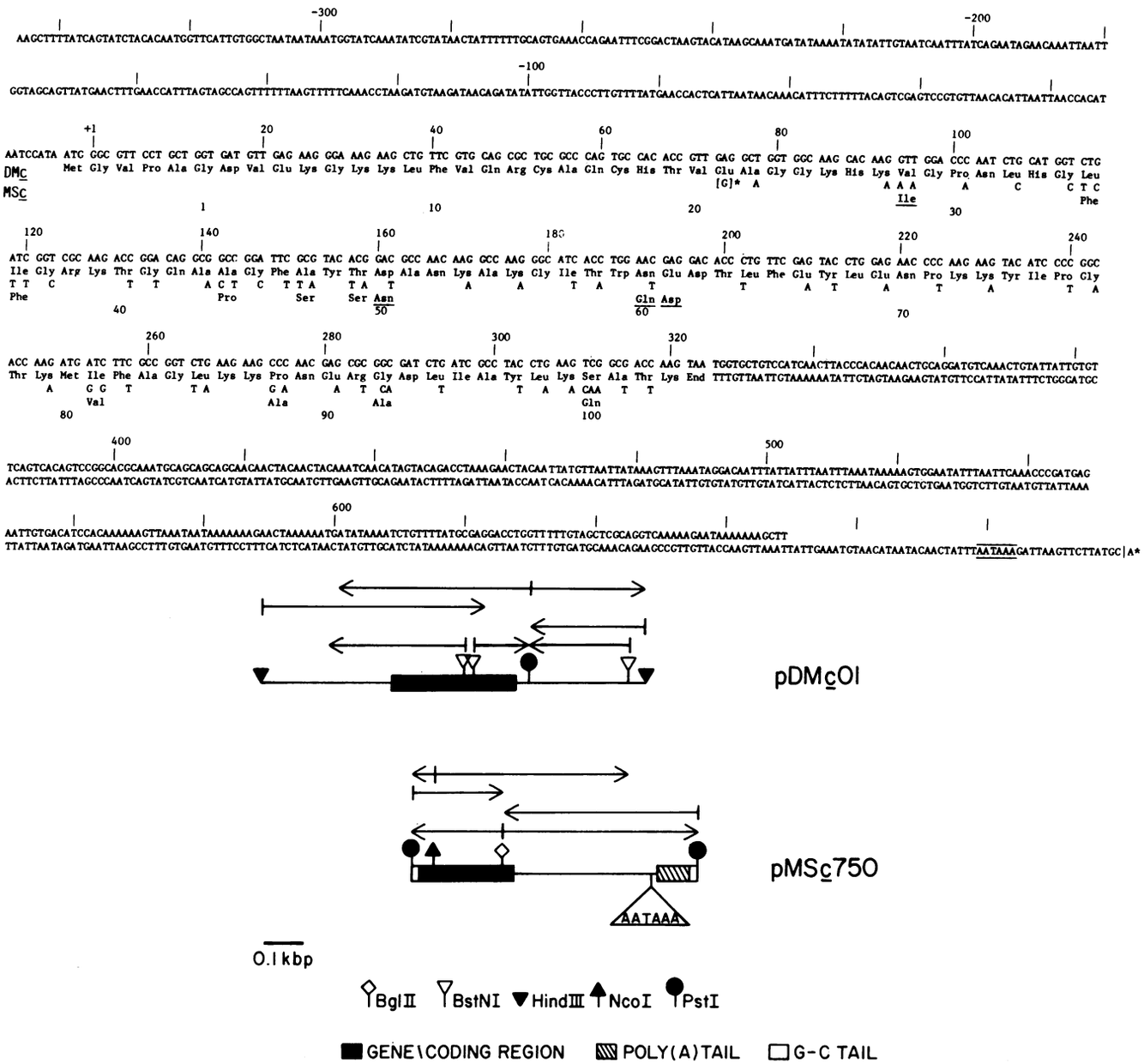

FIG. 6. (*Upper*) Nucleotide sequences of the pMS*c*750 cDNA and pDM*c*01 genomic clones and their derived amino acid sequences. (*Lower*) The partial restriction enzyme digest maps of both clones and the sequencing strategies used. DM*c* and MS*c* in *Upper* refer to the *Drosophila* genomic and *Manduca* cDNA clones, respectively. The *Drosophila Hin*dIII restriction enzyme fragment sequence (DM*c*) is shown in full, while only the differences between it and the *Manduca* cDNA nucleotide and amino acid sequences are shown below the corresponding position. The amino acid positions underlined are those that vary from previously determined amino acid sequences in the case of both *Drosophila* and *Manduca* (unpublished data). The designation [G]* refers to the position at which the tail [G·C] was added at the 5′ end of pMS*c*750, while |A* (last letter in the sequence) refers to the poly(A) tail (85 residues in length) immediately followed by the [G·C] tail at the 3′-end.

This poly(A)$^+$ RNA was used as template in the construction of a cDNA library of 5000 clones from which 48 clones were selected at random for analysis by hybridization selection/translation. Nearly all contained cytochrome *c* cDNA of various lengths. Fig. 3*B* shows an analysis of the clone with the longest insert, pMS*c*750. Although the pBR322 vector alone adsorbs some RNA species, immunoprecipitation demonstrates that pMS*c*750 contains a cytochrome *c* sequence (Fig. 3*B*, lane a_i). First-strand cDNA, made directly from the polysome-immunoadsorbed poly(A)$^+$ RNA, hybridizes to a single species (Fig. 4*A*); this was confirmed by a shorter exposure time. This RNA comigrates with an RNA that hybridizes to pMS*c*750, and this transcript is developmentally regulated, being much less abundant in fifth-instar larvae than in emerging adults (compare lanes a and b in Fig. 4*B*). Interestingly, this cDNA clone is not able to detect the major cytochrome *c* transcript from *Drosophila* under hybridization conditions of high stringency (Fig. 4*B*, lane c). In agreement with the result of *in vitro* translation and immunoprecipitation, this transcript is developmentally regulated during the eclosion period (Fig. 4*C*, lanes a–f), increasing approximately 5-fold between days 14 and 17 and declining to an undetectable level 48 hr after adult emergence (Fig. 4*C*, lane f). In contrast, this transcriptional program was not followed by a muscle-specific actin, as detected with the *Drosophila* actin gene, which hybridized to a unique *Manduca* RNA at high stringency; the actin transcript became progressively less abundant during the day 14–17 period (Fig. 4*D*). These results demonstrate that pMS*c*750 represents a transcript from the major thoracic muscle cytochrome *c* gene.

Isolation of *Drosophila* Cytochrome *c* Genes. The first attempts to isolate *Drosophila* cytochrome *c* genes from phage libraries produced many positively hybridizing plaques with nick-translated pMS*c*750, but the signals were extremely weak even at low stringencies. To circumvent the analysis of many false positives, we recloned the cDNA *Pst* I insert of pMS*c*750 into pSP64 and produced run-off transcripts. Because DNA·RNA hybrids are ≈10°C more thermostable than DNA·DNA hybrids and because there is no competing strand hybridization, these probes produced more intense signals, so that on rescreening of approximately 2×10^6 plaques, only 42 were strongly hybridizing. Southern analysis of genomic DNA from the Canton-S strain with the pMS*c*750 RNA probe indicated several hybridizing species (Fig. 5*A*, lane a) even at high stringency conditions. As commonly observed, RNA probes yielded higher backgrounds only on genomic Southern blots (compare lanes a and b in Fig. 5*A*). Restriction maps demonstrated that many of the recombinant phage represented overlapping genomic fragments. However, one phage, λ DM*c*9/10, contained two *Eco*RI fragments of approximately 1.5 and 3.5 kbp that hybridized to the pMS*c*750 RNA probe, the 3.5-kbp species producing a signal more than 100-fold greater (Fig. 5*B*, lane c). Therefore, a 1-kbp subfragment of the 3.5-kbp fragment was subcloned (pDM*c*01) and used to probe Southern blots of λ DM*c*9/10 (Fig. 5*B*, lanes d–f) and a genomic blot of Canton-S (Fig. 5*A*, lane b).

Only one major *Eco*RI and *Hin*dIII hybridizing fragment was normally visible in both λ DM*c*9/10 and genomic Southern blots. However, as described above, another weakly hybridizing fragment was also detected on much longer exposures. The other sequence, located on λ DM9/10, subcloned as an *Eco*RI subfragment, and termed pDM*c*02, is also a cytochrome *c* gene. It is expressed at almost undetectable levels in adult thoracic muscle and shares only limited nucleotide homology with pDM*c*01 (data not shown). Fig. 5*C*, a blot hybridization of poly(A)$^+$ RNA from emerging *Drosophila* adults, demonstrates that pDM*c*01 hybridizes to a single 900-bp RNA species under high-stringency hybridization conditions. Based upon the greater homology of pMS*c*750 to pDM*c*01 and the relative poly(A)$^+$ RNA concentrations corresponding to the two genes, pDM*c*01 clearly represents the major expressed gene of *Drosophila* thoracic muscle. The restriction maps of pDM*c*01 and pMS*c*750, the sequence strategies used, and the nucleotide sequences are shown in Fig. 6.

In conclusion, the present study has demonstrated that *Drosophila* contains multiple cytochrome *c* genes. The way is now clear for a study of their developmental regulation.

Note Added in Proof. After this work was completed, K. J. Limbach and R. Wu communicated to us a manuscript (43) in which they also report the isolation of several *Drosophila* cytochrome *c* genes.

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